Microbiology and Technology of Fermented Foods

Second Edition

Robert Hutkins

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Preface

When the first edition of *Microbiology and Technology of Fermented Foods* was published in 2006, it was one of only a few books that had recently covered this topic. Moreover, except for specialized courses in wine, brewing, or cheese technology, few food science or microbiology departments offered courses in fermentation science. In the past decade, however, this situation has changed dramatically. Fermented foods and beverages have attracted the attention of scientists from nearly every discipline, including microbiology, chemistry, engineering, nutrition, and biomedicine. Molecular and analytical methods have been developed that provide a basis for understanding the role of specific microorganisms as well as entire microbial communities in producing the unique flavors, textures, and appearances for which fermented foods and beverages are known.

Consumer interests in craft- and artisan-produced foods and ethnic cuisines also account for this renewed attention. The popularity of fermented foods and beverages in the marketplace, as well as the internet and book stores, is readily apparent.

This 2nd edition is intended to serve multiple purposes. First, it is written at a level suitable for use as a textbook for an upper division or graduate level college course in fermentation microbiology. It is also expected to serve as a general reference text that can be used by researchers in academia, as well as by the food and beverage industry. Although readers are expected to have had a basic course in food microbiology, a background in biology and biochemistry should provide the necessary background to understand most of the material.

There are several new and expanded chapters in this edition, including a chapter on distilled spirits and another on coffee, cocoa and cereal fermentations. Many new box entries have been added that, like the 1st edition, are intended to provide interesting diversions from the main text. The 2nd edition also incorporates new material that reflects the many discoveries in fermentation science made in the past decade.

Finally, as in the 1st edition and in an effort to make the text easier to read, the narrative portion of the book has been written with minimal point-by-point referencing. Each chapter includes a bibliography from which most source materials were obtained. The box entries, however, are fully referenced.

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1 Introduction to fermented foods

> You can't just eat good food. You've got to talk about it too. And you've got to talk about it to somebody who understands that kind of food.
>
> Kurt Vonnegut Jr., American writer, from the novel, *Jailbird*

FERMENTED FOODS AND HUMAN HISTORY

Long before there were microbiomes, genome sequences, pure starter cultures, and before Lister and Pasteur, there were fermented foods and beverages. Indeed, fermented foods were very likely among the first "processed" foods consumed by human beings. This was not because early humans had actually planned on or had intended to produce a particular fermented food. Rather, fermentation was simply the inevitable outcome that resulted when raw food materials were left in an otherwise unpreserved state. When, for example, several thousands of years ago, milk was collected from a domesticated cow or goat, either it was consumed within a few hours or else it would ferment. If the latter, the milk soured and curdled, turning into something we might today call yogurt. The milk could also spoil and become unpalatable, an event that must have occurred frequently. Other raw food materials likely experienced a similar fate. Juices expressed from ripe grapes and other fruits would remain sweet for perhaps only a few days before being transformed into a variety of pleasant, intoxicating, and entirely drinkable beverages.

These products provided sustenance, and they were likely appreciated, as well, for their aesthetic benefits, i.e., they tasted good. It must have also been recognized and valued early on that however imperfect the soured milk, wine, olives, and other fermented food products may have been, at least compared to more recent versions, they possessed several noteworthy advantages. They would generally have lasted longer and been less susceptible to spoilage, compared to the raw materials from which they were made. In addition, they were usually, though not always, observed to be safer.

Despite the "discovery" that fermented foods had many virtues, it took centuries for humans to figure out how to control or influence conditions to consistently produce high quality fermented food products. Remarkably, the means for producing so many fermented foods appears to have evolved independently on every continent and on an entirely

Microbiology and Technology of Fermented Foods, Second Edition. Robert W. Hutkins.

empirical basis. Although there must have been countless failures and disappointments, small "industries", skilled in the art of making fermented foods, eventually developed. As long ago as 3000 to 4000 BCE, bread and beer were already being mass produced by Egyptian bakeries and Babylonian breweries. It is clear from historical records that the rise of civilizations, around the Mediterranean and throughout the Middle East and Europe, coincided with the production and consumption of wine and other fermented food and beverage products (Box 1.1). It is noteworthy that the types of fermented foods consumed in China, Japan, and the Far East were vastly different from those in the Middle East. Plants and seafood, rather that animal products, served as the main raw materials. It is also clear that these Asian fermentations evolved and became established around the same time.

Box 1.1 The origins of alcoholic fermentations and the drunken monkey hypothesis

Although the very first fermentations were undoubtedly accidental or inadvertent, it is just as certain that human beings eventually learned how to intentionally produce fermented foods and beverages. When, where, and how this discovery occurred are elusive questions, since written records, alas, do not exist. However, other forms of archaeological evidence do indeed exist. This has made it possible to not only establish the historical and geographical origins of many of these fermentations, but also to describe some of the techniques likely used to produce these products.

Molecular archaeology Most of the early investigations on the origins of food fermentations focused on alcoholic fermentations, namely wine and beer. Led initially by "biomolecular archaeologists" at the University of Pennsylvania Museum of Archaeology and Anthropology (http://www.penn.museum/), these researchers could not use traditional types of physical evidence, since they were often absent. Instead, they relied on chemical and molecular records, obtained from artifacts discovered in locations from around the world (McGovern et al., 2004).

Specifically, their approach involved extracting residues from ancient clay pottery jars and vessels found in excavated archaeological sites, mainly from the Near East and China. These vessels are generally porous, and any organic material would have become adsorbed and trapped within the vessel pores. In a dehydrated state, this material would have been protected against microbial or chemical decomposition. Carbon dating is used to establish the approximate age of these vessels. Then various analytical procedures, including gas chromatography-mass spectroscopy, Fourier transform infrared spectrometry, and other techniques, are used to identify the chemical constituents. The analyses revealed the presence of several marker compounds, in particular, tartaric acid that is present in high concentrations in grapes, and in wine, but generally absent elsewhere (Guash-Jané et al., 2004; McGovern, 2003). Based on these and other studies, it would appear that wine had been produced in the Near East regions around present-day Turkey, Egypt, and Iran, as long ago as the Neolithic Period (8500 to 4000 BCE).

The McGovern Molecular Archaeology Lab group has also ventured to China in an effort to establish when fermented beverages were first produced and consumed (McGovern et al., 2004). As described in Chapter 11, Asian wines are made using cereal-derived starch, rather than grapes. Rice is the main cereal used. In ancient times, other components, particularly, honey and herbs, were added. As before, the investigators analyzed material extracted from pottery vessels that were from the Neolithic Period, ca. 7000 BCE. However, the specific biomarkers would not necessarily be the same as for wine made from grapes. Rather, they would be expected to reflect the different starting materials. Indeed, the analyses revealed the presence of rice, honey, and herbal constituents, but also grapes, in the form of tartaric acid. Although domesticated grapes vines were not introduced into China until about 200 BCE, wild grapes could have been added to the wine as a source of yeast. An alternative explanation is that tartaric acid could have been derived from other native fruits and flowers. Analyses of proto-historic vessels, from ca. 1900–700 BCE, indicates that these later wines were cereal-based using rice and millet. Thus, it now appears clear that fermented beverage technology in China began around the same time as in the Near East, and that the very nature of the fermentation evolved over several millennia.

Drunken monkeys An entirely new way to estimate when humans first began consuming ethanol-containing fermented products is based on the emerging field called "paleogenetics" (Carrigan et al., 2015; Thomson et al., 2005). According to this approach, scientists work backwards in time using ancient genetic material. Then, based on Darwinian principles, they can then predict when a particular genotype emerged in a population.

Ordinarily, ethanol is metabolized in the liver by two enzymes, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). If one of these enzymes is absent, even modest consumption of alcohol will result in alcohol toxicity. For individuals that express these enzymes, if more ethanol reaches the liver than can be handled by the normal metabolic pathway, the toxic intermediate, acetaldehyde, accumulates. This results, as many college students know, in headaches and nausea, i.e., hangovers.

Recently, scientists have shown that our primate ancestors harbored an ADH that was inactive against ethanol (Carrigan et al., 2015). However, a mutation in a specific ADH gene (ADH4) led to expression of a functional ADH. This mutation, they suggest, occurred millions of years ago, and correlated to when dietary sources of ethanol would have begun to appear naturally. This would have coincided with the early days of terrestrial life when fruits that had fallen from trees were subsequently fermented. Primates, that ventured from trees to eat the fallen fruit and that could tolerate the alcohol, had a selective nutritional advantage. Consequently, these researchers concluded that hominids had learned how to "handle" their drinking long before they intentionally began to make and consume alcoholic beverages. Indeed, as one commentator suggested, one reason why alcoholic fermentative technologies evolved in the first place was because of our "adaptive predilection for ethanol" (Dominy, 2015). In other words, our ancestors' acquired ability to tolerate ethanol led, for better or worse, first to human liking of alcohol, and eventually, to the development of brewing, wine, and other ethanol technologies.

References

Dominy, N.J. 2015. Ferment in the family tree. PNAS. 112:308–309.
Carrigan, M.A., O. Uryasev, C.B. Frye, B.L. Eckman, C.R. Myers, T.D. Hurley, and S.A. Benner. 2015. Hominids adapted to metabolize ethanol long before human-directed fermentation. Proc. Natl. Acad. Sci. USA. 112:458–463.
Guasch-Jané, M.R., M. Ibern-Gómez, C. Andrés-Lacueva, O. Jáuregui, and R.M. Lamuela-Raventós. 2004. Liquid chromatography with mass spectrometry in tandem mode applied for the identification of wine markers in residues from ancient Egyptian vessels. Anal. Chem. 76:1672–1677.
McGovern, P.E. 2003. Ancient Wine: the Search for the Origins of Viniculture. Princeton University Press. Princeton, New Jersey.
McGovern, P.E., J. Zhang, J. Tang, Z. Zhang, G.R. Hall, R.A. Moreau, A. Nuñez, E.D. Butrym, M.P. Richards, C.-S. Wang, G. Cheng, Z. Zhao, and C. Wang. 2004. Fermented beverages of pre- and proto-historic China. Proc. Nat. Acad. Sci. 101:17593–17598.
McGovern, P.E., B.P. Luley, N. Rovira, A. Mirzoian, M.P. Callahan, K.E. Smith, G.R. Hall, T. Davidson, and J.M. Henkin. 2013. Beginning of viniculture in France. Proc. Natl. Acad. Sci. USA. 110:10147–10152.
McGovern, P.E., A. Mirzoian, and G.R. Hall. 2009. Ancient Egyptian herbal wines. Proc. Natl. Acad. Sci. USA. 106:7361–7366.
Thomson, J. M., E.A. Gaucher, M.F. Burgan, D.W. De Kee, T. Li, J.P. Aris, and S.A. Benner. 2005. Resurrecting ancestral alcohol dehydrogenases from yeast. Nature Gen. 37:630–635.

Fermentation became an even more widespread practice during the Greco-Roman era, as new raw materials, technologies, and cuisines were adopted from conquered lands, and fermented foods spread throughout the region. Due to their increased storage stability, and in some cases, ease of transport, these foods were also important for distant armies and navies. Cheese, for example, would have been a more stable and compact source of nutrients compared to milk. In addition, beer and wine were often preferred over water. No surprise there, as the latter was often polluted with fecal or other foreign material. During the Roman Empire, the means to conduct trade developed. Thus, cheese and wine, as well as wheat for bread-making, became available around the Mediterranean, Europe, and the British Isles.

Although the organized manufacture of bread had existed even in ancient Egypt, by the Middle Ages, its manufacture, as well that of beer and cheese, became the province of craftsmen and organized guilds. The guild structure involved long training and apprenticeships. Once learned, these skills were often passed on to the next generation. For some products, particularly beer and wine, these craftsmen were often monks operating out of monasteries and churches, a tradition that lasted for hundreds of years. Indeed, many of the technologies and manufacturing practices employed even today were originally developed by monks. For example, the process used to achieve carbonation in Champagne, is credited to the Benedictine monk, Dom Pierre Pérignon, who lived 300 years ago. Eventually, the manufacture of these products became more privatized, although often under some form of state control, which conveniently allowed for taxation.

From the Neolithic Period, to the Middle Ages, to the current era, fermented foods had been among the most culturally and nutritionally important foods consumed by humans (Figure 1.1). An argument can be made that fermented foods, and the subsequent development of technologies for their production, directly contributed to the dietary, cultural, and social evolution of human history. Consider, after all, how integral fermented foods are to the diets and cuisines of nearly all civilizations and cultures and how many fermented foods and beverages are consumed as part of religious customs, rites, and rituals (Box 1.2).

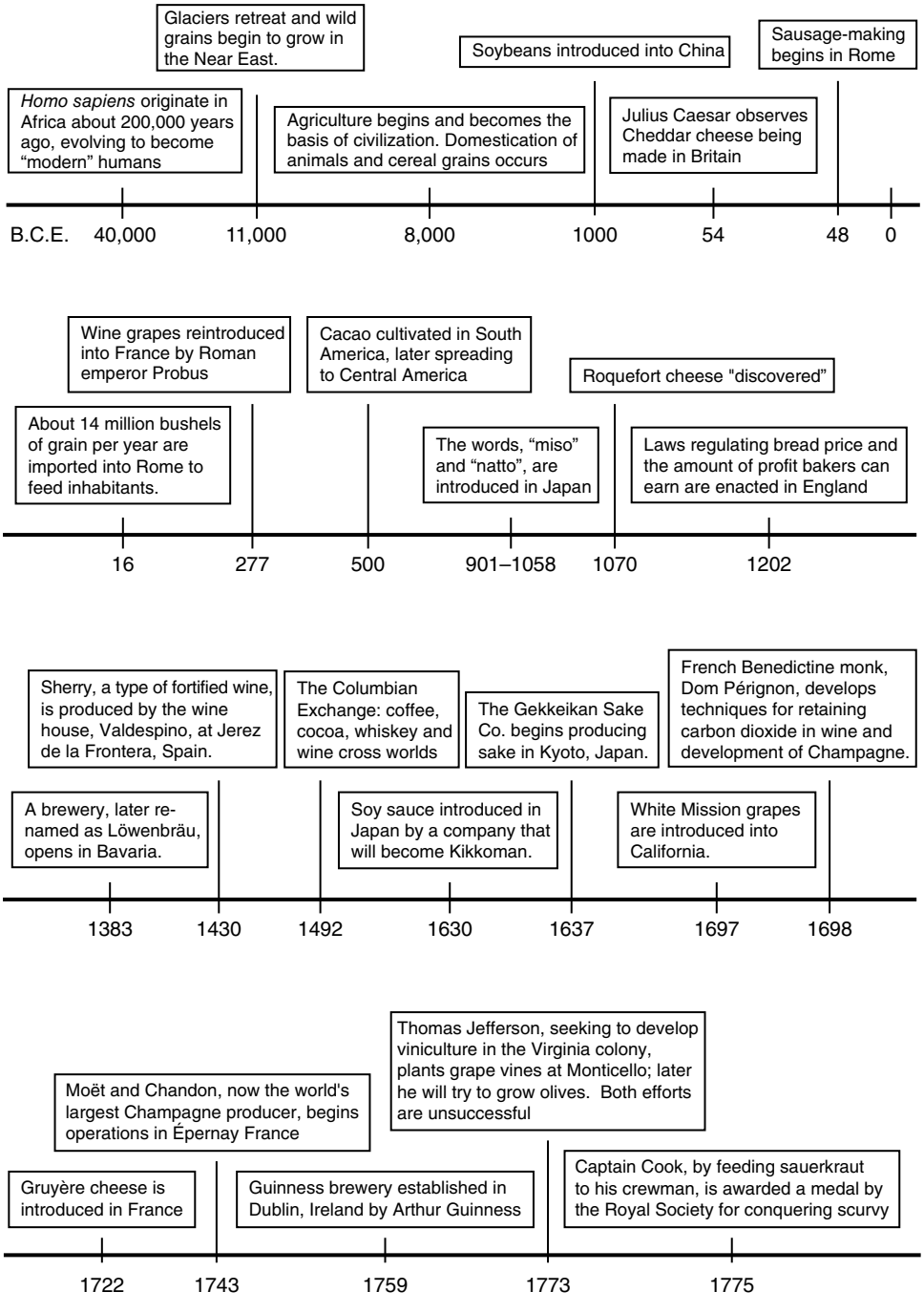


Figure 1.1 Important developments in the history of fermented foods. From Trager, 1995 and other sources.

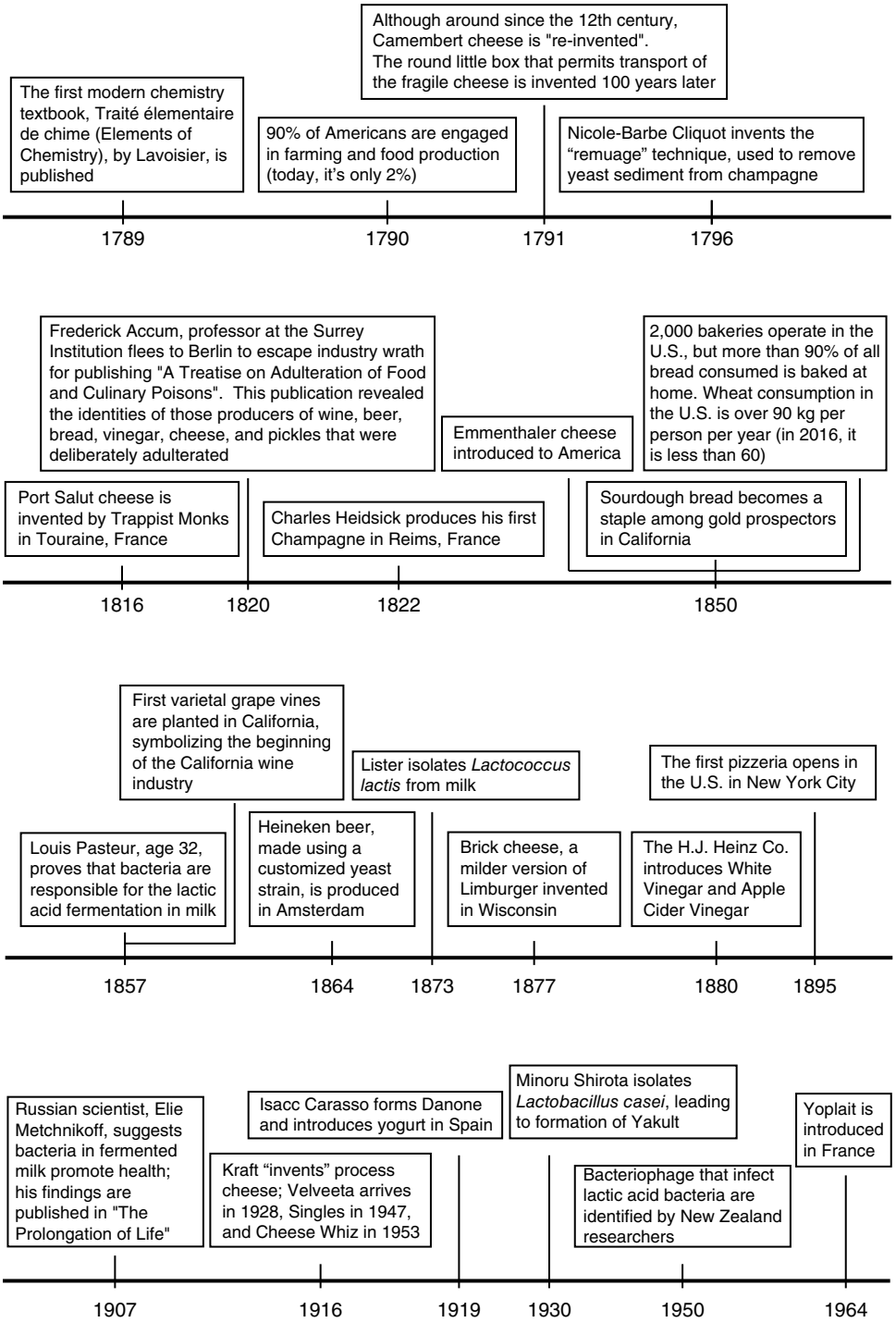


Figure 1.1 (*Continued*)

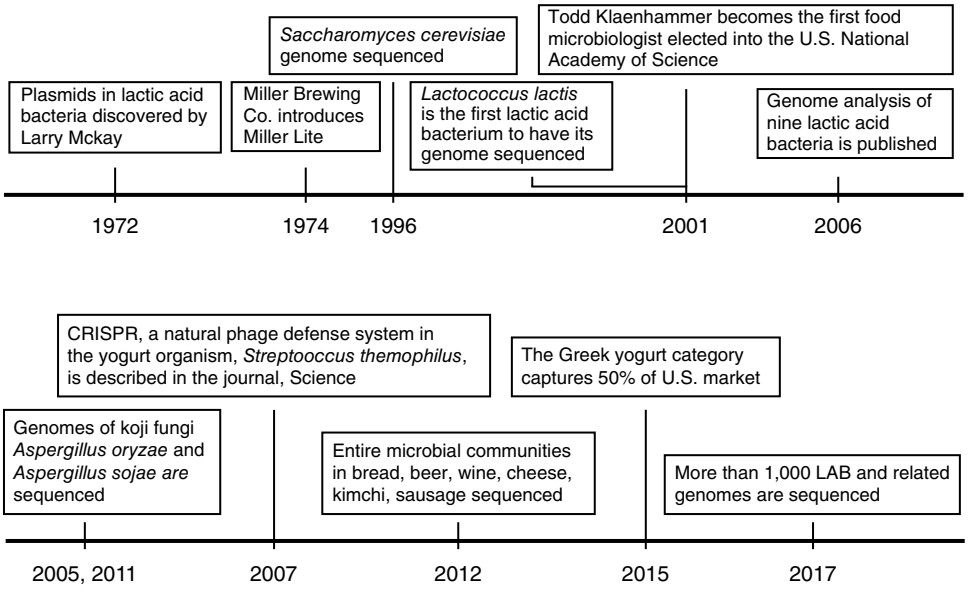


Figure 1.1 (*Continued*)

Box 1.2 Fermented foods and ancient texts

It is not much of an overstatement to argue that fermented foods and beverages have had a major impact on the cultural history of human societies. After all, bread – the "staff of life", and wine, "which cheers man's heart" – are among the earliest fermented foods. No wonder that references to these and other fermented foods are common in the early records of human history and civilization. Notably, the Bible, both Old and New Testaments, and other religious tracts, contain numerous references to fermented foods. In some sections, as indicated below, fermented foods are even the focal point.

Fermented foods, apart from their mention in the Bible, have also served a major role in ancient Eastern and Western mythologies. There were no scientific explanations to account for the unique sensory and often intoxicating properties of fermented foods. Therefore, these products were often described as "gifts of the gods". In Greek mythology, Dionysus was the god of wine, and Bacchus held this position in Roman mythology. The Iliad and the Odyssey, classic poems written by the Greek poet, Homer, in about 1150 BCE, also contain numerous references to wine, cheese, and bread. A hemisphere away, Korean and Japanese mythology also refers to the gods that provided miso and other Asian fermented foods (see Chapter 14).

Fermented foods and the Bible From the Genesis story of Eve and God's warning not to eat from "the tree of knowledge", to the dietary laws described in the books of Exodus and Leviticus, food serves a major metaphoric and thematic role throughout the Old Testament. Fermented foods, in particular, are often mentioned in biblical passages. Clearly, these foods must have already been well known to those

cultures and civilizations that lived during the time the bible was written. In Genesis (Chapter 9: Verse 20), the first action taken by Noah, after the flood waters had receded and he "went forth from the ark", was to plant a vineyard. It is instructive to note in the very next line that Noah drank enough wine to become intoxicated.

Just a few chapters later (Genesis 18:8), Abraham receives three strangers, presumably, angels, to whom he offers various refreshments, including milk "curds". The latter has been popularized as being yogurt, and perhaps Abraham's regular yogurt consumption accounted for his longevity of 175 years. Probably the most relevant reference to fermentation in the Bible is the story of Passover. The events described in Exodus culminate with the hasty departure of the Hebrew slaves (Chapter 12:39). Once Moses had secured their freedom from Pharaoh, they were "thrust out of Egypt, and could not tarry". There was not enough time for microorganisms to ferment, so the bread dough could not rise or become "leavened". Instead, it had to be baked in its "unleavened" state. This product, called matzoh, is still eaten today by people of the Jewish faith to commemorate, symbolically, the Hebrew exodus.

Ritual consumption of other fermented foods is also prescribed in Judaism. On the weekly Sabbath, the egg bread, Challah, is to be eaten, and either grapes or wine is drunk, preceded by appropriate blessings of praise.

Fermented foods are also featured prominently in the New Testament. At the wedding in Cana (John 2:1–11), Jesus' first miracle is to turn water into wine. Later (John 6:1–14), another miracle is performed when five loaves of bread, and two fish, provide sustenance for five thousand men. During the crucifixion, Jesus is given drops of vinegar. In Catholicism, the Sacrament of Holy Communion, described by Jesus during the Last Supper, is represented by bread and wine.

Given that fermented foods were a major part of the human diet during the biblical era, it is no surprise that the Old and New Testaments often refer to these staple foods.

FERMENTED FOODS: FROM ART TO SCIENCE

It may be difficult for the twenty-first century reader to imagine that fermented foods, whose manufacture relies on the intricate and often subtle participation of microorganisms, could have been produced without even the slightest notion that living organisms were involved. Likewise, neither could the early manufacturers of fermented foods and beverages have appreciated the science involved in their production, since it was only in the last 150 to 200 years that microorganisms and enzymes were "discovered". In fact, up until the middle of the nineteenth century, even many of the most prominent members of the scientific community still believed in the concept of spontaneous generation. The very act of fermentation was a subject for philosophers and alchemists, not biologists. Although the Dutch scientist, Antonie van Leeuwenhoek, had first observed microorganisms in his self-made microscope in 1675, the connection between Leeuwenhoek's "animalcules" and their biological or fermentative activities was only slowly realized. It was not until later in the next century when scientists began to address the question of how fermentation occurs.

Initially, chemists were among the first scientists to study the scientific basis for fermentation. With the exception of van Leeuwenhoek, microbiologists, weren't yet on the scene. By the late 1700s and early 1800s, the French chemists Antoine Lavoisier and Joseph Louis

Gay-Lussac had independently described the overall equations for the alcoholic fermentation.

Meanwhile, improvements in microscopy, had allowed Theodor Schwann and others to observe the presence of yeast cells in fermenting liquids, including beer and wine. These observations led Schwann to propose, in 1837 (as recounted by Barnett, 2003), that, "It is very probable that, by means of the development of the fungus, fermentation is started." Still, the suggestion that yeasts were actually responsible for fermentation was not widely accepted. Rather, it was argued by several of Schwann's prominent contemporaries that fermentation was caused by aerobic chemical reactions, with the involvement of suitable catalysts. Yeasts, they claimed, were merely inert precipitates, and had nothing to do with fermentative processes.

The debate over the role of microorganisms in fermentation was brought to an unequivocal conclusion by another chemist, Louis Pasteur. Writing in 1857, Pasteur stated that, "Fermentation, far from being a lifeless phenomenon, is a living process," that "correlates with the development of…cells and plants which I have prepared and studied in an isolated and pure state" (Schwartz, 2001). In other words, fermentation could only occur when microorganisms were present. The corollary was also true – that when fermentation was observed, growth of the microorganisms occurred. In a series of elegant, and now famous publications, Pasteur described details on lactic and ethanolic fermentations, including those relevant to milk, wine, and beer. He also identified the organism that causes the acetic acid (i.e., vinegar) fermentation that was responsible for wine spoilage.

The behavior of yeasts during aerobic and anaerobic growth also led to important discoveries in microbial physiology. For example, it was observed that when yeasts were exposed to air or oxygen during fermentation, glycolytic metabolism was abruptly inhibited, a phenomenon appropriately termed the Pasteur Effect. Eventually, the recognition that fermentations (and spoilage) were caused by microorganisms led Pasteur to work on other microbial problems, in particular, infectious diseases. Nonetheless, it was readily apparent from Pasteur's research that food spoilage and fermentation were both instigated by microorganisms. Future studies on food and beverage fermentations would be left to other scientists who had embraced this new field of microbiology.

Once the scientific basis of fermentation was established, microbiologists quickly realized the value in identifying and cultivating the yeasts and bacteria responsible for performing specific fermentations. Emil Christian Hansen, working at the Carlsberg brewery in Denmark, adopted the techniques and recommendations of Pasteur, Lister, Koch, and others to isolate a specific yeast strain from wild beer cultures. He used this strain to produce beer having consistent quality characteristics. The advantages of this approach were clearly evident, and by the end of the nineteenth century, breweries throughout Europe, the Americas, and Asia were using pure yeast strains.

Other fermented food industries that also relied on cultures appreciated the benefits of pure culture technology. Soon cultures for butter, cheese, and other dairy products became available. One of the main advantages of these cultures was their consistent performance, in contrast to the unpredictable outcomes that occurred when using so-called back-slopping techniques (Chapter 4). However, as the scale of these industries grew, the availability of concentrated cultures became important as well. Eventually, the dairy industry became one of the largest users of commercial cultures.

The specialized dairy culture supply "houses", formed at the turn of the century, not only sold cultures, they also sold enzymes, colors, and other products necessary for the manufacture of cheese and cultured milk products. Many cheese factories continued to propagate

their own "in-house" cultures throughout the first half of the twentieth century. However, as factory size and product throughput increased, the use of commercial dairy starter cultures eventually became commonplace. Likewise, commercial cultures for bread, wine, beer, and fermented meats became the norm for industries producing these products.

THE MODERN FERMENTED FOODS INDUSTRY

Like other segments of the food processing industry, the fermented foods industry has changed dramatically in the past several decades. Not only has the average size of a typical production facility increased many-fold, so has the rate, or throughput, at which raw materials are converted to finished product. Nonetheless, many small, traditional-style facilities still exist and not just in developing countries. They are common throughout Europe where traditional manufacturing procedures are often required or mandated for a variety of fermented foods. Small-to-medium scale manufacturing of these products has also made a comeback in the US, as is evident by the many craft breweries, boutique wineries, and artisanal bakeries and cheese operations that have opened in recent years. Despite these trends, however, the fermented foods industry is dominated by high-volume producers with large production capacity.

As the size and scale of the fermented food industry have increased in the past century, so has the fundamental manner in which fermented foods are produced (Table 1.1). For example, up until the past 60 or so years, most cheese produced world-wide was made using raw milk of Manufacturing or Grade B quality. Now, at least in the US, Canada, New Zealand, Australia, and parts of Europe, pasteurized Grade A milk, meeting higher quality standards, is more commonly used. Open, copper-lined cheese vats still exist, but modern manufacturing tanks and vats are often enclosed and constructed from stainless steel or other materials that facilitate cleaning and even sterilization treatments. From the outset, modern facilities are specifically designed with an emphasis on sanitation requirements so exposure to air-borne microorganisms and bacteriophage is minimized. In fact, the entire plant design, air-handling, and personnel access is considered in order to prevent inadvertent cross-contamination.

Many of the unit operations are now highly mechanized or automated. Other than a few keystrokes from a control panel, computer, or smart phone, modern manufacture of fermented foods often involves minimal human contact. More than ever before, fermented food

Table 1.1 The fermented foods industry: past and present.

Traditional	Modern
Small scale (craft industry)	Large scale (in factories)
Non-sterile medium	Heat-treated medium
Open and exposed	Closed and aseptic
Manual	Automated
Insensitive to time	Time-sensitive
Varying quality	Consistent quality
Safety not a major concern	Safety a major concern
No culture knowledge	Extensive culture knowledge

production is now subject to time and scheduling demands. In the not too distant past, if an industrial cheese or yogurt fermentation was slow or sluggish, it simply meant that the workers (who may have been family members) would be late for supper, and little else. In the current high-volume production environment, a slow or delayed fermentation could mean that workers would need to stay beyond their shift, and be paid overtime. In many cases, the entire production schedule might be affected, since the production vats would not be emptied and refilled as quickly as needed.

Traditional manufacturing practices may not have always yielded products having consistent quality characteristics, although lot sizes were generally small. Thus, economic losses due to an occasional misstep were not likely to be too serious. Besides, it was probably often the case that for every cask of wine or wheel of cheese considered inferior, or not quite up to the expected standard, there would have been an equally spectacular lot compensating for the ones that turned out badly. Consider the absolute worst case scenario – a food poisoning outbreak occurs due to an improperly manufactured product. Such an event, however costly, would likely have been limited in geographical scope, as well as number of cases, due to the narrow distribution range and small production volume. Continuing these practices today, however, is simply beyond consideration for most major food manufacturers. A day's worth of production may be worth tens, if not hundreds of thousands of dollars, in raw materials cost alone. There is simply no way a producer could adsorb or tolerate such losses, even if they occurred only sporadically.

Food safety, in particular, has become an international priority. Minimizing risks associated with food borne pathogens, or other food safety issues, is a major goal for the fermented foods industry. Depending on the country or region, there may be zero tolerance for pathogens and other hazards in fermented foods. Quality assurance programs now exist throughout the entire food industry with the major goal being to produce safe food products on a consistent and predictable basis.

In essence, the fermented foods industry has evolved from a mostly art- or craft-based practice to one that relies on modern science and technology. The emphasis on safety, sanitation, quality, and consistency, applies to all processed foods, not just fermented foods. What is uniquely different about fermented foods, compared to other foods, is that the manufacture of fermented foods relies on biologically alive ingredients, namely the cultures. Therefore, it is the only food processing industry in which product success depends on the presence, growth, and activity of microorganisms. If a particular trait is somehow lost, the cells become less viable, or the culture is infected by viruses or inhibited by other environmental factors, the fermentation may fail and product quality and safety may suffer. For fermented products for which food safety is a concern, many are made from raw substrates without the benefit of a thermal process or "kill step". Although salt and other anti-microbial agents may be added, control of pathogens is primarily achieved biologically. Thus, the implications are highly significant.

In contrast to flavoring, nutritional, or functional food ingredients, microorganisms used to initiate fermentations are not easily standardized. This is because their viability, biochemical activity, and even their concentration (number of cells per unit volume) may fluctuate from lot to lot, or during storage. Although custom-made starter cultures standardized for cell number and activity are widely available, many industrial fermentations still rely on the presence of naturally-occurring microorganisms. The composition and biological activities of this indigenous microbiota are often subject to considerable variation. In addition, microorganisms are often exposed to a variety of inhibitory chemical and biological agents in the food or environment that can compromise their viability and

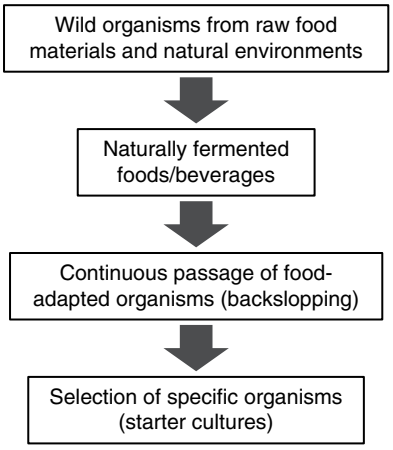


Figure 1.2 Domestication of cultures, from wild to modern. Adapted from Gibbons and Rinker, 2015, with permission.

activity. Since the culture organisms, whether added or indigenous, are often the main means by which spoilage and pathogenic microorganisms are controlled in fermented foods, their role in ensuring food safety and quality cannot be overstated. If the culture fails to perform in an effective and timely manner, the finished product will be subject to spoilage, quality defects, or worse. Thus, the challenge confronting the fermented foods industry is to manufacture products whose very production is subject to inherent variability, yet still satisfy the modern era demands of consistency, quality, through-put, and safety. It is no surprise that fermentation organisms (and starter cultures) have essentially been domesticated such that their performance is predictable and reliable (Figure 1.2). The co-evolution of *Saccharomyces* and fermented foods is a perfect example of microbe domestication, given that similar but distinct strains are used in bread, beer, and wine (Box 1.3).

Box 1.3 Yeasts – Man's best friend

The domestication of plants and animals for the purpose of producing food was arguably one of the most important events in human history. Without the plentiful food supply provided by agriculture, human civilizations would not have developed. Fermented foods, particularly bread, beer, and wine, were among the foods first produced by early civilizations some 10,000 years ago. As will be described in subsequent chapters, all of these products require yeasts, namely *Saccharomyces cerevisiae*, for their production. That the manufacture of these products depends on specific yeasts suggests that relevant strains may have co-evolved in parallel with human activities. Recent research on the evolution of *S. cerevisiae* suggest that domestication of wild strains emerged at nearly the same time when plants and animals were domesticated, and when fermented foods were first produced by humans (Sicard and Legras, 2011; Steensels and Verstrepen, 2014). These foods would rely entirely on natural or

"spontaneous" fermentations. Eventually, the practice of backslopping, intentional passage of "cultures" from one batch to the next, was adopted. This would have led to selection of adapted strains that were truly "domesticated" (Gibbons and Rinker, 2015).

Researchers have also been able to trace the spread of *S. cerevisiae*, geographically and temporally, by comparing the genotypes of hundreds of bread, beer, and wine strains collected around the world (Legras et al., 2007). In this study, genotypes were based on the sequences of microsatellite DNA, regions of yeast DNA that serve as molecular fingerprints. These sequences are also useful for establishing relatedness of different strains. The results showed there were more than 550 distinct genotypes among the 651 strains, but there was considerable relatedness, as well. Nonetheless, strains could still be clustered based on their origin. Thus, it's possible to consider S. cerevisiae strains as bread strains, beer strains, or wine strains, albeit with some overlap. There were also geographical differences, suggesting that some strains had been domesticated locally.

Beyond describing the differences between different strains of *S. cerevisiae*, researchers have also been interested in the evolution of *S. cerevisiae* (Liti et al., 2009). That is, how and when did *S. cerevisiae* strains become specialized or adapted to the different habitats with which they are associated? Researchers have suggested that the phenotypic properties of different extant, i.e., contemporary yeasts strains reflect their adaptation to different environments (Liti et al., 2009). Genetic rearrangements and duplications lead to new alleles and ultimately new phenotypes that are well-suited for growth in specialized habitats (Ibáñez et al., 2014). Although these present-day strains sort within well-defined lineages, they also have a "mosaic" genetic structure. That is, they harbor lots of genes or sequences from many sources, reflecting centuries of cross-breeding.

Ultimately, by practicing fermentation science for several millennia, humans have essentially shaped the genome of *S. cerevisiae* (Ibáñez et al., 2014). In fact, the domestication of wild yeast is not unlike the domestication of man's "other" best friend.

References

Gibbons, J.G., and D.C. Rinker. 2015. The genomics of microbial domestication in the fermented food environment. Curr. Opinion Genet. Develop. 35:1–8.

Ibáñez, C., R. Pérez-Torrado, R. Chiva, J.M. Guillamón, E. Barrio, and A. Querol. 2014.Comparative genomic analysis of *Saccharomyces cerevisiae* yeasts isolated from fermentations of traditional beverages unveils different adaptive strategies. Int. J. Food Microbiol. 171:129–135.

Legras, J.-L., D. Merdinoglu, J.-M. Cornuet, and F. Karst. 2007. Bread, beer and wine: *Saccharomyces cerevisiae* diversity reflects human history. Mol. Ecol. 16:2091–2102.

Liti, G., Carter, D.M., Moses, A.M., Warringer, J., Parts, L., James, S.A., Davey, R.P., Roberts, I.N., Burt, A., Koufopanou, V., Tsai, I.J., Bergman, C.M., Bensasson, D., O'Kelly, M.J.T., van Oudenaarden, A., Barton, D.B.H., Bailes, E., Nguyen, A.N., Jones, M., Quail, M.A., Goodhead, I., Sims, S., Smith, F., Blomberg, A., Durbin, R., Louis, E.J., 2009. Population genomics of domestic and wild yeasts. Nature 458:337–341.

Sicard, D., and J.-L. Legras. 2011. Bread, beer and wine: yeast domestication in the *Saccharomyces sensu stricto* complex. Comptes Rendus Biol. 334:229–236.

Steensels, J. and K.J. Verstrepen. 2014. Taming wild yeast: potential of conventional and nonconventional yeasts in industrial fermentations. Annu. Rev. Microbiol. 68:61–80.

PROPERTIES OF FERMENTED FOODS

As suggested in the previous discussion, fermented foods were likely among the first "processed" foods produced and consumed by humans. The reasons for their popularity, more than 5000 years ago are, for the most part, the same reasons why they continue to be popular today (Table 1.2).

Preservation

Fermented foods were observed to be well preserved, at least compared to the starting materials. This was obviously important thousands of years ago, when few other preservation techniques existed. A raw food material such as milk or meat had to be consumed shortly after it was collected or it would soon spoil, especially in temperate climates. Although salting, smoking, or drying could have been used for some foods, fermentation must have been an attractive alternative, due to other desirable features, noted below. Preservation was undoubtedly one of the main reasons why fermented foods became such an integral part of the human diet and contributed to the cuisines of nearly every culture on every continent. However, even today, preservation and enhanced shelf-life remain as important properties of fermented foods. As described in subsequent chapter, cultures now exist that not only perform the normal fermentation, but that also produce specific antimicrobial agents in the food. Such cultures have the added benefit of providing an extra margin of food safety and extended shelf-life.

Nutrition

Most fermented foods are made from nutritionally-rich raw materials, such as milk, meat, rice, soybeans, and wheat. Additional nutritional value has long been attributed to fermented foods, even though the scientific basis for many of the nutritional claims have, until recently, not been well established. Evidence that fermentation may enhance nutritional properties now exists for several fermented products.

The nutritional case for yogurt is particularly interesting. In much of the world, fluid milk is not regularly consumed because most people are unable to produce the enzyme β-galactosidase which is necessary for digestion of lactose, the main sugar present in milk. Lactose malabsorption is the physiological condition that occurs when individuals deficient in β-galactosidase production consume lactose. A variety of unpleasant, though non-life-threatening symptoms are associated with this disorder. These include mild-to-severe intestinal distress, bloating, diarrhea, and cramps. This condition is especially common among Asian, African, and Middle Eastern populations, although adult Caucasians may also

Table 1.2 Properties of fermented foods.

- Enhanced preservation
- Enhanced nutritional value
- Enhanced functionality
- Enhanced organoleptic properties
- Uniqueness
- Increased economic value

be lactose intolerant, and many individuals from these regions consume dairy products without ill effects. Interestingly, numerous studies have revealed that lactose-intolerant subjects can consume yogurt without any untoward symptoms and can therefore obtain the nutritional benefits (e.g., calcium, high quality protein, and B vitamins) contained in milk. The metabolic and physiological hows and whys to explain these observations will be described in more detail in Chapter 3.

Wine is another great example of a fermented product that appears to be nutritionally superior compared to the raw material from which it was derived. There is now a large volume of literature showing that wine contains components, other than ethanol, that may contribute to enhanced health (Chapter 10). Specifically, resveratrol and several other phenolic compounds have been identified in red wine and shown to have anti-oxidant activities that may reduce the risk of heart disease and cancer. Wine, as well as other fermented foods, are widely consumed in Mediterranean countries where mortality rates due to heart disease are generally lower than in other regions. This has led to the suggestion that a "Mediterranean diet" may be ideal for human health.

Finally, it has been suggested that there may be health benefits associated with consumption of fermented foods that extend beyond the macronutrients or metabolic end-products produced by fermentative microorganisms. Specifically, the very microorganisms that perform the actual fermentation are now thought to contribute to human health, especially in the gastrointestinal tract. Yogurt and cultured dairy products have attracted the most interest as carriers of desirable microorganisms. Many other fermented foods, including fermented vegetables, cereals, and soy products may also harbor beneficial organisms, as well. Even extra-intestinal benefits, including mental health, cognition, and acuity, have been suggested to result from consumption of fermented foods (Box 1.4). The nutritional importance of fermented foods has become so widely appreciated that their consumption is now being recommended as part of a healthy diet. Perhaps the US MyPlate, Canada's Food Guide, the Chinese Food Guide Pagoda, or other guideline will eventually include fermented foods (Figure 1.3). As recently suggested by one group of nutrition researchers, "knowing the general benefits of traditional and supplemented fermented foods, they should be a daily item on most national food guides" (Bell et al., 2017).

Box 1.4 Fermented foods, mental health, and the gut–brain axis

As noted elsewhere in this chapter, there are many reasons accounting for why fermented food have remained popular from ancient times to the present. In particular, there are many nutritional advantages humans have gained by consuming fermented foods. Fermented foods, like cheese, sauerkraut, and sausage provide a stable source of vitamins, minerals and other nutrients. Wine and other fermented beverages are enriched in polyphenol compounds that have antioxidant and other biological activities. Yogurt, miso, and kimchi contain populations of live microorganisms that contribute to intestinal health.

Recently, several research groups have suggested that the nutritional benefits of fermented foods extend beyond physical health (Selhub et al., 2014; Dasha et al., 2015; Hilimire et al., 2015; Logan et al., 2015). Specifically, they have proposed that depression, mood, anxiety, cognition, and behavior are affected by diet and that fermented foods, in particular, can improve mental health. Data to support this hypothesis is based, in part, on epidemiological studies and self-reported symptoms. Several

studies have shown that traditional or Mediterranean diets that are naturally rich in fermented foods reduces the risk of depression, compared to high sugar, high fat Western diets (Lai, et al., 2014; Skarupski et al., 2013; Jacka et al., 2010).

There is also physiological data showing that the molecules produced by the gut microbiota may act as neurological signals to the brain. This gut–brain axis has emerged as one of the most intriguing and exciting new areas in food science and gut biology. It should be noted that the diet–gut–brain connection is not entirely new – suggested linkages date all the way back to Metchnikoff (Orla-Jensen et al., 1945). What is new is that this hypothesis is now supported by considerable evidence, and several possible mechanisms may explain how the microbiota, and fermented food, could influence mental health and well-being (Dinan and Cryan, 2017; Kennedy et al., 2016).

First, fermentation may result in formation of biologically active end-products, *in situ*, including those having anti-oxidant activity (Wang et al., 2006; Wang et al., 2014). Depending on the substrate (i.e., soy, bran, or dairy), fermented foods may also contribute particular dietary components. In other products, it's not the fermented foods, per se, that are responsible for the suggested mental health benefits, but rather the ingested microorganisms that reach the colon. This would include bacteria that are either added directly as starter cultures, like in yogurt, or those present as part of the normal microbiota, like in fermented vegetables. Germ-free mice, i.e., raised without a microbiota, have reduced anxiety-like behavior (Foster and McVey Neufeld, 2013). Bacteria added to fermented foods for their probiotic activities could also contribute to mental health benefits (Tillisch et al., 2013). Likewise, prebiotics naturally present or added to fermented foods could shift the microbiota toward a stress-reducing phenotype (Burokas et al., 2017).

Studies have shown that bacterial products produced in the gut could activate the central nervous system or modulate the immune system. For example, the neurotransmitter, gamma-aminobutyric acid (GABA) is produced by *Lactobacillus* and *Bifidobacterium* species, resulting in lower stress levels in mice (Bravo et al., 2011). Recently, another mechanism has been hypothesized to account for how the gut microbiota affects mental health, and autism, in particular (Reddy and Saier, 2015; Thakur et al., 2014). Briefly, these authors suggested that an altered gastrointestinal microbiota may affect gut epithelial barrier function. Specifically, a dysbiotic microbiota can lead to increased gut permeability, or leaky gut syndrome. Ultimately, microbial end-products reach the bloodstream, resulting in metabolic endotoxemia and inflammation. Leaky gut syndrome has been associated with gastrointestinal disorders, as well as several neurological conditions, including autism.

References

Bravo, J.A., P. Forsythe, M.V. Chew, E. Escaravage, H.M Savignac, T.G. Dinan, J. Bienenstock, and F. Cryan. 2011. Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc. Natl. Acad. Sci. USA. 108:16050–16055.

Burokas, A., S. Arboleya, R.D. Moloney, V.L. Peterson, K. Murphy, G. Clarke, C. Stanton, T.G. Dinan, and J.F. Cryan. 2017. Targeting the microbiota–gut–brain axis: prebiotics have anxiolytic and antidepressant-like effects and reverse the impact of chronic stress in mice. Biol. Psychiatry. 82:472–487.

Dasha, S., G. Clarkeb, M. Berka, and F.N. Jacka. 2015. The gut microbiome and diet in psychiatry: focus on depression. Curr. Opin. Psychiatry 28:1–6.
Dinan, T.G. and J.F. Cryan. 2017. Brain-gut-microbiota axis and mental health. Psychosom. Med.
Foster, J.A., and K.A. McVey Neufeld. 2013. Gut-brain axis: how the microbiome influences anxiety and depression. Trends Neurosci. 36:305–312.
Hilimire, M.R., J.E. Devylder, and C.A. Forestell. 2015. Fermented foods, neuroticism, and social anxiety: an interaction model. Psychiatry Res. 228:203–208.
Jacka, F.N., J.A. Pasco, A. Mykletun, L.J. Williams, A.M. Hodge, S.L. O'Reilly, G.C. Nicholson, M.A. Kotowicz, and M. Berk. 2010. Association of Western and traditional diets with depression and anxiety in women. Am. J. Psychiatry. 167:305–311.
Kennedy, P.J., A.B. Murphy, J.F. Cryan, P.R. Ross, T.G. Dinan, and C. Stanton. 2016. Microbiome in brain function and mental health. Trends Food Sci. Technol. 57: 289-301.
Lai, J.S., S. Hiles, A. Bisquera, A.J. Hure, M. McEvoy, and J. Attia. 2014. A systematic review and meta-analysis of dietary patterns and depression in community-dwelling adults. Am. J. Clin. Nutr. 99:181–197.
Logan, A.C., M.A. Katzman, and V. Balanzá-Martínez. 2015. Natural environments, ancestral diets, and microbial ecology: is there a modern "paleo-deficit disorder"? Part II. J. Physiol. Anthropol. 34:1.
Neufeld, K.M., N. Kang, J. Bienenstock, and J.A. Foster JA. 2001. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. Neurogastroenterol. Motil. 23:255-264 e119.
Orla-Jensen, S, E. Olsen, and T. Geill. 1945. Senility and intestinal flora – a reexamination of Metchnikoff's hypothesis. Ejnar Munksgaard, Copenhagen.
Reddy, M.L. and M.H. Saier, Jr., 2015. Autism and our intestinal microbiota. J. Mol. Microbiol. Biotechnol. 25:51–55.
Selhub, E.M., A.C. Logan, and A.C. Bested. 2014. Fermented foods, microbiota, and mental health: ancient practice meets nutritional psychiatry. J Physiol. Anthropol. 33:2.
Skarupski, K.A., C.C. Tangney, H. Li, D.A. Evans, and M.C. Morris. 2013. Mediterranean diet and depressive symptoms among older adults over time. J. Nutr. Health Aging 17:441–445.
Thakur, A.K., A. Shakya, G.M. Husain, M. Emerald, and V. Kumar. 2014. Gut microbiota and mental health: current and future perspectives. J. Pharmacol. Clin. Toxicol. 2:1016.
Tillisch, K., J. Labus, and L. Kilpatrick. 2013. Consumption of fermented milk product with probiotic modulates brain-response to emotional pictures. Gastroenterology 144:1394–1401.
Wang, Y.C., R.C. Yu, and C.C. Chou. 2006. Antioxidative activities of soymilk fermented with lactic acid bacteria and bifidobacteria. Food Microbiol. 23:128–135.
Wang, C.-Y., S.-J Wu, and Y.T. Shyu. 2014. Antioxidant properties of certain cereals as affected by food-grade bacteria fermentation. J. Biosci. Bioeng. 117:449–456.

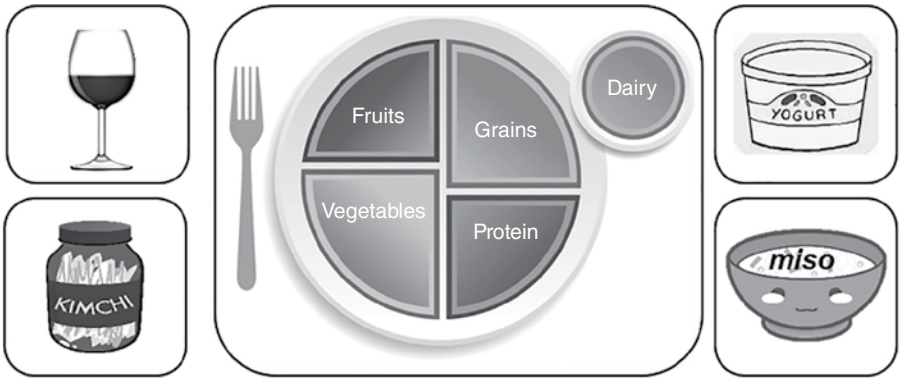


Figure 1.3 Possible future nutritional guideline: "Fermented foods, including those that contain live microorganisms, should be included as part of a healthy diet". Adapted from the USDA's MyPlate promotional program.

Functionality

Most fermented foods differ nutritionally from the raw materials of which they are made. Also, most are dramatically different with respect to their functional properties. Cheese, for example, differs in function, form, and flavor from milk. Functional enhancement is perhaps most evident for bread and beer. When humans first began some 10,000 years ago to use wheat as food there was little they could do with the flour, except to make simple doughs and flat breads. Yet allowing the dough to ferment and leaven prior to baking, would transform the flour into flavorful and expansive breads.

Barley was another widespread grain in the ancient world and was often used in foods. However, compared to wheat, low gluten content of barley limited its functionality in bread. Barley did become important in another fermented product – beer (and later whisky). Given that barley is the main ingredient, other than water, in the manufacture of beer, it can reasonably be argued that beer is the best example of enhanced functionality due to fermentation.

Organoleptic

Although preservation, nutrition, and functionality were likely responsible for the early adoption and eventual spread of fermented foods throughout the world, there was another important reason for the success of these foods. Simply, fermented foods, must have tasted, smelled, and looked dramatically different from the starting raw materials. Many of these differences would have certainly been duly appreciated, then as now. While individuals that do not particularly care for aromatic Limburger cheese, fermented fish sauce, or a viscous soybean product called natto might argue that those differences are for the worse, there is little argument that fermented foods have aroma, flavor, and appearance attributes that are quite unlike the raw materials from which they are made. For those individuals who partake and appreciate Limburger cheese, the sensory characteristics between the cheese and the milk make all the difference in the world.

Uniqueness

With few exceptions (see below), there is simply no way to make fermented foods without the fermentation process. The hole or eyes, as well as complex flavors, of Swiss and Gouda cheese cannot be formed mechanically or by any other means, other than fermentation. Likewise, dry cured salami, Champagne, Pilsner beer, Balsamic vinegar, and kimchi cannot be produced any other way. For many fermented products, even the procedures used for their manufacture are unique, requiring strict adherence (Box 1.5). Parmesan cheese, for example, must be made in a defined region of Italy, from milk obtained from specific breeds of cows, manufactured according to traditional and established procedures, and then aged under specified conditions. The finished product must also meet quality requirements. Any deviation results in forfeiture of the name Parmesan.

It is possible, nonetheless, to manufacture “fermented” foods, such as certain fresh cheeses, sausages, and even soy sauce, without fermentation. The manufacture of these products generally involves direct addition of acids, enzymes, or flavors to simulate the activities normally performed by fermentative microorganisms. These products, which the purist might be inclined to dismiss from further discussion, generally lack the flavor and overall organoleptic properties of their traditional fermented counterparts.

Box 1.5 Fermented foods of protected origins

For thousands of years, the manufacture of fermented foods was based on experience, skill, art, and sometimes luck. Science, and microbiology and biochemistry, in particular, did not enter the scene until the nineteenth century. From identifying the microorganisms responsible for fermentation, to establishing fermentation pathways, to sequencing entire microbial communities found in fermented foods, much has been learned in just over a century. Moreover, engineering and processing applications have led to automation, robotics, and other technologies for producing fermented foods on a large scale with minimal human labor.

Despite these remarkable advances, many fermented foods and beverages are still made via centuries old manufacturing practices. In fact, traditional manufacture of fermented food is codified in several regions, most notably in Europe (see below). Thus, the milk used for traditional cheeses is usually raw, fermented sausages are dried, not cooked, and ingredients are limited to an essential few. For some products, starter cultures cannot even be used. Geography is also of utmost importance, and is often the major determinant for these products.

Official origins The official criteria for such foods depends on the region. In the European Commission, three categories exist. To obtain "Protected Designation of Origin" (PDO) status, the product must be "produced, processed and prepared in a given geographical area using recognized know-how". Products that have Protected Geographical Indication (PGI) status are "closely linked to the geographical area" and "at least one of the stages of production, processing or preparation takes place in the area." Finally, "Traditional Speciality Guaranteed" (TSG) products simply have "traditional character, either in the composition or means of production". To achieve one of the designations, manufacturers must follow a rather long and detailed application process, which is subsequently subject to careful review by experts. Nonetheless, hundreds of products have received PDO status. Foods or beverages that are made similar to a PDO product, but either with production deviations or made outside the designated areas cannot be labeled as the PDO product. Thus, Parmesan cheese can only be made in the designated Parma-Reggio regions in Italy.

The case of wine There are also country-specific designation in Europe and elsewhere. In France, for example, the wine industry has long followed strict regulations, called the "appellation d'origine controlee" (AOC), for naming wine. These regulations are based on region and method of manufacture. Similar systems exist in Italy ("denominazione di origine controllata or DOC") and throughout the EC. In the US, wines can be geographically labeled, and in Tennessee, bourbon whiskey is also a protected name ("Tennessee whisky"), provided it is made in Tennessee according to specified procedures.

Although it might strike twenty-first century readers as rather odd for manufacturers to make cheese, sausage, beer, and wine without taking advantage of modern science and engineering, there are several reasons why these industries, as well as consumers and governments, support the PDO system. First, the PDO system provides consumers with the confidence that the product was made according to strict

quality standards. For example, the Parmesan cheese mentioned above, must be made in small copper-lined vats using whey as the culture and calf rennet as the coagulant. The cheese must be aged at least 12 months, and wheels are inspected and graded. Thus, consumers have a pretty good idea what to expect when they buy Parmesan cheese.

Likewise, PDO, PGI, and TSG designations provide manufacturers (and the region and country) with considerable market advantages. The value for products with terroir or "geographical indications" (GI) is over $50 billion (Capozzi and Spano, 2011). Consider, for example, the branding associated with the aforementioned Parmesan, or Roquefort and Feta cheeses, Black Forest ham, or lambic beer. In the wine industry, there is hardly a brand more valuable than "Champagne". Importantly, in addition to regional protections, many of these GI products are also recognized internationally by treaty or trade agreements.

Terroir The concept of terroir is central to the GI designation, and refers to the collection of environmental factors that contribute to product quality. Temperature, rain, elevation, soil composition, cultivar or breed, and human interventions are among the elements that account for terroir. In the past decade, researchers have proposed that the microbial community residing in foods is a major contributor to terroir, as well (Capozzi and Spano, 2011; Felder et al., 2012; Bokulich et al., 2014). This so-called microbial terroir is as specific to province and as influential to product quality as are other environmental factors. Thus, not just wine, but cheese and other fermented foods have also been suggested to possess a microbial terroir (Paxson and Helmreich, 2014). Recently, researchers examined the microbial communities from cheeses obtained from disparate regions (Wolfe et al., 2014). Even though the microbiotas were similar and reproducible, human interventions, i.e., handling of the cheese by different cheese makers, apparently account for the variations observed in these products.

Ultimately, the tools of molecular ecology can be applied to even traditionally-made fermented food products, providing a basis for understanding and improving product quality (Cocolin and Ercolini, 2015).

References

Bokulich, N.A., J.H. Thorngate, P.M. Richardson, and D.A. Mills. 2014. Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. Proc. Natl. Acad. Sci. USA. 111:E139–E148.

Capozzi, V. and G. Spano. 2011. Food microbial biodiversity and "microbes of protected origin". Front. Microbiol. 2:1–3.

Cocolin, L. and D. Ercolini. 2015. Zooming into food-associated microbial consortia: a "cultural" evolution. Curr. Opin Food Sci. 2:43–50.

Felder, D. D. Burns, and D. Chang. 2012. Defining microbial terroir: the use of native fungi for the study of traditional fermentative processes. Int. J. Gastron. Food Sci. 1:64–69.

Paxson, H. and S. Helmreich. 2014. The perils and promises of microbial abundance: Novel natures and model ecosystems, from artisanal cheese to alien seas. Soc. Stud. Sci. 44:165–193.

Wolfe, B.E., J.E. Button, M. Santarelli, and R.J. Dutton. 2014. Cheese rind communities provide tractable systems for in situ and in vitro studies of microbial diversity. Cell 158:422–433.

ECONOMIC VALUE

For food scientists, "value-added" refers to foods to which inexpensive or "low value" starting materials are converted or processed to yield "higher value" products. Thus, corn plus extrusion, plus sugar, plus other ingredients, becomes corn flakes. Fermented foods, made primarily by the addition and manipulation of the appropriate microorganisms, are the original members of the "value-added" category. While milk is milk, one can add the relevant culture and enzymes, manipulate the curds in a particular manner, age it for just the right time, and the result may be a fine cheese that fetches a price well above the combined costs of the raw materials, labor, and other expenses. Grapes are grapes, but if they are cultivated, harvested, and crushed in a particular environment under precise conditions, and then the juice is allowed to ferment and mature under optimal conditions, then voila – the finished product will be worth far more than the original grapes. In reality, the economic value of fermented foods, especially fermented grapes, can reach dizzying heights. As noted in Chapter 11, there are wines listed on various wine-buying internet sites with price tags of more than $20,000 a bottle. In 2010, a bottle of Cheval Blanc 1947, from the Bordeaux region of France sold for over $300,000. Granted, it was an "imperial" bottle that is the equivalent of eight normal bottles. There are even some specialty vinegars (Chapter 12) that sell for over $200 for just 100 ml.

Not all fermented foods command such a high dollar value. The fermented foods market is just as competitive, and manufacturers are under the same market pressures as other segments of the food industry. For the most part, fermented foods are made from inexpensive commodities – wheat, milk, meat, grapes – and most commercial products have modest profit margins. Some products, such as non-aged cheese, are sold on commodity markets, with very tight margins. Other products have long aging periods with uncertain outcomes. The challenge in making fermented foods a profitable enterprise is perhaps best epitomized by the well-known joke about the wine business – "How do you make a million dollars in the wine industry? Easy, first you start with two million dollars."

Finally, not only is there an economic incentive for both small and large manufacturers of fermented foods, but on an industry-wide basis, fermented foods may have a substantial economic impact on a region, state or even a country. In California, for example, the wine industry was reported to contribute nearly 800,000 jobs and more than $110 billion to the US economy in 2016 (according to a Wine Institute report; www.wineinstitute.org). A similar analysis of the US beer industry (www.beerinstitute.org) reported a combined overall annual impact of more than $350 billion to the US economy in 2016.

FERMENTED FOODS IN THE TWENTY-FIRST CENTURY

Archaeological evidence has shown that for 10,000 years, humans have consumed fermented foods. As noted above, throughout human history, fermentation provided a means for producing safe, nutritious, and well-preserved foods. Even today, fermented foods are still among the most popular type of food consumed. No wonder that about one-third of all foods consumed are fermented. In the United States, beer is the most widely consumed fermented food product, followed by bread, cheese, wine, and yogurt (Table 1.3). While global statistics are incomplete, it can be estimated that alcoholic products are the most popular fermented foods in much of the world. In Asia, soy sauce production and consumption ranks at or near the top. Collectively, the sales volume of fermented foods, on a global basis, exceeds a

Table 1.3 Consumption of selected fermented foods and beverages[1].

Product	United States	International
Wine[2]	11 L	44 L (Croatia)
Beer[3]	75 L	142 L (Czech republic)
Cheese[4]	16 kg	27 kg (France)
Yogurt[5]	7 kg	36 kg (Netherlands)
Fermented meats[6]	0.3 kg	5.5 kg (Germany)
Bread[7]	25 kg	120 kg (Turkey)
Table olives[8]	0.6 kg	1 kg (Albania)
Kimchi[9]	–	35 kg (Korea)
Soy sauce[10]	1 L	6 L (Japan)

[1] Per person, per year.
[2] Wine Institute (updated 2015).
[3] Kirin Beer University Report for 2015.
[4] International Dairy Federation and USDA (data from 2014).
[5] Euromonitor 2013, AC Nielsen.
[6] Lücke and Zangerl, 2014 (data from 2008).
[7] International Association of Plant Bakers Bread Market Report (data from 2013) and the National Association of Manufacturers of Pan (Adepan), 2012.
[8] Data for 2015, International Olive Council.
[9] Data for 2015, Park, J., and H.-J. Lee, 2017. Korean J. Community Nutr. 22:145–158.
[10] Japan Federation of Soy Sauce Manufactures Cooperatives; Nakadai, 2015, and Mikio Bakke, personal communication, data for 2014.

trillion dollars. When jobs, tax revenues, and the effects on rural and agricultural communities are also considered, the overall economic and social impacts are even greater.

Fermented foods have been part of the human diet for thousands of years. As the world becomes more multicultural and cuisines and cultures continue to mix, it is highly likely that fermented foods will assume an even more important dietary and nutritional role. It may surprise younger readers, but prior to the 1940s, yogurt was unknown nearly everywhere, except Europe and the Middle East. It is now one of the most popular fermented food products consumed throughout the world. Foods such as kimchi (from Korea), miso (from Japan), fish sauce (from Thailand), and kefir (from Eastern Europe) are fast becoming part of the Western cuisine. Certainly, the desirable flavor, sensory, and nutritional attributes of traditional, as well as new-generation fermented foods, will drive much of the interest in these foods.

Consumption of these products also will likely be increased as the potential beneficial effects of fermented foods on human health become better established, scientifically and clinically. As noted above, compelling evidence now exists to indicate that red wine may reduce the risk of heart disease and that live bacteria present in cultured milk and other products may positively influence gastrointestinal health. Perhaps the most important development in the past decade has been the availability of genomic information on the microorganisms involved in food fermentations. The composition of entire microbial communities can now be quickly and inexpensively measured and described. The functional role of specific members involved in fermentations can also be assessed. Likewise, the field of metabolomics has advanced to the point where the metabolic products of fermentation, even those produced at very low levels, can be identified and quantified. Thus, it is now becoming possible to link the metabolic activities of individual or collections of microorganisms with their organoleptic contributions. These technological developments may provide opportunities for researchers to custom-produce fermented foods, not only with predictable flavor and other functional characteristics, but that also impart nutritional benefits to consumers.

BIBLIOGRAPHY

Bamforth, C.W. and R.E. Ward, eds. 2014. The Oxford Handbook of Food Fermentations. Oxford University Press, Oxford.

Barnett, J.A. 2003. Beginnings of microbiology and biochemistry: the contribution of yeast research. Microbiology 149:557–567.

Bell, V., J. Ferrão, and T. Fernandes. 2017. Nutritional guidelines and fermented food frameworks. Foods 6:65.

Bourdichon, F., S. Casaregola, C. Farrokh, J.C. Frisvad, M.L. Gerds, W.P. Hammes, J. Harnett, G. Huys, S. Laulund, A. Ouwehand, I.B. Powell, J.B. Prajapati, Y. Seto, E. Ter Schure, A. Van Boven, V. Vankerckhoven, A. Zgoda, S. Tuijtelaars, E.B. Hansen. 2012. Food fermentations: microorganisms with technological beneficial use. Int. J. Food Microbiol. 54:87–97.

Brüssow, H. 2013. Nutrition, population growth and disease: a short history of lactose. Environ. Microbiol. 15:2154–2161.

Chilton, S.N., J.P. Burton, and G. Reid. 2015. Inclusion of fermented foods in food guides around the world. Nutrients 7:390–404.

Douillard, F.P. and W.M. de Vos. 2014. Functional genomics of lactic acid bacteria: from food to health. Microb. Cell Fact. 13:S8.

Gibbons, J.G. and D.C. Rinker. 2015. The genomics of microbial domestication in the fermented food environment. Curr. Opin. Genet. Dev. 35:1–8.

Legras, J.L., Merdinoglu, D., Cornuet, J.M., and Karst, F. 2007. Bread, beer and wine: *Saccharomyces cerevisiae* diversity reflects human history. Mol. Ecol. 16:2091–2102.

Lücke, F.-K. and P. Zangerl. 2014. Food Control 43:217–230.

Marco, M.L., D. Heeney, S. Binda, C.J. Cifelli, P.D. Cotter, B. Foligne, M. Ganzle, R. Kort, G. Pasin, A. Pihlanto, E.J. Smid, and R. Hutkins. 2016. Health benefits of fermented foods: microbiota and beyond. Curr. Opin. Biotechnol. 44:94–102.

Nakadai, T. 2015. J. Soy Sauce Res. Technol. 41:117–138 (in Japanese).

Schlenk, F. 1997. Early research on fermentation – a story of missed opportunities. Trends Biochem. Sci. 10:252–254.

Selhub, E.M., A.C. Logan, and A.C. Bested. 2014. Fermented foods, microbiota, and mental health: ancient practice meets nutritional psychiatry. J. Physiol. Anthropol. 33:2.

Steinkraus, K.H. 2002. Fermentations in world food processing. Comp. Rev. Food Sci. Technol. 1:23-32.

Trager, J., 1995. The Food Chronology. Henry Holt and Co., New York

2 Microorganisms

If you don't like bacteria, you're on the wrong planet. This is the planet of the bacteria.
Craig Venter, American Geneticist, as reported in a Los Angeles Times interview, November, 2007.

INTRODUCTION

As described in general in the preceding chapter and in detail in subsequent chapters in this text, there are thousands of different fermented food products produced and consumed around the world. Thus, it should not be surprising that a wide and diverse assortment of microorganisms is used in their manufacture. Although lactic acid-producing bacteria and ethanol-producing yeasts are responsible for performing most food fermentations, there are many other bacteria, yeast, and fungi that contribute to the flavor, texture, and appearance of the finished foods. Indeed, for most fermented foods, more than one organism or group of organisms is involved in the fermentation.

In the manufacture of blue mold-type cheeses, for example, mesophilic lactic acid bacteria from the genus *Lactococcus*, are required to ferment lactose, produce lactic acid, and acidify the cheese to below pH 5.0. This task occurs during the first 12–48 hours after manufacture. A short time later, the fungus, *Penicillium roqueforti*, begins to grow, producing the characteristic blue-green color. Lipolytic and proteolytic enzymes are also produced by the mold, generating flavor and aroma compounds for which blue cheeses are known.

To expand on the example from above, it should be noted that the *Lactococcus* is added to the milk in the form of a starter culture and *Penicillium* spores are intentionally added to the milk or curds as an adjunct. However, the properties and quality of blue cheese are also influenced by wild or environmental yeast and bacteria. Blue-mold fermented cheeses may contain a wide assortment or bacteria, yeasts, and molds, all contributing to the overall quality of the product. This is true for many fermented foods where organisms other than the actual starter culture often play inadvertent, and sometimes essential supporting roles. Thus, tempeh, a fermented food product that originated in Indonesia (and has now become popular in Europe and the US), is made by inoculating soybeans with a single organism, the fungus, *Rhizopus microsporus* var. *oligosporus*. The manufacturing process lends itself, however, to fortuitous contamination with other microorganisms, including bacteria that synthesize Vitamin B_{12}.

Microbiology and Technology of Fermented Foods, Second Edition. Robert W. Hutkins.

This makes tempeh not only a high quality protein-rich food, but also a good source of a nutrient that might otherwise be absent in the diet of individuals that consume this product.

A PRIMER ON MICROBIAL CLASSIFICATION AND NOMENCLATURE

For many readers, keeping track of the numerous genus, species, and subspecies names assigned to the organisms used in fermented foods can be a challenging task. To make matters even more confusing, nomenclature is an evolving science, such that the names of even well-known organisms are subject to change. Sometimes the change is modest, where one species is reclassified as another species. But in other cases, an organism may be reassigned to an entirely new genus. Still, knowing which organisms are used in specific fermented foods is rather essential for understanding and appreciating the scientific basis for how fermented foods are produced. Therefore, a primer on identification, classification, and nomenclature of the relevant fermentation organisms is necessary, prior to describing the properties and functions these microorganisms have in food fermentations.

Collectively, classification, identification, and nomenclature are all part of the biological discipline called systematics. Assembling organisms into groups or taxa based on similarities and differences (classification), relating an unknown organism to a known organism based on specific characteristics (identification), and assigning names based on international rules (nomenclature) – these are the three goals of systematics. Systematics also provides researchers the means to understand microbial diversity and the evolutionary and ecological relationships of organisms in foods and other ecosystems (a fourth goal perhaps). To the casual student, these goals might seem to be a thankless exercise. One could even argue there are more important biological questions that deserve our attention. However, there are many practical reasons for why it is important to correctly classify, identify, and name the organisms used in fermented foods. Indeed, manufacturers of fermented foods now expect culture suppliers to provide accurate information on the species and even the strains used in their products. Regulatory agencies have also become more vigilant. In Europe, for example, the first criteria for establishing a health claim for a given organism is that it be properly classified and named. The European Food Safety Authority (EFSA) has rejected several health claim applications based in part, on inadequate identification and characterization of the purported probiotic organisms.

As noted above, rules for classification, identification, and nomenclature are not fixed, but rather can be amended and re-defined in response to new, more discriminating methods and technologies. For the most part, these new methods are based on molecular properties and the small subunit of ribosomal RNA (16S rRNA in prokaryotes and an internal transcribed spacer region in fungi), in particular. Sequencing this region in individual organisms or entire communities provides a basis for determining identity as well as phylogenetic or evolutionary relationships between related organisms. However, whole genome sequencing has recently become easy and affordable, and this approach may provide additional opportunities for identifying and classifying strains.

The three domains of life

According to modern taxonomy, life on planet Earth can be grouped into three branches or domains – the *Eukarya*, the *Bacteria*, and the *Archaea* (Figure 2.1). This organizational structure was proposed in the late 1970s by Carl Woese, and was based on the relatedness of

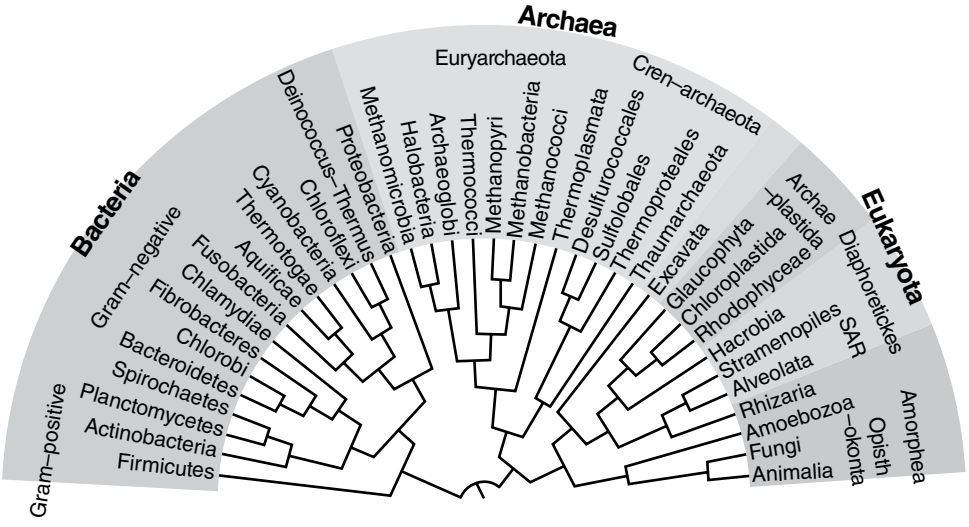


Figure 2.1 Phylogenetic tree of life. Image courtesy of S. Gribaldo on Wikimedia Commons (https://commons.wikimedia.org/wiki/File:Phylogenetic_Tree_of_Life.png).

16S rRNA sequences. This scheme displaced the classical taxonomy that had recognized only two groups, Eukaryotes and Prokaryotes and that was based primarily on morphology and biochemical attributes. While taxonomy based on 16s RNA sequences was a revolutionary concept in 1977, it has now become the standard means by which organisms are placed into taxonomic groups.

All of the microorganisms relevant to fermented foods (and food microbiology, in general) belong to either the *Eukarya* or *Bacteria*. The *Archaea*, while interesting to biologists for a number of reasons, are rarely associated with foods. Rather, they consists of organisms that mostly live and grow in extreme environments (e.g., very high temperature, very low pH, or very high salt). However, some *Archaea* live in the gastrointestinal tract and produce methane; their role in human health has become the subject of much current interest.

As noted above, the classification of organisms as eukaryotic or prokaryotic is based on a variety of characteristics. For example, The Ninth Edition of *Bergey's Manual of Determinative Bacteriology* lists more than 50 cytological, chemical, metabolic, molecular, and reproductive properties. However, the traditional distinguishing feature between eukaryotic and prokaryotic organisms is the presence of a nuclear membrane in eukaryotes and its absence in prokaryotes. Accordingly, *Bacteria* and the *Archaea* are considered as prokaryote. In contrast, the *Eukarya* domain consists of animals, plants, protists, and fungi. The latter are represented by the Kingdom Fungi, which includes both yeasts and molds.

Fungi

Although the exact number of species within the Kingdom Fungi is unknown, fungal taxonomists have estimated there may be as many as 1.5 to 5 million species. In the Dictionary of Fungi (2008), however, fewer than 100,000 have been described. Thus, only about 1 to 5% of fungal species are currently recognized. There is also not much consensus among taxonomists on how to organize fungi into higher taxa (e.g., Kingdom, Phyla, Sub-Phyla, etc). Finally, there are many redundant and synonymous names in the fungal literature. While approximately 400,000 species names appear in the literature, the current taxonomy has

accepted only 100,000. Thus, it is not surprising that fungal taxonomy has been referred to as a "chaotic discipline". A new movement among fungal taxonomists has even emerged to sort out these issues. Called the "one fungus = one name", their goal is to "stabilize" fungal taxonomy and facilitate a more consistent system of nomenclature.

In the current (2011) system of fungal taxonomy, seven major groups or phyla and ten sub-phyla are recognized among the true fungi or Eumycota. Only those relevant in foods, along with their main properties, will be discussed here.

The Zygomycota group contains as many as 900 species, but those important in foods and food fermentations are found primarily in the *Rhizopus* and *Mucor* genera. In particular, some strains produce proteolytic enzymes that mimic chymosin – the enzyme used to coagulate milk during cheese manufacture. Others are used in the tempeh fermentation.

The Ascomycota is a large phylum of fungi, containing as many as half of all fungal species. The ascomycetes (members of the Ascomycota) have septate mycelia, with cross walls or septa, and produce sexual spores, called ascospores within a structure known as an ascus. The ascomycetes also produce asexual reproductive states, where reproduction occurs by production of asexual spores known as conidia. These spores are released freely from the end of special aerial fertile hyphae (conidiophores).

One particularly important group of ascomycetes are the single celled organisms known as yeasts. Yeasts may produce ascospores within the yeast cell, but they also reproduce asexually by a process known as budding. Yeasts, because of their ability to produce ethanol and carbon dioxide, are very important in food and beverage fermentations (see below).

Bacteria

Organization of the *Bacteria* domain has been no less challenging than for the *Fungi*. In fact, it is appropriate to start this section with a warning: there is no official classification of prokaryotes (see below)! Currently (May, 2017), 30 phyla are listed in the "List of prokaryotic names with standing in nomenclature" (http://www.bacterio.net/index.html). Beyond the phyla, bacteria can be further divided into classes (and sub-classes), orders (and sub-orders), families, genera, and ultimately species. One very important caveat should be noted–while microbiologists often refer to various phyla, there is no official recognition of any organizational group above the rank of Class. Thus, these phylum designations, while still useful, are not covered by The Rules of the Bacteriological Code (see below). Nonetheless (and despite this stipulation), most of the bacteria relevant in fermented foods, including lactic acid bacteria, belong to a single phylum, the Firmicutes.

Nomenclature

Like all living organisms, microorganisms are named according to Latinized binomial nomenclature, meaning they are assigned two names, a genus and a species. By convention, both the genus and species names are italicized, but only the genus is capitalized. Thus, the name of the common food yeast is written as S*accharomyces cerevisae*. For some organisms, a trinomial system is applied to indicate a subspecies epithet, as is the case for the dairy organism, *Lactococcus lactis* subsp. *lactis*.

Microorganisms are named according to the rules established by the appropriate governing body. Indeed, nomenclature is the one part of systematics, unlike classification and identification that actually has rules. The International Association for Plant Taxonomy defines the nomenclature rules for fungi. These rules are then published in the International

Code of Nomenclature for algae, fungi, and plants. For prokaryotic organisms, nomenclature rules are established by International Committee on Systematics of Prokaryotes (ICSP), formerly the International Committee on Systematic Bacteriology.

In addition, a running list, called the List of prokaryotic names with standing in nomenclature (www.bacterio.cict.fr), is maintained that provides updated bacterial nomenclature. To be included in these lists (which implies "official" recognition), a name must have been "validly" published in the relevant scientific literature (as prescribed by the Code), along with a detailed description and relevant supporting data.

In some cases, a validly named organism may be referred to by another name, indicated as a synonym. In other instances, the name of an organism may have been replaced by a new name. In situations where a name was unofficially assigned and does not appear on the List, that name is considered to be illegitimate and its use should be discontinued. However, to the consternation of students, as well as taxonomists, such names often remain in the scientific literature. In addition, even if the name assigned to a particular organism is supported by a valid publication, this does not mean that the name is or must be accepted by the scientific community. Although names are indeed based on official rules (where each taxon has a valid name), the utility of a given classification scheme, on which a given organism is named, is left up to the scientists that use it. That is, a researcher may propose that a given organism be assigned a new name, and have the supporting evidence published in a valid journal, but other microbiologists are entitled to disagree with the taxonomy and reject the proposed classification.

The nomenclature issue is neither trivial nor inconsequential; indeed, it is relevant to raise these issues for several reasons. First, for practitioners in fermentation science or any other area of microbiology, using the proper genus and species names is essential; otherwise, clear and accurate scientific communication cannot occur. Moreover, many of the organisms used in food fermentations have been reclassified into new taxa and therefore new names have been assigned. For example, the original name of the common dairy organism mentioned above, *L. lactis* subsp. *lactis* was *Bacterium lactis* (the first organism isolated in pure culture and named by Joseph Lister in 1873). It was then renamed as *Streptococcus lactis* in 1909, which is how this organism was known for more than 70 years before the new genus, *Lactococcus*, was adopted.

In other cases, a name was proposed, only to later be rescinded (see below). There are also instances of organisms that had been assigned an unofficial name, and through frequent use, had acquired some level of validity, however undeserved. An example of the latter situation was for Lactobacillus sporogenes, an organism that is clearly misnamed, as the genus *Lactobacillus* includes no members that form spores. Rather, these spore-forming bacilli belong to the genus *Bacillus.* Yeast nomenclature, although under the authority of the International Code of Nomenclature, is also subject to taxonomical challenges and changes in classification.

Microbial taxonomy and methods of analysis

If microbiology began in the middle of the nineteenth century, then for the next 120 years, microbial classification was primarily based on morphologic and phenotypic characteristics. Although many of these traits remain useful as diagnostic tools, by far, the most powerful means of classifying microorganisms is now via molecular techniques. In particular, DNA–DNA (and DNA–RNA) hybridization has long been considered the gold standard for establishing a species and the proximal relationships of one species to another. Organisms that

share high DNA homology (usually greater than 70%) are regarded as members of the same species. However, in the past 20–25 years, the nucleic sequence of the 16S rRNA region has become the most common way to distinguish between organisms, to show relatedness, and ultimately to classify an organism to the genus or species level. These sequences can be easily determined from the corresponding DNA after PCR amplification. By comparing the sequence from a given organism to those obtained from reference organisms in the 16S rRNA database, it is possible to obtain highly significant matches or to infer relationships if the sequence is unique. In addition, DNA probes, based on genus- or species-specific 16S rRNA sequences from a particular reference organism, are also useful for identifying strains in mixed populations.

Other techniques that provide a molecular fingerprint and that can be used to distinguish between strains of the same species have also become common. These include restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), multi-locus sequence analysis (MLSA), pulsed field gel electrophoresis (PFGE), and DNA microarray technology. Other methods, including various mass spectroscopy techniques, rely on chemical fingerprints to identify and speciate strains. Remarkably, the more recent availability of several next generation sequencing platforms has made whole genome sequencing a quick, easy, and low-cost alternative for strain identification and classification.

The precision of modern molecular identification methods and the rapidity at which species identification can be established may be under-appreciated for some students. Much of the classic microbiological hardware – culture tubes, Petri dishes, and microscopes, are hardly needed to analyze samples or to determine who's who (Box 2.1).

Finally, it should be noted that nucleic acid-based methods for classification, whether based on 16S rRNA sequences, DNA–DNA hybridization values, or even whole genome analyses, are not necessarily the be-all, end-all of microbial taxonomy. Rather, a more holistic or integrative taxonomy, referred to as polyphasic taxonomy, has been widely adopted and used to classify many different groups of bacteria. Polyphasic taxonomy is based on genotypic, as well as phenotypic and phylogenetic information, and is considered to represent a consensus approach for bacterial taxonomy.

Box 2.1 From the Pasteur era to the genome era

At the end of the previous century, the American Society of Microbiology published a chronology of "Microbiology's 50 most significant events during the past 125 years" (ASM News, 1999). The list started with Pasteur's discovery in 1861 that yeast produced more ethanol during anaerobic fermentative growth compared to growth under aerobic, respiring conditions (the aptly called "Pasteur effect"). The list ended with the report in 1995 that described, for the first time, the complete genome sequence of a bacterium (Fleischmann et al., 1995).

The latter event marked, perhaps, the end of one era and the start of another. In the decade that followed (i.e., from 1995 to the publication of the first edition of this book in 2006), about 250 microbial genomes had been sequenced. Remarkably, over the next decade (through January, 2016), the number of sequenced genomes listed in the National Center for Biotechnology Information (NCBI) database has reached over 100,000, representing 50 different bacterial and 11 different archaeal phyla.

Several reasons account for this extraordinary scientific development. First, the actual sequencing is now fast, automated, and above all, cheap. The cost of a genome sequencing project in 1995 (using first generation sequencing technologies) was about $1 million. In 2005, just before so-called second generation sequencing had emerged, the cost for a sequenced genome had dropped to $50,000 (Land et al., 2015). Currently, a genome can be sequenced for as little as $50 and take only a few hours (Bentley and Parkhill, 2015). At the same time, the development of bioinformatics and various computational tools used to analyze and compare genome information has also advanced at an equally rapid rate. The availability of so many published genomes also meant that draft genomes could be mapped or scaffolded onto existing genomes, making sequence finishing somewhat unnecessary. The latter has generally been time-consuming and expensive, but even the cost of finishing a genome has also become more affordable.

Genomes of food fermentation organisms

Among organisms involved in food fermentations, the first sequenced genome was published in 1996 for the common yeast, *Saccharomyces cerevisiae* (Goffeau et al., 1996). Although the sequence strain was a lab strain, and was quite different from the typical baker's or brewer's yeasts used in industry, the sequence nonetheless provided valuable information on yeast biology and genetics. Several years later, the genome sequence of the lactic acid bacterium, *Lactococcus lactis* subsp. *lactis*, was reported (Bolotin et al., 2001). A few years later, the genomes of eleven commercially important bacteria were sequenced as part of a collaborative effort (Makarova et al., 2006). Currently (May, 2017), there are more than 2,500 sequenced genomes in the NCBI database for lactic acid and related bacteria (Figure 2.1.1). Of course, whole genome sequences are really only the first steps toward understanding phylogeny and evolution and how pathways and regulatory networks are constructed. The ultimate goal, perhaps, is to use genome information to predict how these organisms will behave during food fermentations.

The age of the genome: what have we learned? From the outset, researchers discovered that the genomes of the lactic acid bacteria were generally small, with several less than 2 Mb. Accordingly, the genomes reflect the rather specialized metabolic capabilities of these bacteria and the specific environmental niches in which they live (Makarova et al., 2006). For example, most species contain genes encoding for only a few of the carbon-utilization pathways (i.e., homo- and heterofermentation; pyruvate dissimulation; and pentose, citrate, and malate fermentation). These bacteria are auxotrophic for several amino acids, and, therefore, contain many genes encoding for protein and peptide catabolism. In fact, given their overall dependence on obtaining nutrients from the environment, it is not surprising that the genomes are replete (between 13% and 18%) with transport system genes.

Genome evolution analyses have revealed that the loss of biosynthetic genes over time, and the corresponding acquisition of catabolic genes, are consistent with the view that the lactic acid bacteria have recently adapted from nutritionally-poor, plant-type habitats to milk and other nutritionally-complex food environments. This suggestion is supported by several observations. First, some of the lactic acid

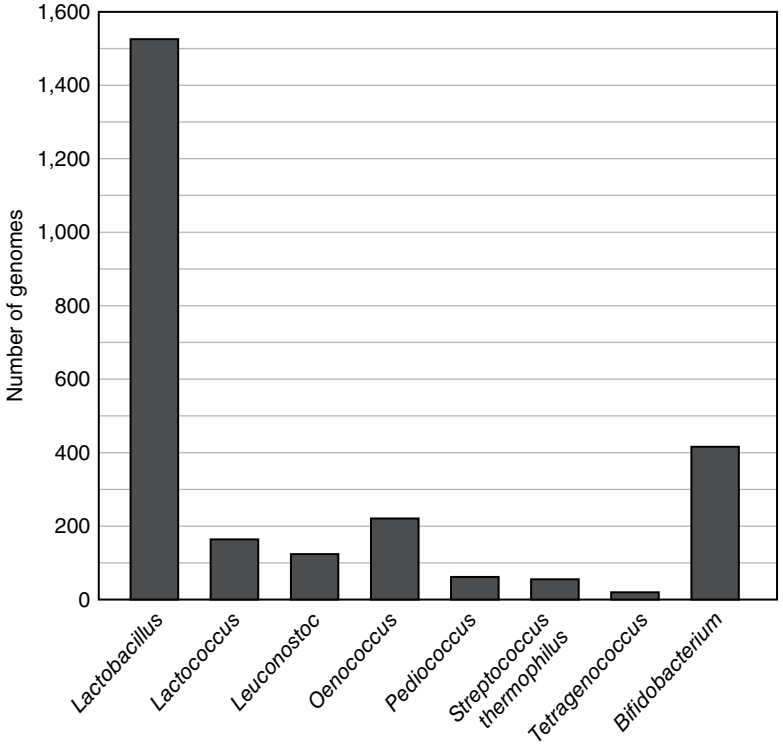


Figure 2.1.1 Number of sequenced genomes from lactic acid and related bacteria (from NCBI, as of December, 2017).

bacteria contain a large number of plasmids and transposons that accelerate horizontal gene transfer (genetic exchange between related organisms) and that promote adaptation to new environments. Second, there are a large number of non-functional pseudogenes (containing mutations or truncation) within the genomes that likely represent "gene decay" or loss of genes that are no longer needed (Makarova et al., 2006). For example, 13% of the genome of the nutritionally-fastidious *Streptococcus thermophilus* (strain LMD-9) are classified as pseudogenes (Goh et al., 2011). As lactic acid bacteria have evolved, therefore, significant genome reduction, with modest genome expansion, i.e., with closed pan genomes, has apparently become the norm (Kelleher et al., 2017).

References

ASM News. Microbiology's fifty most significant events during the past 125 years. Poster supplement. 65.

Bolotin, A., P. Wincker, S. Mauger, O. Jaillon, K. Malarme, J. Weissenbach, S.D. Ehrlich, and A. Sorokin. 2001. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. Genome Res.11:731–753.

Fleischmann, R.D., M.D. Adams, et al. (38 other authors). 1995. Whole-genome random sequencing and assembly of *Haemophilus influenza* Rd. Science 269:496–512.

Goh, Y.J., C. Goin, S. O'Flaherty, E. Altermann, and R. Hutkins. 2011. Specialized adaptation of a lactic acid bacterium to the milk environment: the comparative genomics of *Streptococcus thermophilus* LMD-9. Microb. Cell Fact. 10, Suppl. 1: S22.

Kelleher, P., F. Bottacini, J. Mahony, K.N. Kilcawley, and D. van Sinderen. 2017. Comparative and functional genomics of the *Lactococcus lactis* taxon; insights into evolution and niche adaptation. BMC Genomics 18:267.

Klaenhammer, T., E. Altermann, et al. (35 other authors). 2002. Discovering lactic acid bacteria by genomics. Antonie van Leeuwenhoek 82:29–58.

Bentley, S.D., and J. Parkhill. 2015. Genomic perspectives on the evolution and spread of bacterial pathogens. Proc. R. Soc. B. 282:20150488.

Land, M., L. Hauser, S.-R. Jun, I. Nookaew, M.R. Leuze, T.-H. Ahn, T. Karpinets, O. Lund, G. Kora, T. Wassenaar, S. Poudel, and D.W. Ussery. 2015. Insights from 20 years of bacterial genome sequencing. Funct. Integr. Genomics 15:141–161.

Makarova, K., A. Slesarev, Y. Wolf, A. Sorokin, (and 26 others). 2006. Comparative genomics of the lactic acid bacteria. Proc. Natl. Acad. Sci. USA 103:15611–15616.

BACTERIA USED IN THE MANUFACTURE OF FERMENTED FOODS

Despite the diversity of bacteria involved directly or indirectly in the manufacture of fermented foods, nearly all are currently classified in one of three phyla, the *Firmicutes*, the *Proteobacteria*, and the *Actinobacteria*. The most important of these are the *Firmicutes.* They include the lactic acid bacteria, a cluster of Gram-positive bacteria that are the main organisms used in the manufacture of fermented foods. Also included in this phylum are the genera, *Bacillus* and *Staphylococcus.* They consist of species used in the manufacture of only a few selected fermented foods. The *Proteobacteria* contains Gram-negative bacteria that are involved in one main process – the vinegar fermentation, but they also are important in the spoilage of wine and other alcoholic products. Finally, the *Actinobacteria* contains several genera relevant to fermented foods, including *Propionibacterium*, *Kocuria*, *Micrococcus*, and *Bifidobacterium*.

It is worth emphasizing that fermented foods may contain many other microorganisms, whose presence occurs as a result of inadvertent contamination. However, the section below describes only those bacteria whose contribution to fermented foods manufacture is well established.

THE LACTIC ACID BACTERIA

From the outset, it is important to recognize that the very term, lactic acid bacteria, has no official status in taxonomy. Rather, it is a general term of convenience used to describe a group of functionally and genetically related bacteria. However, like coliforms or psychrotrophs, the term carries rather significant meaning among microbiologists and others who study food fermentations, and, therefore, will be used freely in this text. Accordingly, the lactic acid bacteria are generally defined as a cluster of lactic acid-producing, low %G+C, non-spore-forming, Gram-positive rods and cocci that share many biochemical, physiological, and genetic properties (Table 2.1; Figure 2.2). They are distinguished from other Gram-positive bacteria that also produce lactic acid (e.g., *Bacillus, Listeria*, and *Bifidobacterium*), by virtue of numerous phenotypic and genotypic differences.

Table 2.1 Common characteristics of lactic acid bacteria.

- Gram-positive
- Fermentative
- Catalase negative
- Facultative anaerobes
- Non-sporeforming
- Low mol% G+C
- Non-motile
- Acid-tolerant

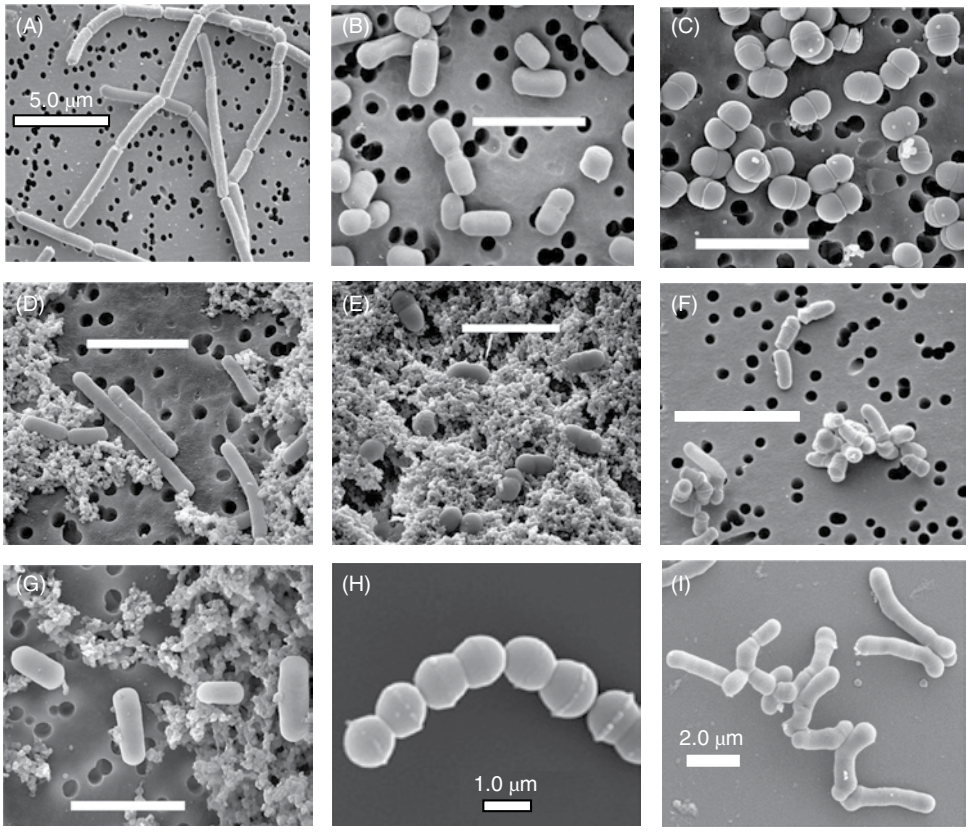


Figure 2.2 Electron micrographs of lactic acid and related bacteria. A, *Lactobacillus delbrueckkii* subsp. *bulgaricus*; B, *Lactobacillus brevis*; C, *Pediococcus pentosaceus*; D, *Lactobacillus casei*; E, *Lactococcus lactis*; F, *Brevibacterium linens*; G, *Lactobacillus helveticus*; H, *Streptococcus thermophilus*; and I, *Bifidobacterium adolescentis*. Scale bars are 3.0 μm, unless otherwise indicated. Photos courtesy of J. Broadbent and B. McManus, Utah State University and with permission from the American Society for Microbiology (ASM News, March, 2005, pp. 121–129).

Other important traits and properties are also characteristic of the lactic acid bacteria, although exceptions occasionally exist. Most lactic acid bacteria are catalase-negative, acid-tolerant, aero-tolerant, facultative anaerobes. Based on their carbon and energy needs, they

are classified as heterotrophic chemoorganotrophs, meaning they require pre-formed organic carbon both as a source of carbon and energy. Until relatively recently, it was thought that all lactic acid bacteria lack cytochrome or electron transport proteins and, therefore, could not derive energy via respiratory activity. This view, however true for many lactic acid bacteria, has been revised, based on findings that indicate that several species may indeed respire, provided the medium contains the necessary nutrients (see Chapter 3). Still, the substrate level phosphorylation reactions that occur during fermentative pathways (see below) are the primary means by which ATP is obtained during fermentation.

The lactic acid bacteria as a group are often described as being fastidious, with complex nutritional requirements. Certainly, there are species that will grow only in nutrient-rich, well-fortified media (or food) under optimum conditions. However, there are also species of lactic acid bacteria that are quite versatile with respect to the growth conditions or environment and that can grow reasonably well even when the nutrient content is less than ideal. Furthermore, some lactic acid bacteria are actually known for their ability to grow in inhospitable environments, including those that often exist in fermented foods. This is reflected by the diverse habitats occupied by lactic acid bacteria. Thus, these organisms can be isolated not only from plant material, milk, and meat, but also salt brines, low pH foods, and ethanolic environments. This also means that lactic acid bacteria, in addition to their positive attributes, are occasionally responsible for spoilage of fermented foods.

Perhaps the most relevant properties of lactic acid bacteria are those related to nutrient metabolism. After all, one of the main reasons why lactic acid bacteria are used in fermented foods is due to their ability to metabolize sugars and make lactic acid and other metabolic end-products. Two fermentative pathways exist. In the homofermentative pathway, more than 90% of the sugar substrate is converted exclusively to lactic acid. In contrast, the heterofermentative pathway results in four end-products–lactic acid, acetic acid, ethanol, and carbon dioxide. For the most part, lactic acid bacteria possess either one or the other of these two pathways (i.e., they are obligate homofermentative or obligate heterofermentative). However, there are some species that have the metabolic wherewithal to perform both pathways (referred to as being facultative heterofermentative). These pathways will be described in detail in the next chapter.

The genera of lactic acid bacteria

According to current taxonomy, the lactic acid bacteria group consists of 40 genera, although only about 12 are considered relevant in fermented foods (Table 2.2). Based on 16S rRNA sequencing and other molecular techniques, the lactic acid bacteria can be grouped into a broad phylogenetic cluster, positioned not far from other low G+C Gram-positive bacteria (Figure 2.3). Five sub-clusters are evident from this tree, including: (1) a *Streptococcus-Lactococcus* branch; (2) a *Lactobacillus* branch; (3) a separate *Lactobacillus-Pediococcus* branch; (4) an *Oenococcus-Leuconostoc-Weissella* branch; and (5) a *Carnobacterium-Enterococcus-Tetragenococcus-Vagococcus* branch. It is worth noting that this phylogeny is not entirely consistent with regard to the morphological and physiological characteristics of these bacteria. For example, *Lactobacillus brevis* and *Pediococcus pentosaceus* are in the same sub-cluster, yet the former are rods and heterofermentative, whereas the latter are homofermenting cocci. Likewise, *Carnobacterium* are obligate heterofermentative rods, and *Enterococcus* are homofermentative cocci.

Seven genera of lactic acid bacteria, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, and *Tetragenococcus*, are used directly in

Table 2.2 Genera of lactic acid bacteria and their properties[1,2].

Genus	Cell morphology	Fermentation route	Growth at: 10°C	Growth at: 45°C	Growth in NaCl at: 6.5%	Growth in NaCl at: 18%	Growth at pH: 4.4	Growth at pH: 9.6	Lactic acid isomer
Lactobacillus	rods	homo/hetero[3]	±[4]	±	±	–	±	–	D, L, DL[5]
Lactococcus	cocci	homo	+	–	–	–	±	–	L
Leuconostoc	cocci	hetero	+	–	±	–	±	–	D
Oenococcus	cocci	hetero	+	+	±	–	±	–	D
Pediococcus	cocci (tetrads)	homo	±	±	±	–	+	–	D, L, DL
Streptococcus	cocci	homo	–	+	–	–	–	–	L
Tetragenococcus	cocci (tetrads)	homo	+	–	+	+	–	+	L
Aerococcus	cocci (tetrads)	homo	+	–	+	–	–	+	L
Carnobacterium	rods	hetero	+	–	–	–	–	–	L
Enterococcus	cocci	homo	+	+	+	–	+	+	L
Vagococcus	cocci	homo	+	–	–	–	±	–	L
Weissella	coccoid	hetero	+	–	±	–	±	–	D, L, DL

[1] Adapted from Von Wright and Axelsson, 2012.
[2] Refers to the general properties of the genus; exceptions may exist.
[3] Species of *Lactobacillus* may be homofermentative, heterofermentative, or both.
[4] This phenotype is variable, depending on the species.
[5] Some species produce D-, L-, or a mixture of D- and L-lactic acid.

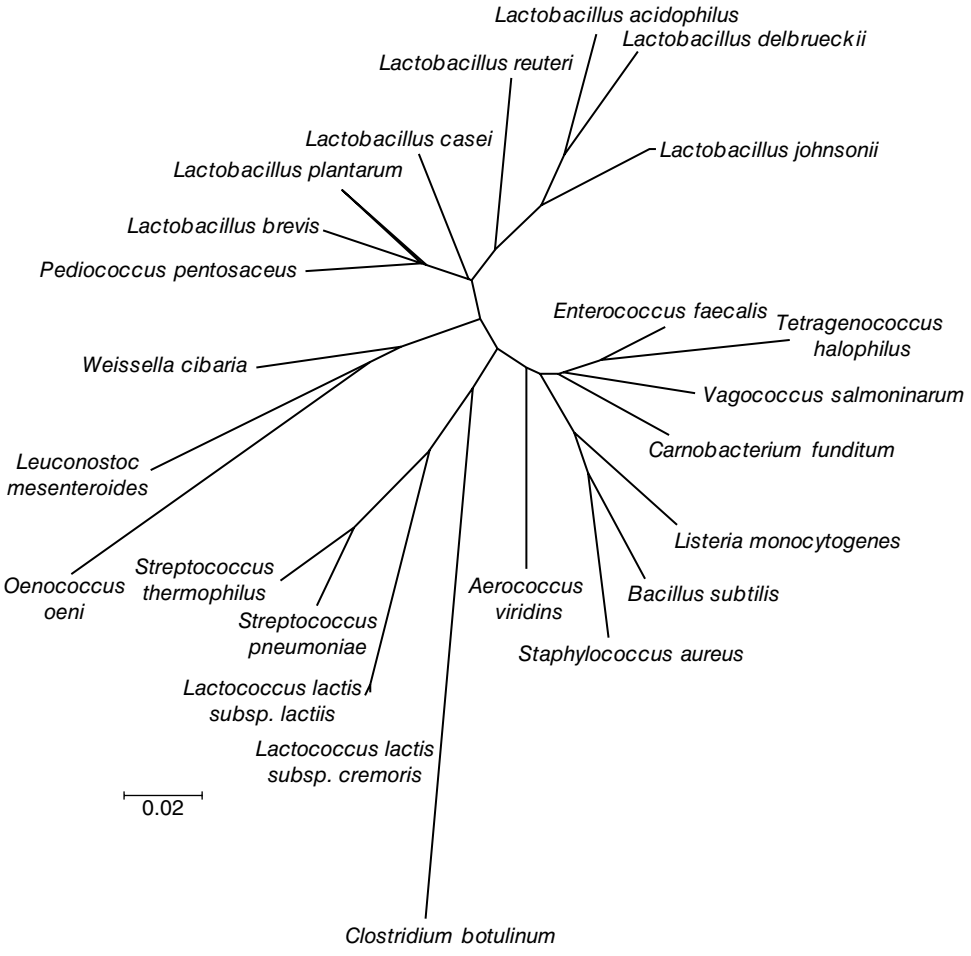


Figure 2.3 Phylogeny of lactic acid and other Gram-positive bacteria (based on 16s RNA). The tree (un-rooted) was generated using the neighbor-joining method.

food fermentations. Although *Enterococcus* spp. are often found in fermented foods (e.g., cheese, sausage, fermented vegetables), except for a few products, they are not added directly. In fact, their presence can be viewed as undesirable, in part, because enterococci are sometimes used as indicators of fecal contamination. Also, some strains may harbor mobile antibiotic-resistance genes, which can trigger regulatory issues. Importantly, some strains of *Enterococcus* may be capable of causing opportunistic infections in humans. Likewise, *Carnobacterium* are also undesirable, mainly because they are considered as spoilage organisms in fermented meat products. Finally, species of *Aerococcus, Vagococcus*, and *Weissella* are not widely found in foods, and their overall significance in food is unclear. In the section to follow, the most current nomenclature, general properties, habitats, and practical considerations of the genera and species having the most relevance to food fermentations will be described. Information on the genetics of these bacteria, based on genome sequencing and functional genomics, will be presented later in this chapter.

Lactococcus

The genus *Lactococcus* consists of seven phylogenetically-distinct species, *Lactococcus lactis*, *Lactococcus garviae*, *Lactococcus piscium*, *Lactococcus plantarum*, *Lactococcus fugiensis*, *Lactococcus chungangensis*, and *Lactococcus raffinolactis* (Figure 2.4). They are all non-motile, obligately homofermentative (producing L-lactic acid), facultative anaerobes, with an optimum growth temperature near 30°C. They have a distinctive microscopic morphology, usually appearing as ovoid cocci in pairs or short chains.

One particular species, *L. lactis*, is among the most important of all lactic acid bacteria (and perhaps one of the most important organisms involved in food fermentations, period). This is because *L. lactis* is the work horse of the dairy products industry, as it is used as a starter culture for most of the hard cheeses and many of the cultured dairy products produced around the world. There are four *L. lactis* subspecies: *L. lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *hordinae*, and *Lactococcus lactis* subsp. *tructae*. Only *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*, however, are used as starter cultures. Despite their genetic similarity to the other subspecies, *L. lactis* subsp. *hordinae* and *L. lactis* subsp. *tructae* are associated with insets and fish, respectively, and have no relevance in fermented food manufacture.

A commercially important variant of *L. lactis* subsp. *lactis*, formerly named *L. lactis* subsp. *diacetilactis* (or *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*), is distinguished based on its ability to metabolize citrate. This species is not included in the current

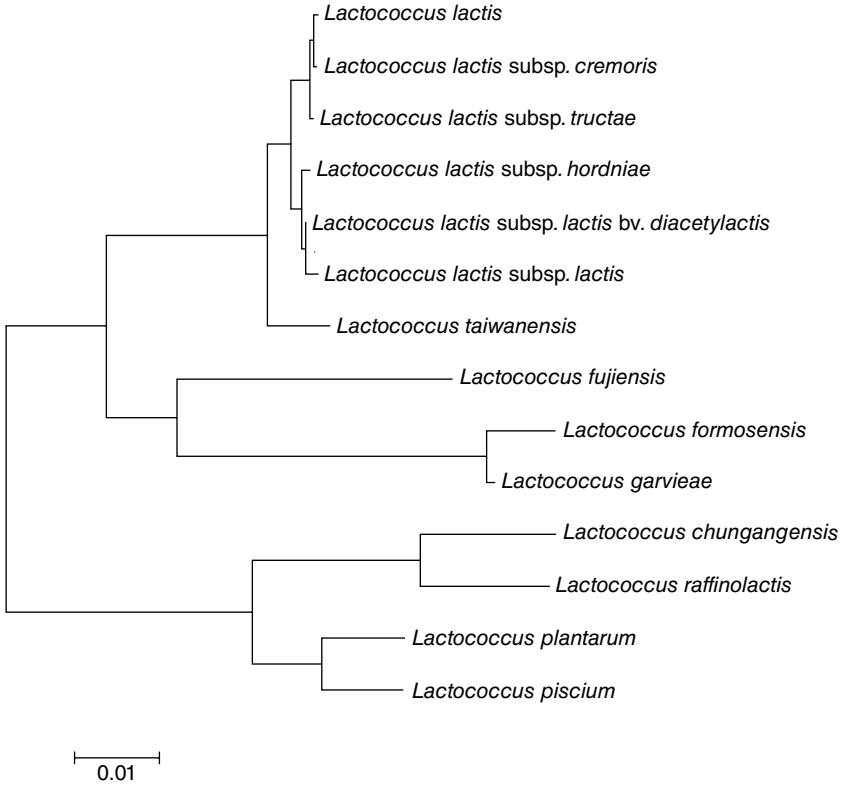


Figure 2.4 Phylogeny of *Lactococcus* based on 16S rRNA sequence analysis.

List of Bacterial Names, and instead is encompassed within *L. lactis* subsp. *lactis* (see below). However, *Lactococcus lactis* subsp. *diacetilactis* is listed in *Bergey's Taxonomical Outline of the Prokaryotes* (2004 version), and in *Bergey's Manual of Systematic Bacteriology* (second edition, 2009) *Lactococcus lactis* subsp. *diacetylactis* is mentioned, but only for "practical" reasons.

Plant material has long been considered to be the original habitat of *L. lactis*. The suggestion, however, that milk has become their new habitat is supported by several observations. First, they are readily isolated from raw milk; in fact, it is difficult to find *L. lactis* subsp. *cremoris* anywhere but milk. Second, both subspecies grow rapidly in milk, producing lactic acid and lowering the pH to below 4.5. Thus, they generally will out-compete most potential competitors in milk. Third, although plant-associated strains contain genes encoding for utilization of plant carbohydrates, dairy strains are adapted to utilize milk proteins. Similarly, the genomes of *L. lactis* reflect gene decay, implying that genes useful in their original habitat (i.e., plant) are no longer essential in the new environment (i.e., milk). Finally, in contrast to genome decay, the genes that are required for growth in milk are, for many strains, located on plasmids (extrachromosomal DNA), indicating they were acquired recently (relatively speaking). Specifically, plasmid-borne genes in lactococci encode for proteins involved in lactose transport and metabolism and casein hydrolysis and utilization. Moreover, plasmid cured derivatives grow poorly in milk. Plasmids, however, are common in lactococci, even in strains isolated from non-dairy sources.

Although *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* differ in just a few seemingly minor physiological respects, at least two of these differences are significant during milk fermentations. For example, the highest temperature at which most strains of *L. lactis* subsp. *lactis* are able to grow is at 40°C, whereas most *L. lactis* subsp. *cremoris* strains do not grow above 38°C. In addition, *L. lactis* subsp. *lactis* has greater tolerance to salt (up to 4%) than does *L. lactis* subsp. *cremoris*. What makes these differences relevant is that both temperature and salt are among the primary means by which the activity of the starter culture is controlled during cheese manufacture. Moreover, *L. lactis* subsp. *cremoris* is generally considered to be less tolerant to the environmental stresses encountered during both culture production and during dairy fermentations. Thus, if *L. lactis* subsp. *cremoris* is used as a culture for cheesemaking (and it is generally accepted that this organism makes better quality products), then temperature and salt concentrations must be adjusted to account for this organisms' particular growth requirements. Complicating this discussion on the differences between these two organisms, it has recently been noted that there are strains of *L. lactis* subsp. *lactis* that have a *lactis* genotype, but a *cremoris*-like phenotype. Likewise, there are strains of *L. lactis* subsp. *cremoris* that have a *cremoris* genotype, but a *lactis*-like phenotype (Box 2.2). The use of genomics (discussed later in the chapter), will help to determine the genetic basis for these industrially relevant differences.

Streptococcus

The genus, *Streptococcus*, contains many diverse species that inhabit a wide array of habitats. Included in this genus are human and animal pathogens, oral commensals, intestinal commensals, and one (and only one) species, *Streptococcus thermophilus*, that is used in the manufacture of fermented foods. Streptococci are non-motile, facultative anaerobes, with an obligate homofermentative metabolism. Since the mid-1980s, there have been several major taxonomical revisions. Some of these changes were especially relevant for food microbiologists and illustrate the importance of nomenclature in food products. For the

Box 2.2 *Lactococcus lactis* – workhorse to the cheese industry

Yeasts and lactic acid bacteria are, without doubt, the most widely used microorganisms in the manufacture of fermented foods and beverages. Among the lactic acid bacteria, *Lactococcus lactis* has long been considered the main starter culture organism for cheese and fermented milk (Cavanagh et al., 2015). Growth of yogurt and Italian cheese industries have certainly led to increased use of thermophilic cultures (i.e., *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*), but *L. lactis* is still probably the main species used. Even apart from its practical use in dairy fermentations, *L. lactis* biology and genetics are so well know that it has become a model organism for biotechnological applications (Song et al., 2017).

History As described earlier, there are nine recognized species of *Lactococcus,* with *L. lactis* representing the only species used as a starter culture. Prior to 1985, strains of *L. lactis* had been classified as *Streptococcus lactis* or *Streptococcus cremoris*. Reclassification led to these species being renamed as *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris,* respectively. Two other subspecies, *Lactococcus lactis* subsp. *hordniae* and *Lactococcus lactis* subsp. *tructae*, have also been recognized, but these subspecies are not associated with milk or fermented dairy products.

Diversity Although these organisms are very similar, several phenotypic differences exist. These differences have some practical relevance (i.e., ability to grow at 40°C or at 4% salt), but they have also long been used diagnostically to distinguish between the two subspecies (Table 2.2.1). Differences also exist at the molecular level, and these differences are now commonly used to assign strains to the appropriate subspecies. While the 16S rRNA gene has commonly been used to distinguish between *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*, high sequence similarity can make such analyses difficult. Nonetheless, discriminating 16S primer pairs have been developed (Xu et al., 2015). Others primer sets, based on various functional genes, have also been used for subspecies identification (Cavanagh et al., 2015). Several molecular finger-printing methods, including amplified fragment length polymorphism (AFLP) and multilocus sequence typing (MLST) are also used to

Table 2.2.1 Distinguishing characteristics of dairy *Lactococcus*[1].

Property	***Lactococcus lactis* subsp. *lactis***	***Lactococcus lactis* subsp. *cremoris***	***Lactococcus lactis* subsp. *lactis* var. *diacetylactis***
Growth at 40°C	+	–	+
Growth in 4.0% NaCl	+	–	+
Growth at pH 9.2	+	–	+
Citrate fermentation	+/–	–	+/–
Arginine hydrolysis	+	–	+
Acid from maltose	+	–	+
Acid from ribose	+	–	+

[1] Adapted from Kim, 2014.

differentiate the subspecies (Le Bourgeois et al., 2015). Recently, MLST was also used to distinguish between wild and domesticated strains of between *L. lactis* subsp. *lactis* (Laroute et al., 2017).

Interestingly, it appears that classification of *L. lactis* by both phenotypic and genotypic criteria is not so clear-cut (Odamaki et al., 2011). One research group analyzed 500 strains of *L. lactis* and organized them into one of five different groups (Kelly and Ward, 2002). Group 1 consists of strains having a *L. lactis* subsp. *lactis* genotype and a "lactis phenotype". These strains, considered as the common or typical *L. lactis* subsp. *lactis*, are able to grow at 40°C, grow at 4% salt, and degrade arginine. Group 2 strains also have a *L. lactis* subsp. *lactis* genotype, but have one particular distinguishing phenotypic property, namely the ability to ferment citrate and to produce diacetyl. As noted previously, these citrate-fermenting strains had, for a long time, held subspecies status. Strains in Group 3 have a cremoris phenotype, but a *L. lactis* subsp. *lactis* genotype. They are not commonly found. In contrast, Group 4 strains, having a *L. lactis* subsp. *cremoris* genotype but a lactis phenotype, are frequently isolated from milk and plant sources. Their lactis-like phenotype makes it difficult to distinguish them from other lactis strains. Finally, Group 5 strains have a *L. lactis* subsp. *cremoris* genotype and phenotype. They are found almost exclusively in milk and dairy environments, and are the most common and preferred strains for manufacture of cheese.

On a practical basis, it is probably easier to establish the genotype of a particular lactococcal strain than it is to determine its phenotype (at least with regard to traits relevant to fermentations). However, it is the phenotypic properties of a given strain that ultimately dictate whether that strain will be useful in fermented foods manufacture. In some cases, properties beyond those normally considered as part of a phenotype will be relevant, such as bacteriophage sensitivity or the ability to produce good cheese flavor. Interestingly, the phenotypic of the strain may depend on its original source. Thus, while *L. lactis* strains isolated from plant material may be genetically similar to milk-derived strains, their phenotypes will likely be very different. In general, the former strains ferment a wider range of carbohydrates, and they have enhanced stress tolerance (Nomura et al., 2006).

Other studies lend support for the suggestion that genotype-based classifications may not be reliable for identifying or selecting strains having a desired phenotype (Passerini et al., 2010). Thus, the association of specific genes, rather than genotype per se, with phenotype may be more informative. Matching gene to phenotype also provides an experimental basis for screening culture collections for strains having desired phenotypes (Bayjanov et al., 2013).

Finally, the phenotypes and genotypes of wild *Lactococcus* strains were recently compared using PCR-based genotyping methods combined with various bioinformatics tools (Cavanagh et al., 2015). Although the eight non-dairy strains had a "lactis" phenotype (see above), most had a "cremoris" genotype. Indeed, the differences between the two subspecies was large enough that the authors of this study suggested they should be reclassified as two separate species. This finding was supported more recently by Kellehere et al. (2017) who used whole genome comparisons to show that a clear phylogenetic division exists between *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*.

References

Bayjanov, J.R., M.J. Starrenburg, M.R. van der Sijde, R. Siezen, and S.A. van Hijum. 2013. Genotype-phenotype matching analysis of 38 *Lactococcus lactis* strains using random forest methods. BMC Microbiol. 13:68.

Cavanagh, D., G.F. Fitzgerald, and O. McAuliffe. 2015. From field to fermentation: the origins of *Lactococcus lactis* and its domestication to the dairy environment. Food Microbiol. 47:45–61.

Cavanagh, D., A. Casey, E. Altermann, P.D. Cotter, G.F. Fitzgerald, and O. McAuliffe. 2015. Evaluation of *Lactococcus lactis* isolates from nondairy sources with potential dairy applications reveals extensive phenotype-genotype disparity and implications for a revised species. Appl. Environ. Microbiol. 81: 3961–3972.

Kelly, W. and L. Ward. 2002. Genotypic vs. phenotypic biodiversity in *Lactococcus lactis*. Microbiology 148:3332–3333.

Kelleher, P., F. Bottacini, J. Mahony, K.N. Kilcawley, and D. van Sinderen. 2017. Comparative and functional genomics of the *Lactococcus lactis* taxon; insights into evolution and niche adaptation. BMC Genomics 18:267.

Kim, W. 2014. The genus *Lactococcus*. In: W.H. Holzapfel, B.J.B. Wood, eds. Lactic Acid Bacteria: Biodiversity and Taxonomy. Wiley Blackwell, Oxford, pp. 429–443.

Laroute, V., H. Tormo, C. Couderc, M. Mercier-Bonin, P. Le Bourgeois, M. Cocaign-Bousquet, and M.-L. Daveran-Mingot. 2017. From genome to phenotype: an integrative approach to evaluate the biodiversity of *Lactococcus lactis*. Microorganisms 5: 27.

Le Bourgeois, P., D. Passerini, M. Coddeville, M. Guellerin, M.-L. Daveran-Mingot, and P. Ritzenthaler. 2015. PFGE protocols to distinguish subspecies of *Lactococcus lactis*. pulse field gel electrophoresis: methods and protocols. In: K. Jordan and M. Dalmasso, eds. pp. 213–224. [Methods in Molecular Biology, vol. 1301, Springer Science + Business Media New York].

Lu, W., W. Kong, P. Yang, and J. Kong. 2015. A one-step PCR-based method for specific identification of 10 common lactic acid bacteria and *Bifidobacterium* in fermented milk. Int. Dairy J. 41:7–12.

Makarova, K., A. Slesarev, Y. Wolf, A. Sorokin (and 26 others). 2006. Comparative genomics of the lactic acid bacteria. Proc. Natl. Acad. Sci. USA 103:15611–15616.

Nomura, M., M. Kobayashi, T. Narita, H. Kimoto-Nira, and T. Okamoto. 2006. Phenotypic and molecular characterization of *Lactococcus lactis* from milk and plants. J. Appl. Microbiol. 101:396–405.

Odamaki, T., S. Yonezawa, H. Sugahara, J. Xiao, T. Yaeshima, and K. Iwatsuki. 2011. A one step genotypic identification of *Lactococcus lactis* subspecies at the species/strain levels. Syst. Appl. Microbiol. 34:429–434.

Passerini, D., C. Beltramo, M. Coddeville, Y. Quentin, P. Ritzenthaler, M.L. Daveran-Mingot, and P. Le Bourgeois. 2010. Genes but not genomes reveal bacterial domestication of *Lactococcus lactis*. PLoS ONE 5: e15306.

Song, A.A.-L., L.L.A. In, S.H.E. Lim, and R.A. Rahim. 2017. A review on *Lactococcus lactis*: from food to factory. Microb. Cell Fact. 16:55.

previous 50 years, the streptococci had been divided into four main groups, pyogenic, enterococcus, viridans, and lactic. These groupings, which were based on the so-called Sherman scheme (published in 1937, as described in Chapter 3), were revised in subsequent years, but generally served as the primary means for organizing streptococcal species.

Starting in 1984, however, two major revisions were proposed and adopted. First, the enterococci, which included *Streptococcus faecalis*, *Streptococcus faecium*, and *Streptococcus durans*, were moved to a new genus, *Enterococcus*. Then, in 1985, two species that had long been referred to as lactic streptococci (*Streptococcus lactis* and *Streptococcus cremoris*), were also assigned to a new genus, *Lactococcus* (see above). Thus, *S. thermophilus* is now the only member of this genus used in food fermentations (mainly yogurt and

cheese). That is not to say that this organism has been exempt from taxonomical considerations. Originally, this species was part of the Sherman viridans group (which also included oral streptococci). In the 1986 edition of Bergey's manual, it was given the rather ignominious treatment of being listed as an Other Streptococci. At about the same time, based on its physiological, structural, and genetic similarities to *Streptococcus salivarius*, researchers recommended that it be reclassified as a subspecies of *S. salivarius*. Although this name change was adopted in the 1980s (and *Streptococcus salivarius* subsp. *thermophilus* is still seen in the literature), subsequent DNA–DNA homology studies eventually led to the restoration of its original name. It is still considered a member, however, of the salivarius streptococci group. Nonetheless, labels on yogurt products can justifiably claim the presence of *Streptococcus thermophilus* rather than the less appetizing *Streptococcus salivarius*.

In several respects, *S. thermophilus* is not that different from the mesophilic dairy lactococci (*L. lactis* subsp. *lactis* and *L. lactis* subsp. cremoris), as evidenced by their close phylogenetic position. Like the lactococci, *S. thermophilus* is highly adapted to a milk environment, in that it ferments lactose rapidly and produces lactic acid in homolactic fashion. The route by which lactose is metabolized by *S. thermophilus*, however, is quite different from how *L. lactis* ferments this sugar (discussed in Chapter 3). Also, *S. thermophilus* has a higher temperature optima (40–42°C), a higher maximum growth temperature (52°C), and a higher thermal tolerance (above 60°C). Its nutritional requirements are somewhat more demanding that lactococci, in that *S. thermophilus* is weakly proteolytic and, therefore, needs pre-formed amino acids for maximum growth rates. Salt tolerance, bile sensitivity, and a limited metabolic diversity are also characteristic of *S. thermophilus*. In fact, the statement made by Sherman in 1937 that *S. thermophilus* is "marked more by the things which it cannot do than by its positive reactions" describes this organism quite well. Finally, in contrast to lactococci, most *S. thermophilus* strains contain few plasmids. When plasmids are found, they are generally small and cryptic (i.e., having no known function).

Leuconostoc

The *Leuconostoc* belong to the *Leuconostocaceae* Family, which also contains the closely related genera, *Oenococcus* and *Weissella*. The latter had not been given much consideration until relatively recently – they are now the subject of considerable interest (Box 2.3).

Leuconostocs are mesophilic, with optimum growth temperatures ranging from 18 to 25°C. Some species are capable of growth at temperatures below 10°C. Microscopically, they appear coccoid or even somewhat rod-like, depending on the composition and form of the growth medium (liquid versus solid). The leuconostocs, in contrast to the obligate homofermenting lactococci and streptococci, are obligately heterofermentative. Accordingly, they are missing an intact glycolytic pathway, and instead rely on the phosphoketolase pathway for metabolism of sugars (described in Chapter 3). With respect to atmospheric requirements, Leuconostocs are truly facultative, as some species grow better aerobically, while for other species, a reduced or anaerobic environment will enhance growth. Plasmids are common in *Leuconostoc* species, and, when present, may encode for important functions, including lactose and citrate metabolism and bacteriocin production.

The genus, *Leuconostoc*, has also been subject to taxonomical revision, as several species have been moved to other existing or newly-formed genera. For example, *Oenococcus oeni* was formerly classified as *Leuconostoc oenos*. Other *Leuconostoc* species have been reclassified as *Weissella*. Currently, the genus consists of 12 species (Figure 2.5).

Box 2.3 The curious case of *Weissella* in fermented foods

When the genus *Weissella* was first established in 1993, only five species were recognized (Collins et al., 1993). All had previously been classified as *Lactobacillus* or *Leuconostoc*, and all had been isolated from fermented sausage. Although the initial observations that led researchers to propose the new genus were based on physiological and phenotypic tests, subsequent molecular analyses (based on 16S rRNA sequence similarities) confirmed that genus status was warranted. Since 1993, the genus has expanded to 21 species that are currently recognized (Fessard and Remize, 2017).

As noted previously, *Weissella* are Firmicutes, belonging to the Family Leuconostocaceae. Like other lactic acid bacteria, they are non-spore-forming, Gram-positive, and catalase-negative (Table 2.3.1). *Weissella* are mesophilic, obligate heterofermentors, and share other phenotypic properties with the *Leuconostoc*. Microscopically, their shape can be a source of confusion, as they can appear as cocci or rods, or have a coccoid morphology.

Weissella, along with *Lactobacillus*, are among the most ecologically diverse of the lactic acid bacteria. They are found in plant material, lakes, soil, and sediments. Like lactobacilli, they have also been isolated from humans and other animals. Thus, there are *Weissella* associated with the oral cavity, the gastrointestinal tract, and the urogenital tract. Although in some of these reports, they were isolated by cultural methods, in other cases they are detected based on 16S sequencing. Finally, many *Weissella* can also be food-borne and have often been isolated from fresh, spoiled, and fermented foods. In addition, some strains produce bacteriocins, suggesting their possible use as anti-microbial agents (Fusco et al., 2015).

The association of several *Weissella* species with human habitats is significant for several reasons. First, there are a few reports suggesting that *Weissella* (mainly *Weissella confusa* and *Weissella cibaria*) may be opportunistic pathogens, both in immunocompromised and immune-competent individuals (Lee et al., 2013; Salimnia et al., 2011). Most of these reports involved infections of blood (septicemia) and were not life-threatening. The isolated strains were usually vancomycin-resistant, which may have contributed to their presence in the patients.

A second reason for why *Weissella* may be important in humans is due to their potential probiotic activity. In one study a powder containing *Weissella koreensis* promoted weight gain and performance in pigs (Wang et al., 2014). Interestingly, a similar study revealed the opposite results (Park et al., 2012). In the latter, mice were fed a high fat diet

Table 2.3.1 Characteristics of *Weissella*.

- Gram-positive
- Heterofermentative
- Non-motile, non-spore forming
- Catalase negative
- Complex nutritional requirements
- Mesophilic
- 37–47% G+C
- Ovoid shape
- Facultative anaerobes

From Björkroth et al., 2014 and Fusco et al., 2015.

supplemented with kimchi containing *W. koreensis* OK1-6 (Park et al., 2012). Compared to the control mice (kimchi without *W. koreensis*), the treatment group gained less weight, and other biomarkers were improved, suggesting that *W. koreensis* had anti-obesity effects.

Another strain, *Weissella cibaria* WIKIM28, had important immune effects in mice, suppressing the allergic Th2 response, inducing the Treg response, and inhibiting allergic responses, including atopic dermatitis (Lim et al., 2017). Finally, *W. cibaria* has also been suggested to confer oral health benefits (Jang et al., 2016). In this report, *W. cibaria* CMU inhibited biofilm formation by cariogenic streptococci and had antibacterial activities (>97%) against important oral pathogens, including *Fusobacterium nucleatum* and *Porphyromonas gingivalis*. Ultimately, the use of *Weissella* as a probiotic should be carefully considered, since, as noted above, some species may be opportunistic pathogens (Abriouel et al., 2015; Fairfax et al., 2014).

Finally, the functional properties of *Weissella* has led some researchers to suggest these organism should be considered as potential starter cultures (Fessard and Remize, 2017). In particular, their ability to produce exopolysaccharides and bacteriocins could provide texture and biopreservation functions to fermented foods.

References

Abriouel, H., L.L.Lerma, M. del C. C. Muñoz, B.P. Montoro, J. Kabisch, R. Pichner, G.-S Cho, H. Neve, V. Fusco, C.M. A. P. Franz, A. Gálvez, and N. Benomar. 2015. The controversial nature of the *Weissella* genus: technological and functional aspects versus whole genome analysis-based pathogenic potential for their application in food and health. Front. Microbiol. 6.

Björkroth, J.A., L.M.T.D. Dicks, and A. Endo. 2014. The genus *Weissella.* In: Lactic Acid Bacteria, Biodiversity and Taxonomy. W.H. Holzapfel and B.J. B. Wood, eds. Wiley Blackwell, Chichester, pp. 418–428.

Collins, M.D., J. Samelis, J. Metaxopoulos, and S. Wallbanks. 1993. Taxonomic studies on some *Leuconostoc*-like organisms from fermented sausages: description of a new genus *Weissella* for the *Leuconostoc paramesenteroides* group of species. J. Appl. Bacteriol. 75:595–603.

Fairfax, M.R., P.R. Lephart, and H. Salimnia. 2014. *Weissella confusa*: problems with identification of an opportunistic pathogen that has been found in fermented foods and proposed as a probiotic. Front. Microbiol. 5:254.

Fessard, A. and F. Remize. 2017. Why are *Weissella* spp. not used as commercial starter cultures for food fermentation? Fermentation 3:38.

Fusco, V., G.M. Quero, G.-S. Cho, J. Kabisch, D. Meske, H. Neve, W. Bockelmann, and C.M. A. P. Franz. 2015. The genus *Weissella*: taxonomy, ecology and biotechnological potential. Front. Microbiol. 6:155.

Jang, H.-J., M.-S. Kang, S.-H. Yi, J.-Y. Hong, and S.-P. Hong. 2016. Comparative study on the characteristics of *Weissella cibaria* CMU and probiotic strains for oral care. Molecules 21:1752.

Lee, W., S.-M. Cho, M. Kim, Y.-G. Ko, D. Yong, and K. Lee. 2013. *Weissella confusa* bacteremia in an immune-competent patient with underlying intramural hematomas of the aorta. Ann. Lab. Med. 33:459–462.

Lim, S.K., M.-S. Kwon, J. Lee, Y.J. Oh, J.-Y. Jang, J.-H. Lee, H.W. Park, Y.-D. Nam, M.-J. Seo, S.W. Roh, and H.-J. Choi. 2017. *Weissella cibaria* WIKIM28 ameliorates atopic dermatitis-like skin lesions by inducing tolerogenic dendritic cells and regulatory T cells in BALB/c mice. Sci. Rep. 7:40040.

Park, J.-A., P.B. Tirupathi Pichiah, J.-J. Yu, S.-H. Oh, J.W. Daily III, and Y.-S. Cha. 2012. Anti-obesity effect of kimchi fermented with *Weissella koreensis* OK1-6 as starter in high-fat diet-induced obese C57BL/6J mice. J. Appl. Microbiol. 113:1507–1516.

Salimnia, H., G.J. Alangaden, R. Bharadwaj, T.M. Painter, P.H. Chandrasekar, and M.R. Fairfax. 2011. *Weissella confusa*: an unexpected cause of vancomycin-resistant gram-positive bacteremia in immunocompromised hosts. Transpl. Infect. Dis. 13:294–298.

Wang, J.P., J.S. Yoo, H.D. Jang, J.H. Lee, J.H. Cho, and I.H. Kim. 2011. Effect of dietary fermented garlic by *Weissella koreensis* powder on growth performance, blood characteristics, and immune response of growing pigs challenged with *Escherichia coli* lipopolysaccharide. J. Anim. Sci. 89:2123–2131.

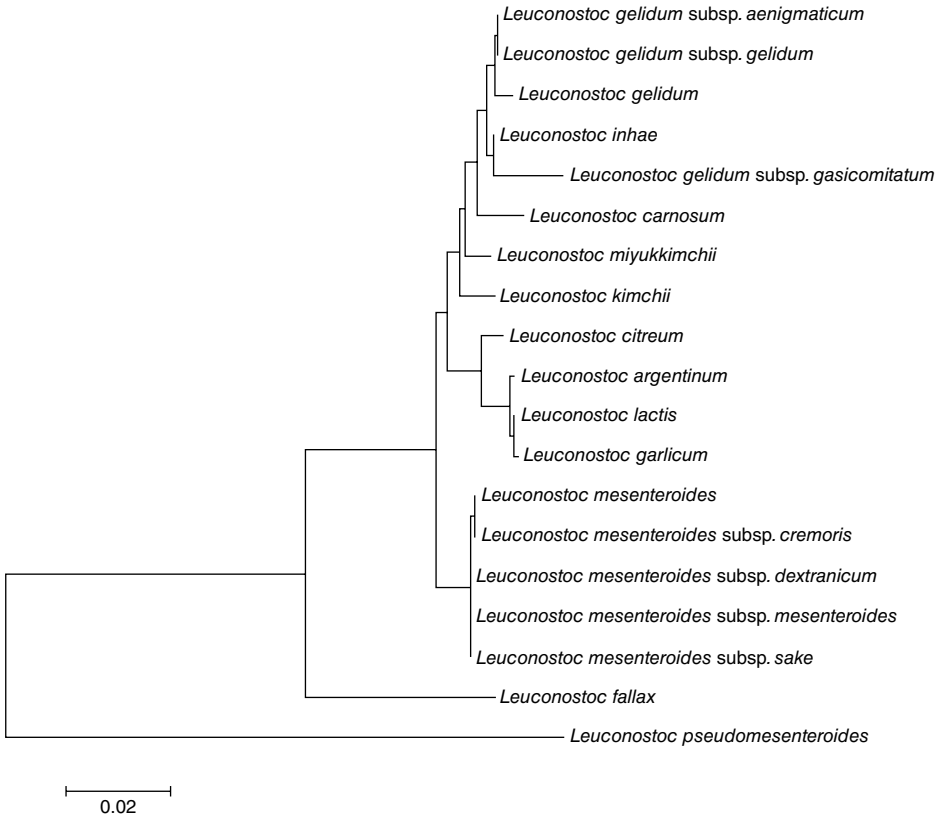


Figure 2.5 Phylogeny of *Leuconostoc* based on 16S rRNA sequence analysis.

Speciation is based on both genetic and phenotypic characteristics. The latter includes carbohydrate fermentation profiles, the ability to produce the polysaccharide, dextran, resistance to the antibiotic vancomycin, and various physiological properties (Table 2.3).

Most species are associated with specific habitats, including plant and vegetable material, milk and dairy environments, and meat products. In addition, some species are involved in food spoilage (e.g., *Leuconostoc gasicomitatum*), whereas others are important in fermented foods. The latter include *Leuconostoc mesenteroides* subsp. *cremoris* and *Leuconostoc lactis*, used in dairy fermentations, and *Leuconostoc mesenteroides* subsp. *mesenteroides, Leuconostoc kimchii*, and *Leuconostoc fallax* that are associated with vegetable fermentations. In general, the habitats occupied by these strains is reflected by the particular carbohydrates they ferment. Plant-associated strains, such as *L. mesenteroides* subsp. *mesenteroides*, ferment plant sugars (i.e., fructose, sucrose, arabinose, trehalose), whereas dairy strains (*L. mesenteroides* subsp. *cremoris* and *L. lactis*) are more likely to ferment lactose, galactose, and glucose. It is also possible for otherwise useful species to cause spoilage, particularly in the case of polysaccharide-producing strains that form slime.

Heterofermentative metabolism of sugars by leuconostocs results in formation of a mixture of end-products, including lactic acid, acetic acid, ethanol, and carbon dioxide. The presence of CO_2 is readily detected and can be used diagnostically to separate these bacteria from homofermentative streptococci, lactococci, and pediococci. Also, while leuconostocs

Table 2.3 Characteristics of *Leuconostoc* and *Oenococcus*[1].

	***Leuconostoc mesenteroides* subsp.**				
Property	***cremoris***	***mesenteroides***	***dextranicum***	***Leuconostoc lactis***	***Oenococcus oeni***
% G+C	38–44	28–32	28–32	37–42	37–42
Growth at 37°C	–	±[2]	+	+	±
Growth at pH 4.8	–	–	–	–	+
Dextran from sucrose	–	+	+	–	–
Growth in 10% ethanol	–	–	–	+	+
Acid from:					
arabinose	–	+	–	–	±
fructose	–	+	+	+	+
maltose	±	+	+	+	–
melibiose	±	+	+	±	±
salicin	–	±	±	±	±
sucrose	–	+	+	+	–
trehalose	–	+	+	–	+

[1] Adapted from Bergey's Manual of Systematic Bacteriology, Vol. 3, 2011 and The Prokaryotes, 2014.
[2] Variable reaction, depending on strain.

generally decrease the pH in the growth medium to between 4.5 and 5.0, acid production by some species may be relatively modest, especially when compared to homofermentative lactic acid bacteria. Thus, acidification is not necessarily the major function of these bacteria during fermentations. In sauerkraut and other vegetable fermentations, for example, they produce lactic and acetic acids and lower the pH during the very early manufacturing stages and also produce enough CO_2 to reduce the redox potential in the food environment. These metabolic events then create an acidic and anaerobic environment conducive for growth of lactobacilli that are responsible for more significant acid development. In addition, the heterofermentative end-products results in a more diverse flavor and aroma profile in fermented foods. The *Leuconostoc* species used in dairy fermentations are particularly important for flavor, in part, for the same reasons as the vegetable-associated strains. Dairy leuconostocs also produce the four-carbon volatile, diacetyl, which imparts a desirable buttery aroma to cultured dairy products (discussed below).

Oenococcus

This genus was established as recently as 1995, and contains only two species, *Oenococcus oeni* and *Oenococcus kitaharae*. The latter species was first described in 2003; however, unlike *O. oeni*, *O. kitaharae* does not appear to be involved in fermented foods. Not surprisingly, both species are located phylogenetically within the *Leuconostocaceae* branch, and based on 16S rRNA sequences, are positioned between *Leuconostoc* and *Weissella*.

Although *O. oeni* shares many phenotypic properties with *Leuconostoc* spp. (e.g., obligate heterofermentative metabolism, mesophilic growth range), several important physiological differences exist. In particular, *O. oeni* is more acid-tolerant than *Leuconostoc*, being able to

commence growth in medium having a pH below 5.0 and able to grow at pH below 4.0. In addition, *O. oeni* is one of the most ethanol-tolerant of all lactic acid bacteria and can grow in the presence of 10% ethanol. In general, however, most strains of *O. oeni* are slow-growing and ferment a limited number of sugars.

As implied by the etymology of its name (oenos is the Greek word for wine), the only known habitat *O. oeni* is wine. Thus, its application in fermented foods is restricted to only one application, namely wine making. Despite its limited use, however, the importance of *O. oeni* during the wine fermentation cannot be overstated. This is because *O. oeni* has the ability to de-acidify wine via the malolactic fermentation, whereby malic acid is decarboxylated to lactic acid (described in detail in Chapter 11). Moreover, given the ability of *O. oeni* to ferment glucose and fructose, but few other sugars, and its high tolerance to low pH and high ethanol, wine or juice would appear to be the natural niche for this organism.

Pediococcus

The pediococci are similar, in many respects, to other coccoid-shaped, obligate homofermentative lactic acid bacteria, with one main exception. When these bacteria divide, they do so in perpendicular fashion and in two planes. Thus, tetrads are formed which can be observed microscopically. Cells may appear as pairs (and always spherical in shape), but chains are not formed, as for lactococci, streptococci, and leuconostocs. Pediococci are, like other lactic acid bacteria, facultative anaerobes, with complex nutritional requirements. They have optimum growth temperatures ranging from 25 to 40°C, but some species can grow at temperatures as high as 50°C. Several of the pediococci are distinguished from other lactic acid bacteria by their ability to tolerate high acid (growth at pH 4.2) and high salt (growth above 5% NaCl) environments (Table 2.4). Phylogenetically, they are located within the *Lactobacillaceae* family and are closely related to the *Lactobacillus casei* sub-cluster.

Table 2.4 Characteristics of *Pediococcus*[1].

Property	***Pediococcus acidilactici***	***Pediococcus pentosaceus***	***Pediococcus damnosus***
% G+C	38–44	37	37–42
Growth at:			
35°C	+	+	–
40°C	+	+	–
50°C	+	–	–
Optimum growth temperature °C)	40	28–32	28–32
Growth at:			
pH 4.2	+	+	+
pH 7.0	+	+	–
Growth in			
4.0% NaCl	+	+	–
6.5% NaCl	+	+	–
Arginine hydrolysis	+	+	–

[1] Adapted from Bergey's Manual of Systematic Bacteriology, Vol. 3, 2011.

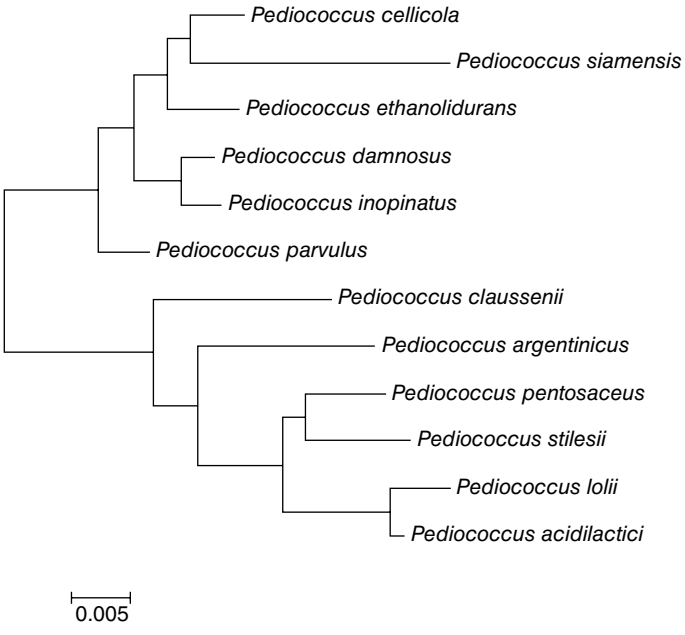


Figure 2.6 Phylogeny of *Pediococcus* based on 16S rRNA sequence analysis.

The pediococci can be found in diverse habitats, including plant material, milk, brines, animal urine, and beer. There are currently 12 recognized species of *Pediococcus* (Figure 2.6); several are important in food fermentations. Two species, *Pediococcus acidilactici* and *Pediococcus pentosaceus* are naturally present in raw vegetables, where, under suitable conditions, they play a key role in the manufacture of sauerkraut and other fermented vegetables. These same species may also be added to meat to produce fermented sausages. Despite their inability to ferment lactose, *P. acidilactici* and *P. pentosaceus* are occasionally found in cheese, where they may participate in the ripening process. Other strains are important in fermented cereal products (Chapter 14). Pediococci are also important as spoilage organisms in fermented foods, in particular, beer, wine, and cider. One species, *Pediococcus damnosus* is especially a problem in beer, where it produces diacetyl, which in beer is a serious defect. This strain also may be involved in wine spoilage, where it has been implicated in the formation of biogenic amines.

Plasmids are frequently present in pediococci. The genes located on these plasmids may encode for functions involved in sugar metabolism (e.g., raffinose and sucrose) and production of bacteriocins. The latter (described in Chapter 7) are defined as anti-microbial proteins that inhibit closely related bacteria, including the meat-associated pathogens, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridium botulinum*. Bacteriocin production is a particularly important trait in pediococci, since these bacteria are used as starter cultures for sausage fermentations. Thus, bacteriocin-producing pediococci could provide an additional level of preservation when used in sausage manufacture.

Tetragenococcus

Like the pediococci, *Tetragenococcus* are homofermentative, tetrad-forming, facultative anaerobes. They are mesophilic and neutraphilic, with temperature optima generally between 25 and 30°C and pH optima between 6.5 and 8.0. Indeed, *Tetragenococcus* are

unique among other lactic acid bacteria in having an optimum growth pH in the alkaline range. The genus was first recognized in 1990, when *Pediococcus halophilus* was reclassified as *Tetragenococcus halophilus*. In 1997, a second species, *Tetragenococcus muriaticus*, isolated from salty fish sauce, was proposed, and in 2005, *Enterococcus solitarius* was reclassified as *Tetragenococcus solitarius*. *Tetragenococcus koreensis*, isolated from kimchi, and *Tetragenococcus osmophilus*, isolated from sugar thick juice, were described in 2005 and 2012, respectively.

Based on 16S rRNA sequences, these bacteria are phylogenetically rather distant to *Pediococcus,* which is surprising given their morphological and physiological similarities. All of these species are characterized by their remarkable tolerance to salt. Not only is growth possible in media containing up to 25% salt, but maximum growth rates require the presence of 3 to 10% NaCl, and no growth occurs when salt is absent. They also grow well when the water activity is low, presumably due to their ability to maintain osmotic balance by accumulating betaine, carnitine, and other compatible solutes. However, it should be noted that some species (*T. halophilus*, in particular) grow well at high salt concentrations but not at high sugar concentrations. Thus, the response to high osmotic and high salt environments are not the same, at least for this species.

Lactobacillus

When the first edition of this book was published in 2006, the genus, *Lactobacillus*, consisted of more than 80 validly described species. In the last decade, microbial taxonomists have been very busy, proposing and validating many new taxa. Currently (May, 2018), there are more than 250 species and subspecies of *Lactobacillus* listed in the "List of prokaryotic names with standing in nomenclature". In the International Journal of Systematic and Evolutionary Microbiology, nearly 20 new species were described just in the past 3 years.

The genus is marked by its heterogeneity. About all that is common about the species within this genus is that they are all rods, but even this description is not wholly satisfactory. There are species that appear rather short (<1.5 μm), whereas others are more than 5 μm in length (some are reportedly up to 10 μm). They may also have a slender, curved, or bent appearance. Thus, when viewed microscopically, it is difficult to be sure that a given strain is even a rod. On agar plates, colony morphology is also variable, with some strains producing large round colonies, and others producing small or irregular colonies. Although the lactic acid bacteria, as a group, generally have a low %G+C (35–40), some lactobacilli have %G+C as low as 32 and others are as high as 55. Not only does this genus contain far more species, with extensive morphological heterogeneity, than any other genera of lactic acid bacteria, but this group is also the most ecologically, physiologically, biochemically, and phylogenetically diverse (Figure 2.7).

Ecologically, lactobacilli occupy a wide range of habitats. Except for very extreme environments, there are few locales where lactobacilli are not found, and they are often described as being ubiquitous in nature. Some species are normal inhabitants of plant and vegetable material, and they are frequently found in dairy and meat environments, in juice and fermented beverages, and in grains and cereal products. Their presence in the animal and human gastrointestinal tract (as well as in the stomach, mouth and vagina) has led to the suggestion (which has gained substantial scientific support) that these bacteria contribute to intestinal, as well as extra-intestinal health. In foods, they not only are key participants in numerous

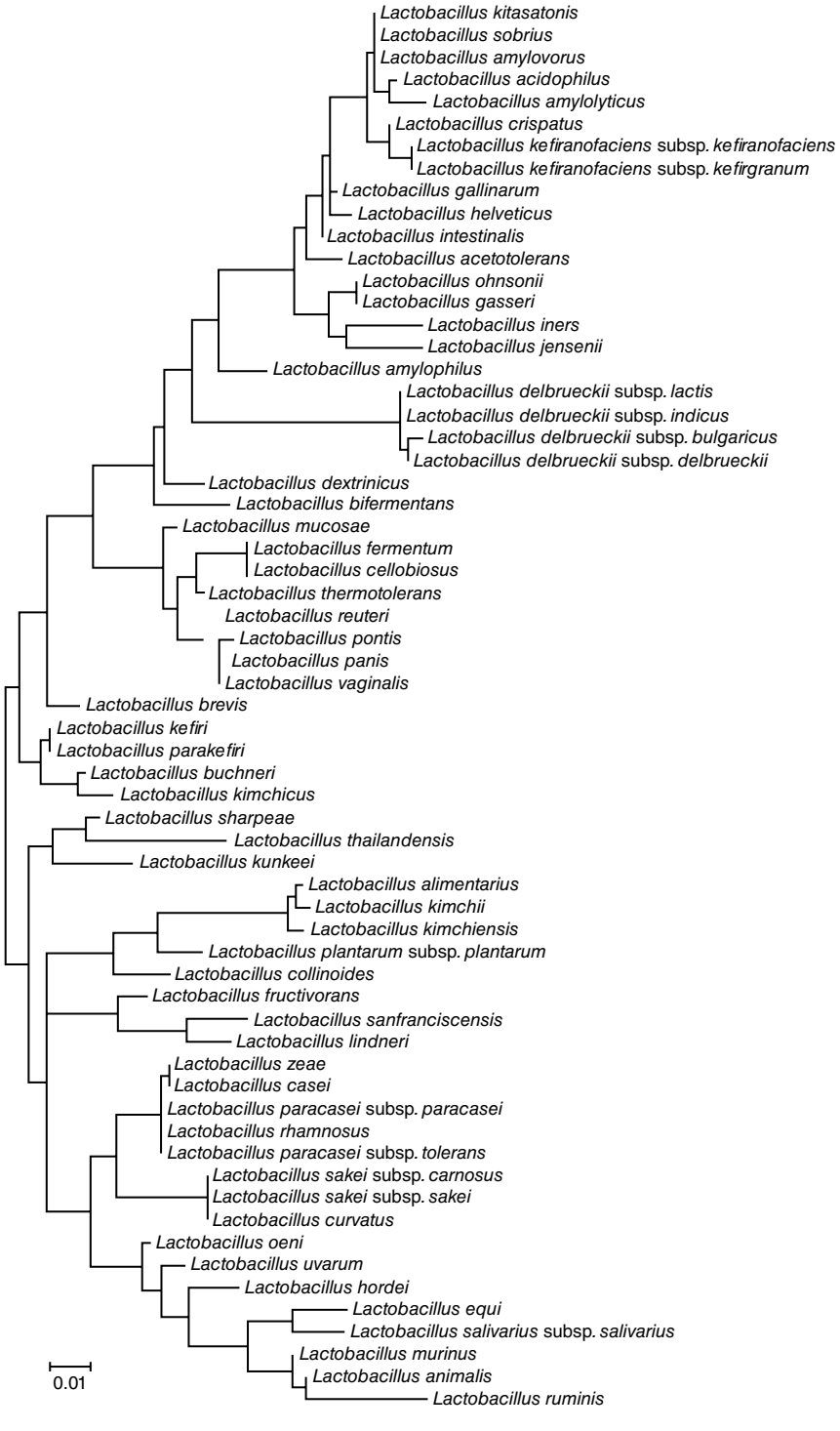


Figure 2.7 Phylogeny of *Lactobacillus* based on 16S rRNA sequence analysis. Not all of the species are listed.

fermentations, but are also frequently implicated in spoilage of fermented and non-fermented foods.

The ability of lactobacilli to grow and persist in so many diverse environments and conditions is a reflection of their diverse physiological properties. Although most species are mesophilic, the genus also contains species that are psychrotrophic, thermoduric, or thermophilic. Their temperature optima varies widely, from 30 to 45°C. Some species show tolerance to salt, osmotic pressure, and low water activity. Acid-tolerance is a common trait of lactobacilli, and many strains actually prefer an acidic environment. Some lactobacilli are also ethanol-tolerant or bile-tolerant. Most species are aerotolerant, whereas others require more strict anaerobic conditions.

Like all lactic acid bacteria, lactobacilli are fermentative, but, again, they are also more metabolically diverse than other lactic acid bacteria. In fact, one major way to subdivide the species is based on the pathways they use to ferment sugars. As noted previously, lactic acid bacteria ferment sugars in either homofermentative or heterofermentative fashion. However, some species of *Lactobacillus* have the genetic and physiological capacity to ferment sugars by either pathway. Accordingly, Group I lactobacilli consists of obligate homofermentative species, Group II contains facultative heterofermentative species (i.e., both pathways present), and Group III contains obligate heterofermentative species (Table 2.5). In addition, the ability to grow aerobically and to perform respiration (described in Chapter 3) has been suggested for some species.

Despite their metabolic diversity, some lactobacilli can be quite fastidious, and many species require nutrient-rich environments. They are not especially proteolytic or lipolytic, and amino acids, peptides, and fatty acids are usually required for rapid growth. Some strains are particularly demanding, requiring an array of vitamins, nucelotides, and other nutrients. Most lactobacilli also need fermentable carbohydrates, and, depending on their source or habitat, they ferment a wide array of sugars. Thus, not only are most common sugars (e.g., glucose, fructose, lactose) fermented, but many plant-derived carbohydrates, such as cellobiose, amygdalin, and trehalose, can also be utilized. Several species even ferment starch and other polysaccharides.

There are numerous species of *Lactobacillus* that are relevant in fermented foods. Some are added directly in the form of starter cultures (Chapter 4), but there are even more endogenous lactobacilli present in the raw material or equipment surfaces that indirectly contribute to the manufacture or to the finished properties of fermented foods. Starter culture lactobacilli are used primarily in dairy and sausage applications. There are two main species used as dairy starter cultures (mainly for cheese and yogurt), *Lactobacillus helveticus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. Other common dairy-related species include *L. casei* and *Lactobacillus acidophilus* (both used frequently as probiotics). Starter cultures for sausage fermentations often contain *Lactobacillus plantarum* or *Lactobacillus sakei* subsp. *sakei*.

Sourdough breads are also made using heterofermentative *Lactobacillus sanfranciscensis*, *L. brevis*, and other lactobacilli. Pure starter cultures for sourdough breads are available, but wild cultures, propagated in house, are still commonly used (Chapter 9). For the production of sauerkraut, kimchi, and other fermented vegetables, the natural microflora is all that is necessary to initiate and perform the fermentation, although pure starter cultures, containing *L. plantarum* and *L. brevis* and related species have become more common for so-called controlled pickle fermentations.

Finally, it is very important to emphasize, despite the general association of a particular species with a particular fermented food, that a given species will often be used or appear in

Table 2.5 Fermentation characteristics of *Lactobacillus*[1].

Group	Representative strains	% G+C	Growth at 15°C	Fermentation of: Cel[2]	Fru	Gal	Lac	Mal	Starch	Suc	Arginine Hydrolysis
Group I Obligate homofermentative	*Lactobacillus acidophilus*	34–37	–	+	+	+	+	+	nd[3]	+	–
	Lactobacillus delbrueckii	49–51	–	±[4]	+	–	+	±	nd	±	±
	Lactobacillus helveticus	38–40	–	+	±	+	+	±	nd	–	–
	Lactobacillus amylophilus	44–46	+	–	+	+	–	+	+	–	nd
	Lactobacillus amylovorus	40–41	–	+	+	+	–	+	+	+	nd
	Lactobacillus crispatus	35–38	–	+	+	+	+	+	nd	+	–
	Lactobacillus gasseri	33–35	–	+	+	+	±	±	nd	+	–
	Lactobacillus jensenii	35–37	–	+	+	+	–	±	nd	+	+
Group II Facultative heterofermentative	*Lactobacillus paracasei*	46	nd	+	+	+	±	+	nd	+	–
	Lactobacillus curvatus	43	nd	+	+	+	±	+	nd	–	–
	Lactobacillus plantarum	45	+	+	+	+	+	+	nd	+	–
	Lactobacillus sakei	43	nd	+	+	+	+	+	nd	+	–
	Lactobacillus bavaricus	43	nd	+	+	+	+	+	nd	+	–
	Lactobacillus homohiochii	36	+	±	+	–	–	+	nd	–	–
	Lactobacillus coryniformis	36	+	–	+	+	±	+	nd	+	–
	Lactobacillus alimentarius	36	nd	+	+	+	–	+	nd	+	–
Group III Obligate heterofermentative	*Lactobacillus fermentum*	53	+	±	+	+	+	+	nd	+	+
	Lactobacillus sanfranciscensis	37	nd	–	–	+	–	+	nd	–	–
	Lactobacillus reuteri	41	nd	–	+	+	+	+	nd	+	+
	Lactobacillus buchneri	45	nd	–	+	±	±	+	nd	±	+
	Lactobacillus brevis	45	nd	–	+	±	±	+	nd	±	+
	Lactobacillus kimchii	35	+	+	+	w[5]	–	+	–	+	–
	Lactobacillus kefiri	41	nd	–	+	–	+	+	nd	–	+
	Lactobacillus divergens	34	+	+	+	±	–	+	nd	+	+

[1] Adapted from Bergey's Manual of Systematic Bacteriology, Vol. 3, 2011.
[2] Cel = cellulose; Fru = fructose; Gal = galactose; Lac = lactose; Suc = sucrose.
[3] Not determined.
[4] Variable reaction, depending on strain or subspecies.
[5] Weak positive reaction.

quite dissimilar situations. In many cases, the species name may even add to the confusion. Thus, *L. sakei* was originally isolated from sake, Japanese rice wine, yet this same organism can be isolated from fermented sausage and is even used in sausage starter cultures. Likewise, *L. plantarum*, is not just found in plant material or in pickle brines, as its name might indicate, but has been suggested to be a normal inhabitant of the human intestinal tract.

As one would expect, the lactobacilli are genetically very diverse. Even genetically similar species, however, can have markedly dissimilar phenotypic properties. This is illustrated by a phylogenetic analysis in which 150 different members of the genus were organized into 19 phylogenetic groups. The largest of these, the *Lactobacillus delbrueckii* group, contained 27 species, including *L. delbrueckii* subsp. *bulgaricus* (one of the organisms involved in the yogurt fermentation) and *L. acidophilus* (well-known for its probiotic applications). While both of these species are obligate homofermenters associated with milk, other members of this phylo-group include *Lactobacillus acetolerans* and *Lactobacillus iners*, both of which are facultative heterofermentors and neither are able to ferment lactose. Neither does habitat necessarily dictate the phylogenetic position. For example, three species are associated with the kimchi fermentation, *Lactobacillus kimchii, Lactobacillus kimchiensis*, and *Lactobacillus kimchicus*. Yet the latter is clustered within the *collinoides* group, whereas *L. kimchi* and *L. kimchiensis* are located, some distance away, in the alimentarius group.

Plasmids are common in the lactobacilli, although for many of these plasmids, the functions of the encoded genes are not established. Lactose metabolism is encoded on at least one plasmid, and several bacteriocin production and antibiotic-resistant systems are also plasmid-encoded.

OTHER BACTERIA IMPORTANT IN FOOD FERMENTATIONS

In addition to the lactic acid bacteria, species from several other genera are involved in fermented foods, either as the primary culture or as culture adjuncts. In most cases, these bacteria are used for a singular purpose. That is, they are involved in just one application and to perform only one major function. These non-lactic acid bacteria represent several different genera and include both Gram-positive and Gram-negative bacteria. Their taxonomical position and general properties are given below. How they are actually used and the particular functions they perform in fermented foods are described in detail in the relevant chapters.

Acetobacter, Gluconobacter, and *Gluconoacetobacter*

The only Gram-negative bacteria used intentionally in the manufacture of fermented foods are the acetic acid-producing rods belonging to the genera, *Acetobacter*, *Gluconobacter*, and *Gluconoacetobacter* (in the *Proteobacteria* phylum, Family *Acetobacteraceae*). These bacteria are obligate aerobes, with a respiration-only metabolism. Their function is to make acetic acid via oxidation of ethanol. Some species may also have the capacity to further oxidize acetic acid completely to CO_2 and water. The acetic acid bacteria are mesophilic, with optimum growth temperatures about 25–30°C. Members of this group are acid-tolerant, however, their preferred growth pH is generally between 5.3 and 6.3. Some species of *Acetobacteraceae* are known to produce surface film and pigments.

Although *Acetobacter* were long considered to be the primary organisms involved in the vinegar fermentation, changes in the taxonomy of the acetic acid bacteria resulted in the

transfer of several important species from *Acetobacter* to *Gluconoacetobacter* and *Komagataeibacter*. However, *Acetobacter aceti* is still considered to be one of the main vinegar-producing bacteria, as it is the most commonly used species in pure culture processes and is also found in natural vinegar fermentations. Other species, however, are also frequently isolated from, or are used in vinegar fermentations, including *Acetobacter orleanensis*, *Acetobacter pasteurianus* subsp. *pasteurianus, Gluconoacetobacter europaeus*, and *Gluconoacetobacter xylinus.* In contrast to their more positive applications, in wine, beer, cider, and other ethanol-containing products, *Acetobacter*, *Gluconobacter*, and *Gluconoacetobacter* can also act as spoilage organisms. Finally, it is important to note that other bacteria, including species of *Acetobacterium* and *Clostridium*, also make acetic acid as a primary metabolic end-product, but these bacteria are obligate anaerobes and rely on reductive pathways (i.e., reduction of CO_2).

Bacillus

Species of the genus *Bacillus* are ubiquitous in foods, serving either as benign contaminants, as spoilage organisms, or occasionally as the cause of food poisoning syndromes. Strains of *Bacillus subtilis*, however, are also used for the production of an Asian fermented soybean product called natto (Chapter 12). These natto strains are closely related to the widely used wild-type laboratory strain, *B. subtilis* Marburg 168, except that the former contain several biochemical and physiological properties that are required for the natto fermentation (Chapter 14).

Bifidobacterium

It is arguable whether species of *Bifidobacterium* should be considered as food fermentation organisms. They certainly have a fermentative metabolism, producing lactic and acetic acids. However, these bacteria are phylogenetically distant from lactic acid bacteria, and perhaps more to the point, are not used in the manufacture of any fermented food. In fact, bifidobacteria are rarely, if ever, found in raw food materials. Nonetheless, bifidobacteria are very important in certain foods, mostly fermented dairy products, because they are added as probiotics to deliver health benefits. The intestinal tract is their primary habitat, and their elevated presence in the human gastrointestinal tract is correlated with a reduced incidence of enteric infections and improvements in intestinal health. Bifidobacteria are now so frequently used as probiotic adjuncts in foods that they have become one of the most commercially important product lines for starter culture companies, being included in yogurt and other dairy culture formulations (Chapter 5), as well as a wide range of other products.

There are currently 50 recognized species and subspecies of *Bifidobacterium* (Figure 2.8), although only about 15 are ordinarily used commercially as probiotics. These include *Bifidobacterium bifidum*, *Bifidobacterium adolescentis*, *Bifidobacterium breve*, *Bifidobacterium longum,* and *Bifidobacterium animalis*. The widespread use of bifidobacteria in probiotic applications has also led to misappropriation of more popular species names and other nomenclature errors, intentional or otherwise. For example, the less-appetizing *B. animalis* supsp. *lactis*, is often shortened to as the more appealing *Bifidobacterium lactis.*

For many years (until the 1970s), bifidobacteria were classified in the genus *Lactobacillus*. It eventually became clear that they were phylogenetically distinct from the lactic

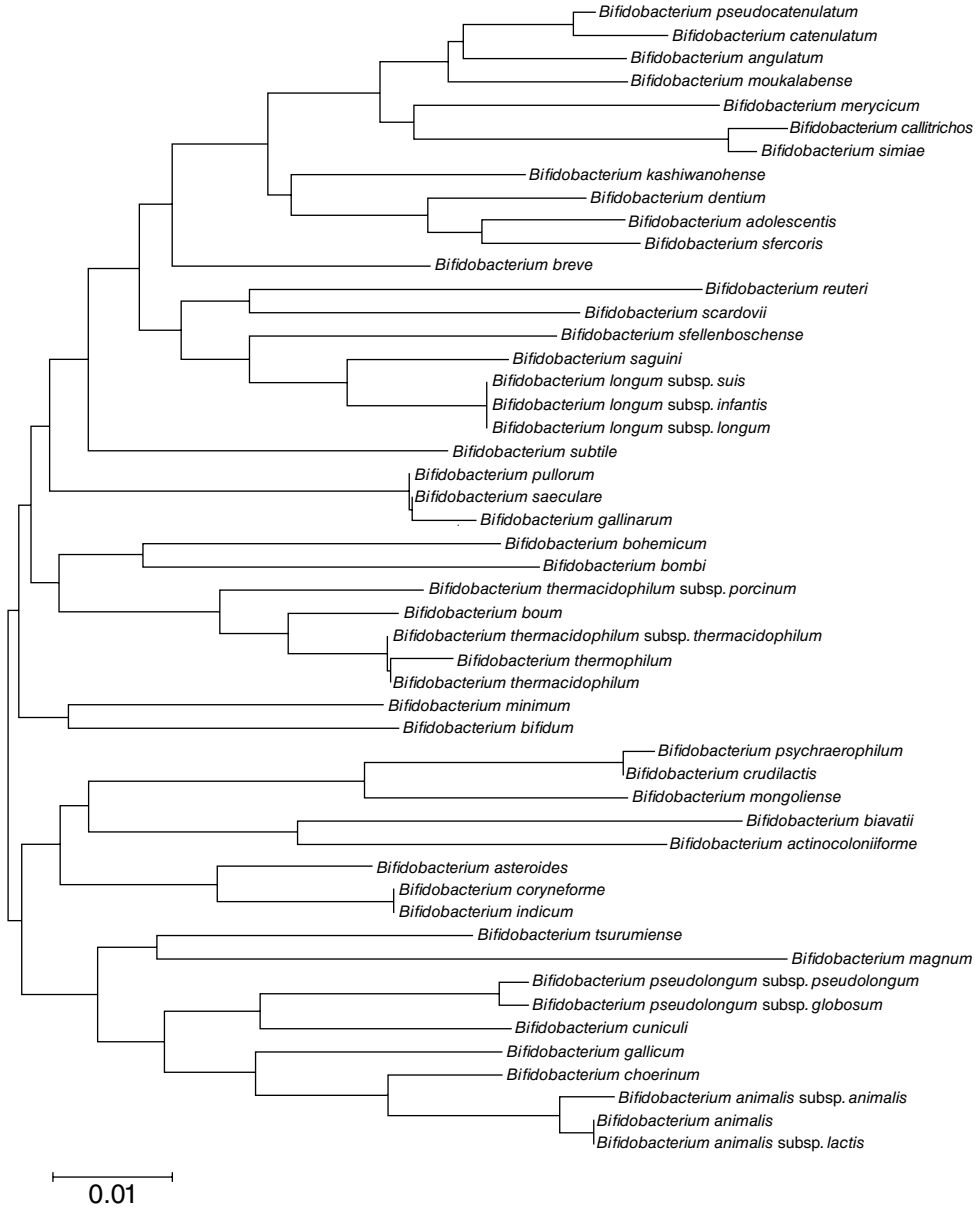


Figure 2.8 Phylogeny of *Bifidobacterium* based on 16S rRNA sequence analysis.

acid bacteria, being classified in an entirely different Phylum (*Actinobacteria*). Bifidobacteria are somewhat similar to the lactobacilli in that they are Gram-positive, non-motile, nonsporing rods, but they have a much higher G+C content (55–67 mol%). They also differ morphologically, with cells occurring in pairs with a V- or Y-like appearance. Bifidobacteria are strictly anaerobic and catalase negative, with a temperature optima between 37 and 41°C and a pH optima (for growth initiation) between 6.5 and 7.0. Most bifidobacteria are nutritionally fastidious and require vitamins and other nutrients for

growth. Their ability to utilize a wide array of carbohydrates, especially non-digestible oligosaccharides found in milk, may provide selective advantages in the colonic environment (discussed in Chapter 4). Sugar metabolism occurs primarily via the well-studied bifidum fermentation pathway that yields acetic acid and lactic acid. Bifidobacteria (except for *B. longum*) rarely contain plasmids.

Brevibacterium

The genus, *Brevibacterium* (Phylum, *Actinobacteria*) are described as non-motile, non-sporing, non-acid-fast, irregular-shaped organisms that belong to the coryneform group. Like other coryneform bacteria, *Brevibacterium* spp. are Gram-positive rods, but both their staining pattern and shape can vary, depending on the age and condition of the cells. They also are high G+C organisms (60–67 mol%). *Brevibacterium* are strictly aerobic, catalase-positive mesophiles, with an optimum growth temperature between 20 and 35°C. Most species are salt-tolerant (> 10%) and able to grow over a wide pH range. Currently, there are 35 recognized species. Several are of medical importance and have been considered as opportunistic pathogens. One species, *Brevibacterium linens*, however, is important in fermented foods, mainly because it is involved in the manufacture of bacterial, surface-ripened cheeses, such as Limburger and Munster. In these products, *B. linens* produces a yellow-orange-red pigment on the cheese surface that gives these cheeses their characteristic appearance. Their ability to hydrolyze proteins and metabolize amino acids, especially the sulfur-containing amino acids, also contributes to cheese ripening and flavor development in a variety of cheeses.

Kocuria, Micrococcus, and *Staphylococcus*

These organisms consist of Gram-positive, catalase positive, non-motile, non-sporeforming, aerobic cocci. Despite these similarities, *Kocuria and Micrococcus* belong to the *Actinobacteria,* and *Staphylococcus* is a Firmicute. Importantly, they are all coagulase negative, which distinguishes them from coagulase positive cocci (such as *Staphylococcus aureus*) that are associated with enterotoxin production. Only a few species are relevant to fermented foods. For the most part, their involvement is limited to the manufacture of dry fermented sausages (although some may show up as inadvertent contaminants in cheese and other products). In fermented meats, these bacteria are not used for acid formation (as they are non-fermentative); rather their function is to enhance flavor and color development. In particular, they produce aroma compounds, hydrolyze lipids and proteins, and reduce nitrate to nitrite. The latter property is important in European- or traditional style dry-cured sausages in which the curing agent contains nitrate salts, rather the active, nitrite form used in conventional sausage manufacture. This function is discussed in more detail in Chapter 6.

Propionibacterium

The propionibacteria are also in the phylum *Actinobacteria*. They are high G+C (53–68 mol%), non-sporing, Gram-positive, non-motile rods, often with a characteristic club-shaped appearance. They may also appear coccoidal or branched. Propionibacteria are catalase

positive (or variable), anaerobic to aerotolerant mesophiles. They are neutraphilic and grow slowly at pH 5.0–5.2, which happens to be the pH that exists in Swiss-type cheese.

In general, the propionibacteria are associated with two quite different habitats, the human skin and dairy products. The former group contains the cutaneous species, *Propionibacterium acnes* and *Propionibacterium avidum*, the organisms that cause acne. The dairy group consists of several species that are important in food fermentations, due to their use in the manufacture of Swiss-type cheeses. The most frequently used dairy species include *Propionibacterium freudenreichii* subsp. *shermanii*, *Propionibacterium freudenreichii subsp. freudenreichii*, *Propionibacterium acidopropionici*, and *Propionibacterium jensenii*. They have a mol% G+C content of 66–68% and are distinguished from other propionibacteria based mainly on morphological and biochemical characteristics, in that they are usually shorter rods and ferment fewer sugars. The ability to ferment lactose varies among strains, but in the absence of lactose, dairy strains are quite capable of using lactate as a carbon and energy source. Metabolic end-products include propionic acid, acetic acid, and carbon dioxide. Small amounts of Vitamin B_{12} may also be produced. During growth in a peptide-rich medium, such as cheese, propionibacteria release the amino acid, proline, via peptide hydrolysis. They also are lipolytic, releasing free fatty acids from triglycerides. Despite their role as eye-formers in Swiss-type cheese, they are not particularly prolific CO_2-producers.

YEASTS AND MOLDS USED IN THE MANUFACTURE OF FERMENTED FOODS

As many as 1.5 million different species of fungi are known to exist, but fortunately for this discussion, relatively few are involved in food fermentations. As described previously, yeasts and molds are in the Eukarya domain and belong to the Kingdom Fungi. Of the phyla within this Kingdom, most of the fungi relevant to fermented foods are classified as Zygomycota and Ascomycota. Included within these groups are several important genera of yeasts, in particular, *Saccharomyces*, *Kluyveromyces*, and *Zygosaccharomyces*. Several genera of mold are also important in fermented foods, including *Aspergillus*, *Penicillium*, and *Rhizopus.*

Fungi are eukaryotes and differ from prokaryotes in several major respects. Specifically, fungi have a much more complex physical structure and they reproduce in an entirely different manner compared to bacteria. Although molds and yeasts are both fungi, molds are multicellular and filamentous, whereas yeast are unicellular and non-filamentous. In addition, molds can produce asexual and sexual spores, while yeast produce only sexual spores or are non-sporulating.

The manner in which spores are formed is often used by mycologists to classify fungi. Mold spores are produced asexually and are usually referred to as conidia. These are the type of spores produced by *Penicillium* and *Aspergillus*. Conidia are borne on conidiophores, and the nature and shape of these structures can be used to help identify the mold. The other primary fermentation molds, *Rhizopus* and *Mucor*, produce sporangiospores in sporangia. Less commonly, a mold may fragment into smaller pieces for dispersal, and these are called arthrospores. Yeasts, in addition to producing ascospores, also reproduce asexually by budding, a process in which part of the cytoplasm bulges from the cell and eventually separates as a new cell. Budding can be either multilateral or polar, and is characteristic of the species.

Saccharomyces

A good argument could be made that yeasts belonging to the genus *Saccharomyces* are among the most important of all organisms used in fermented foods, perhaps more so than even the lactic acid bacteria. These yeasts are required, after all, for the production of beer, wine, and spirits (not to mention bread). These yeast-fermented products are not only popular for social, cultural, and aesthetic reason, but they also have a combined, world-wide economic impact in the trillions of dollars. In addition, the main species, *Saccharomyces cerevisiae*, is widely used as a model organism in biology and genetics, and its physiological and biochemical properties, as well as its genome sequence, have been well studied. At the gene level, *Saccharomyces* and other yeasts are much more complicated than are the bacteria. They have, for example, 16 chromosomes, and they can be diploidal or polyploidal (i.e., more than one set of chromosomes). Thus, the size of their genomes is several times larger than bacteria, with more than 6000 protein-encoding genes compared to about 2000 in most lactic acid bacteria. Nonetheless, the genomes of the common food fermentation strains, domesticated thousands of years ago, are perhaps among the best annotated of all eukaryotic genomes.

The taxonomy and nomenclature of *Saccharomyces* has been subject to rather regular and frequent revisions for over a century. This was especially true prior to molecular typing methods, when yeast taxonomy was based primarily on morphology and physiology (Hittinger, 2013). Although *S. cerevisiae* has long been one of the major species used in wine, brewing, and bread-making applications, the specific strains involved in the manufacture of these products are clearly different from each other and from laboratory strains of *S. cerevisiae*. The frequent changes in nomenclature have made it even more difficult to keep track of the particular species associated with a given fermentation. Several examples illustrate this point. Prior to the 1970s, *Saccharomyces ellipsoideus* was recognized as one of the primary wine yeasts and was accorded species status. It was then reclassified as *S. cerevisiae* (and demoted to synonym status), although the earlier name continues to be used. Similarly, another group of wine strains classified as *Saccharomyces uvarum* were reclassified as *Saccharomyces bayanus* (or sometimes listed as *Saccharomyces bayanus* subsp. *uvarum*). Finally, for many years, the species used in the lager fermentation (where lager is one of the two styles of beer) was classified as *Saccharomyces carlsbergensis*, which was also referred to by its synonym, *Saccharomyces uvarum*. Both species then merged as *S. uvarum*, only to be reclassified yet again, first as *Saccharomyces bayanus* and then as *Saccharomyces pastorianus*. The latter is now considered the valid species name for the lager yeast. In the most recent edition (2011) of *The Yeasts, A Taxonomic Study*, there were eight accepted species of *Saccharomyces* and two varieties. These correspond to the seven so-called "naturally occurring" species recognized by Hittinger (2013): *S. arboricola*, *Saccharomyces eubayanus, S. cerevisiae*, *Saccharomyces kudriavzevii*, *Saccharomyces mikatae*, *Saccharomyces paradoxus*, and *S. uvarum* (with the addition of *S. eubayanus*, published after *The Yeasts* went to press), plus *S. bayanus* and *S. pastorianus.* Genome sequencing of the latter species has revealed they are actually hybrids – strains that have combined genomes (Figure 2.9).

As noted above, distinguishing between species of *Saccharomyces* has historically been based on morphological, physiological, and biochemical properties. These yeasts usually have a round spherical or ovoid appearance, but they may be elongated with a pseudohyphae. The sugar fermentation patterns and the assimilation of carbon sources are key factors for speciation (Table 2.6; also see Chapter 10 for beer yeast speciation). Other specific diagnostic tests include hyphae formation, ascospore formation, resistance to cycloheximide, and

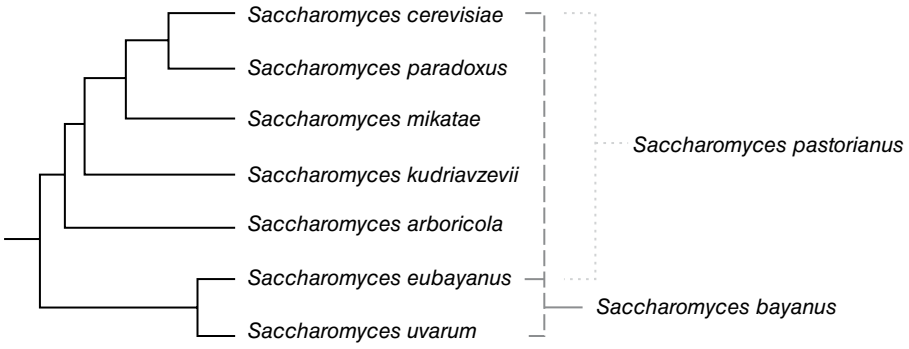


Figure 2.9 Phylogeny of seven natural species of *Saccharomyces* and their key industrial hybrids. Adapted from Hittinger, 2013, with permission.

growth temperatures. Several physiological traits vary among the *Saccharomyces* and are useful not only for classification, but are important for strain selection. Some strains, for example, are very osmophilic and halotolerant, and can grow in foods containing high concentrations of carbohydrates (e.g., high sugar grapes) or salt (soy sauce).

Nowhere, perhaps, does the species of yeasts influence the fermentation as much as in brewing. As noted above, there are two general styles of beer, ales and lagers. Each requires a specific yeast, *S. cerevisiae* for ales and *S. pastorianus* for lagers. These yeasts vary in several respects (reviewed in Chapter 10), but mainly on the basis of their flocculation properties in beer. Hence, ale yeasts are referred to as top-fermenting yeasts because they tend to flocculate at the top of the fermentation vessel, and lager yeasts are referred to as bottom-fermenting because they settle at the bottom of the fermentor. It is important to note that however useful these phenotypic characteristics are for classification, they are not always consistent with species assignments based on the sequences of 18S rRNA and other regions.

Ecologically, *Saccharomyces* and other yeasts are ubiquitous in foods. In some fermented foods, such as wine, their natural presence on grapes and equipment is sufficient to initiate fermentation. Several different yeasts may be involved in natural or spontaneous wine fermentations, usually in a successive manner, where one species is dominant for a time, then gives way to others (see Chapter 11). However, in most modern wine fermentations, as well as beer and bread fermentations, starter culture yeasts are used, selected on the basis of their physiological and biochemical properties. Yeasts also contribute flavor and quality attributes to other fermented foods, including fermented meats, soy-fermented foods, and a wide variety of cheeses. Finally, yeasts are also important in spoilage of fermented (and non-fermented) foods. For example, one of the serious defects in sauerkraut is caused by the pink pigment-producing yeast, *Rhodotorula*.

Penicillium and *Aspergillus*

As previously mentioned, these molds are among the most common and widespread genera of molds founds in foods. In the older literature, they were often referred to as Fungi Imperfecti or Deuteromycetes due to the absence of a sexual stage in their life cycle. Both *Penicillium* and *Aspergillus* are of concern to food microbiologists, in part due to their role in food spoilage and as potential producers of mycotoxins. However, some species of *Penicillium* and *Aspergillus* are also used to produce fermented foods. In fact, one of the

Table 2.6 Distinguishing characteristics of *Saccharomyces* species[1].

	Fermentation of:			Assimilation of:							Growth on		
Species	**Gal[2]**	**Mal**	**Mel**	**Me-glu**	**Gal**	**Mal**	**Tre**	**Mel**	**Inu**	**Rib**	**Gtl**	**Me-glu**	**10% NaCl/5% Glu**
S. bayanus	–	–	–	–	+	+	+	–	–	+	–	+	–
S. cariocanus	+	–	–	–	+	–	–	–	l[3]	–	–	–	l
S. cerevisiae	–	–	–	–	+	+	+	–	–	+	–	+	–
S. kudriavzevii	–	s[4]	–	+	–	–	–	–	+	l	+	+	–
S. mikatae	+	–	+	+	+	+	l	l	–	+	+	+	–
S. paradoxus	–	–	–	–	+	+	+	–	–	+	–	+	–
S. pastorianus	–	–	–	–	+	+	+	–	–	+	–	+	+

[1] Adapted from Naumov et al., 2000.
[2] Abbreviations: Gal. galactose; Mal, maltose; Mel, melibiose; Me-glu, methyl glucoside; Tre, trehalose; Inu, inulin; Rib, ribitol; Gtl, galactitol; Glu, glucose.
[3] Latent (delayed positive).
[4] Slow.

most famous of all organisms used in fermented foods is *Penicillium roqueforti.* This mold produces blue-pigmented mycelia that gives Roquefort and other blue-veined cheeses their characteristic color. The mold is also responsible for much of the flavor and aroma properties of these cheeses. A related white-mycelia producing species, *Penicillium camemberti*, is equally important (and no less famous), due to its involvement in the manufacture of Camembert and Brie cheeses. Various *Penicillium* spp. are also important in mold-ripened meat products.

Although the role of *Aspergillus* in fermented foods manufacture is somewhat less prominent (at least in foods popular in Western cultures), species of *Aspergillus* are involved in some of the world's most widely-consumed fermented foods. Specifically, two main species, *Aspergillus oryzae* and *Aspergillus sojae*, serve a critical function in the manufacture of several fermented foods associated with Asian cuisines, including soy sauce, miso, and rice wines. The fermentation of these foods, which are consumed literally by billions of people (Chapter 14), begins with the production of koji. The latter is prepared by inoculating *A. oryzae* or *A. sojae* on the surface of rice. As they grow, they express proteolytic and amylolytic enzymes necessary for subsequent fermentations.

There are more than 350 different species of *Penicillium* and more than 330 species of *Aspergillus*. Like the yeasts, speciation is based on morphology, structure, and spore type. Biochemical and physiological properties of the fungi, however, are generally less diagnostic. These fungi are heterotrophic and saprophytic, and are usually very good at

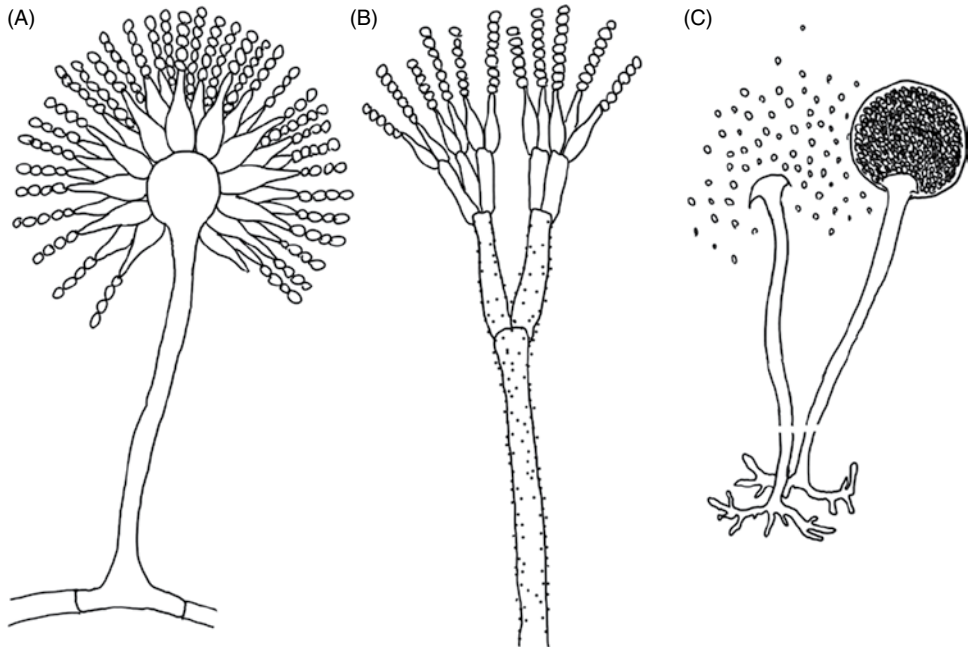


Figure 2.10 Common molds associated with food fermentation and spoilage. A. Aspergillus: flask-shaped phialides produce chains of spores and radiate outwards from a conidiophore that ends in a basal foot cell. B. *Penicillium*: flask-shaped phialides which produce chains of spores commonly arise from one or more branching structures. C. *Rhizopus*: spores are enclosed in a sporangium (right), which often ruptures to release the spores, leaving behind the central columella (left). Not drawn to scale. Figure courtesy of Heather Hallen-Adams, University of Nebraska.

breaking down complex macromolecules (e.g., protein, polysaccharides, lipids) via secretion of proteinases, amylases, and lipases. The hydrolysis products can then be used as substrates for growth. Despite their readily observed structural differences, *Penicillium* and *Aspergillus* do share some common features. Both are filamentous and produce conidiospores, and are probably related. Typical spore-bearing structures of *Penicillium* and *Aspergillus* are shown in Figure 2.10. Finally, *Rhizopus oligosporus* species are used to produce a fermented soybean product known as tempeh that is popular in Indonesia and other Southeast Asia countries (Chapter 14). Typical morphological structures of *Rhizopus* are shown in Figure 2.10C.

MICROORGANISMS IN FERMENTED FOODS – IT TAKES A COMMUNITY!

Much of the above discussion on the microorganisms involved in fermented foods focused on individual species and genera. Although this might give the impression that individual organisms play the major role in food and beverage fermentations, this is only partially true. Rather, for many fermented products, successful fermentation relies on groups of microorganisms acting in concert and subject to basic ecological principles. Thus, many food and beverage fermentations depend on succession events, and in others mutualism or synergism are important. Competition and antagonism are also common.

Understanding the dynamic nature of food fermentations has been especially enhanced in just the past few years due to next generation sequencing capability and application of bioinformatics tools. No longer is it necessary to cultivate organisms on Petri plates, a task of admittedly limited value, given that many relevant organisms do not grow well on artificial media or under laboratory conditions. However, thanks to culture-independent, molecular-based methods it is now possible to identify and quantify the microbial members of these complex food communities. Moreover, changes that occur over time, and even, where appropriate, by location within a food, can also be assessed. Thus, the ecological relationships that occur during fermentation can now be better understood, as noted in subsequent chapters. Certainly, the accessibility of entire microbiomes provides opportunities to explore food fermentations to an extent early microbiologists could hardly have imagined.

BIBLIOGRAPHY

De Oliveira, T.B., E. Gomes, and A. Rodrigues. 2015. Thermophilic fungi in the new age of fungal taxonomy. Extremophiles 19:31–37.

Gibbons, J.G. and D.C. Rinker. 2015. The genomics of microbial domestication in the fermented food environment. Curr. Opin. Genet. Dev., 35:1-8.

Hittinger, C.T. 2013. *Saccharomyces* diversity and evolution: a budding model genus. Trend Genet. 29:309–317.

Holzapfel, W.H. and B.J.B. Wood, eds. 2014. Lactic Acid Bacteria: Biodiversity and Taxonomy, 1st edn. John Wiley & Sons, Oxford.

Jones, A.L. 2012. The future of taxonomy. Adv. Appl. Microbiol. 80:23–35.

De Vos, P., G.M. Garrity, D. Jones, N.R. Krieg, W. Ludwig, F.A. Rainey, K.-H. Schleifer, and W.B. Whitman. 2009. Bergey's Manual of Systematic Bacteriology, 2nd edn, Vol. 3. The Firmicutes. Springer, New York.

Kirk, P.M., P.F. Cannon, D.M. Minter, and J.A. Stalpers. 2008. Dictionary of Fungi, 10th edn. CAB International, Oxford.

Kurtzman, C.P., J.W. Fell, and T. Boekhout. 2011. Definition, classification and nomenclature of the yeasts. In: Kurtzman, C.P., J.W. Fell, and T. Boekhout, eds. The Yeasts, a Taxonomic Study. Volume 1, 5th edn. Elsevier, New York, NY, pp. 3–5.

Ludwig, W., K.-H. Schleifer, and W.B. Whitman. 2009. Taxonomic outline to the phylum Firmicutes. Bergey's Manual of Systematic Bacteriology, vol. 3. Springer-Verlag, New York.

Naumov, G.I., S.A. James, E.S. Naumova, E.J. Louis, and I.N. Roberts. 2000. Three new species in the *Saccharomyces sensu stricto* complex: *Saccharomyces cariocanus*, *Saccharomyces kudriavzevii* and *Saccharomyces mikatae*. Int. J. Syst. Evol. Microbiol. 50:1931–1942.

Samson, R.A., C.M. Visagie, J. Houbraken, S.-B. Hong, V. Hubka, C.H.W. Klaassen, G. Perrone, K.A. Seifert, A. Susca, J.B. Tanney, J. Varga, S. Kocsube, G. Szigeti, T. Yaguchi, and J.C. Frisvad. 2014. Phylogeny, identification and nomenclature of the genus *Aspergillus*. Stud. Mycol. 78:141–173.

Sneath, P.H.A. 1957. Some thoughts on bacterial classification. J. Gen. Microbial. 17:184–200.

Steensels, J. and K.J. Verstrepen. 2014. Taming wild yeast: potential of conventional and nonconventional yeasts in industrial fermentations. Annu. Rev. Microbiol. 68:61–80.

Vaughan-Martini, A. and A. Martini. 2011. A taxonomic study. In: The Yeasts, 5th edn. C. Kurtzman, J.W. Fell, and T. Boekhout, eds. Elsevier, Oxford.

Visagie, C.M., J. Houbraken, J.C. Frisvad, S.-B. Hong, C.H.W. Klaassen, G. Perrone, K.A. Seifert, J. Varga, T. Yaguchi, and R.A. Samson. 2014. Identification and nomenclature of the genus *Penicillium*. Stud. Mycol. 78:343–371.

3 Metabolism and physiology

> If an alien visited Earth, they would take some note of humans, but probably spend most of their time trying to understand the dominant form of life on our planet – microorganisms like bacteria and viruses.
>
> Nathan Wolfe, Virologist (as quoted in National Geographic)

FERMENTATION BASICS

For those students fresh from a general biochemistry course, they might recall that "fermentation" was defined in their biochemistry text as something like "energy-yielding reactions in which an organic molecule is the electron acceptor". The definition might have also included wording about fermentation being an anaerobic process and that the starting substrate is glucose or some other simple sugar. Thus, in the context of the lactic acid fermentation, the pyruvic acid that is generated from glucose via the anaerobic or glycolytic pathway would serve as the electron acceptor to form lactic acid. Likewise, in the ethanolic pathway, acetaldehyde, formed by decarboxylation of pyruvate, is the electron recipient (forming ethanol). This biochemical definition is certainly true for many of the fermentations that occur in foods. However, it is not totally adequate. For several fermented foods, important end-products are produced via non-fermentative pathways (as classically defined). For example, the malolactic fermentation that is very important in wine making is really just a decarboxylation reaction that does not conform, strictly speaking, to the definition stated above. Nor does this definition apply in a range of other fermented foods, such as tempeh and koji. In these fermentations, proteins and polysaccharides are degraded and metabolized by fungi, but without production of glycolytic end-products. Therefore, in this book, we will not be confined to the classic, narrower definition of fermentation. Rather, the term fermentation will be used in a broader sense, accounting for the many metabolic and enzymatic processes that occur during the course of food fermentations.

For the microorganisms that actually perform fermentations, the process is merely the means by which they obtain energy or satisfy their nitrogen or carbon requirements. Microorganisms, after all, need energy to perform work (e.g., nutrient transport and biosynthesis), to maintain chemical and physical homeostasis (e.g., ionic and osmotic), and to grow

Microbiology and Technology of Fermented Foods, Second Edition. Robert W. Hutkins.

and replicate. For the most part, the energy generated by fermentation is in the form of ATP, and usually it is produced via metabolism of sugars (although, exceptions, as suggested above, for both of these claims exist). Whether metabolism by microorganisms also results in the conversion of milk into yogurt or juice into wine is of no concern to the organism. Indeed, the same metabolic pathway may also lead to formation of end-products that cause milk or juice to become spoiled, which matters little to the offending organism. However, these outcomes do matter to yogurt and wine manufacturers. Knowing how to control and manipulate microorganisms and their metabolic activities can mean the difference between a pleasant-tasting container of yogurt or soured milk that is tossed down the drain, between success and failure, and between profit and loss. Therefore, understanding the biochemical basis for metabolism of sugars, as well as other substrates, is essential for consistent production of fermented foods having the expected biological, physical, chemical, nutritional, and sensory characteristics.

SUGAR METABOLISM

Fermentation biochemistry and microbial physiology were first studied by Pasteur, Buchner, Schwann, and other early microbiologists and biochemists. That food-related sugar fermentations, in particular, had attracted the attention of these scientists was actually quite reasonable. After all, fermented milk products, beer, and wine were culturally and industrially-important products whose manufacture depended on fermentation of sugars by microorganisms. Thus, identifying the organisms and pathways was essential to understanding how the manufacture of these products could be controlled or improved and to prevent or delay spoilage. In the sections to follow, metabolism and transport of sugars by lactic acid bacteria and ethanol-producing yeasts will be reviewed.

Homofermentation

Lactic acid bacteria are, in general, obligate fermentors, and do not obtain energy by oxidative or respiratory processes, albeit with one major exception (Box 3.1). Technically, metabolic processes do exist, such as the precursor-product exchange systems described below, that provide an alternative way for these organisms to earn ATP "credits". Evidently, microorganisms have evolved these pathways in order to conserve the energy units that would ordinarily be required to perform metabolic work. Nonetheless, the substrate level phosphorylation reactions that occur during fermentation are the major means by which these cells make ATP.

For homofermentative lactic acid bacteria, hexoses are metabolized via the enzymes of the glycolytic Embden-Meyerhof-Parnas (EMP) pathway. One of the key enzymes of this pathway is aldolase, which commits the sugar to the pathway by splitting fructose-1,6-diphosphate into the two triose phosphates that eventually serve as substrates for ATP-generating reactions. The EMP pathway yields two moles of pyruvate and two moles of ATP per mole of hexose (Figure 3.1). The pyruvate is then reduced to L- or D-lactate by the enzyme, lactate dehydrogenase. More than 90% of the substrate is converted to lactic acid during homofermentative metabolism. Importantly, the NADH formed during the glyceraldehyde-3-phosphate dehydrogenase reaction must be re-oxidized by lactate dehydrogenase, so that the [NADH]/[NAD^+] balance is maintained. Many important lactic acid bacteria are homofermentative, including *Lactococcus lactis*, *Streptococcus thermophilus*, *Lactobacillus helveticus*, and *Lactobacillus delbrueckii* subsp. *bulgaricus* (used as dairy starter

Box 3.1 Respiration in lactic acid bacteria: From discovery to application

Lactic acid bacteria were among the first bacteria studied by microbiologists. Certainly this was because, in large part, of their practical importance in fermented foods. Thus, their physiological and biochemical properties, established nearly a century ago (Orla-Jensen, 1919), were relevant for diagnostic as well as functional purposes. Although advances in microbial physiology, biochemistry and genetics significantly enhanced understanding of these bacteria, their fundamental phenotype remained consistent.

Among the tenets of lactic acid bacteria physiology was that these bacteria lacked cytochromes and a functional electron transport system. Therefore, they were described as being unable to grow aerobically or produce ATP via respiration. Despite a few reports to the contrary (Ritchey and Seely, 1976; Sijpesteijn, 1970), this was dogma for generations of microbiology students and researchers who studied these bacteria. One can imagine the surprise, even disbelief, when researchers in France provided biochemical and genetic evidence that this view was not correct (Duwat et al., 2001; Gaudu et al., 2002). Indeed, they showed that an intact and functional respiratory pathway existed in *Lactococcus lactis*. Later, other lactic acid bacteria were shown to have similar pathways (Pederson et al., 2012). This discovery was not merely of academic interest. As described below, its significance to the field and its impact in the starter culture industry cannot be overstated.

Respiratory metabolism: A short review

Respiration, or oxidative metabolism, is the major means by which aerobic microorganisms obtain energy. During respiration, electrons generated during carbon metabolism (e.g., citric acid cycle) are transported across the cytoplasmic membrane via a series of electron carrier proteins. These proteins are arranged such that the flow of electrons (usually in the form of $NADH^{+}$) is toward increasing oxidation-reduction potentials. Along the way, protons are translocated across the membrane, effectively converting an oxidation-reduction potential into a proton electrochemical potential. This proton potential or proton motive force (PMF) can drive transport, operate flagella motors, or perform other energy-requiring reactions within the membrane. It can also be used to make ATP directly via the ATP synthase. This latter reaction (called oxidative phosphorylation) provides aerobes with most of their ATP and is the coupling step between the respiratory electron transport system and ATP formation.

Respiration in lactic acid bacteria

For respiration to occur in lactococci, several requirements must be met. First, the relevant genes encoding for electron transport proteins must be present and expressed and the functional proteins must be made. Analysis of genome data revealed that electron transport genes were present in a wild-type strain of *L. lactis* (Vido et al., 2004). It also appeared that the respiratory pathway was mostly intact (Figure 3.1.1A). The pathway was comprised of several proteins, including dehydrogenases, menaquinones, and cytochromes. In particular, cytochrome oxidase (encoded by *cydAB*) serves as the terminal oxidase and is essential (Gaudu et al., 2002). The only component missing from a fully functional electron transport system was a system for making

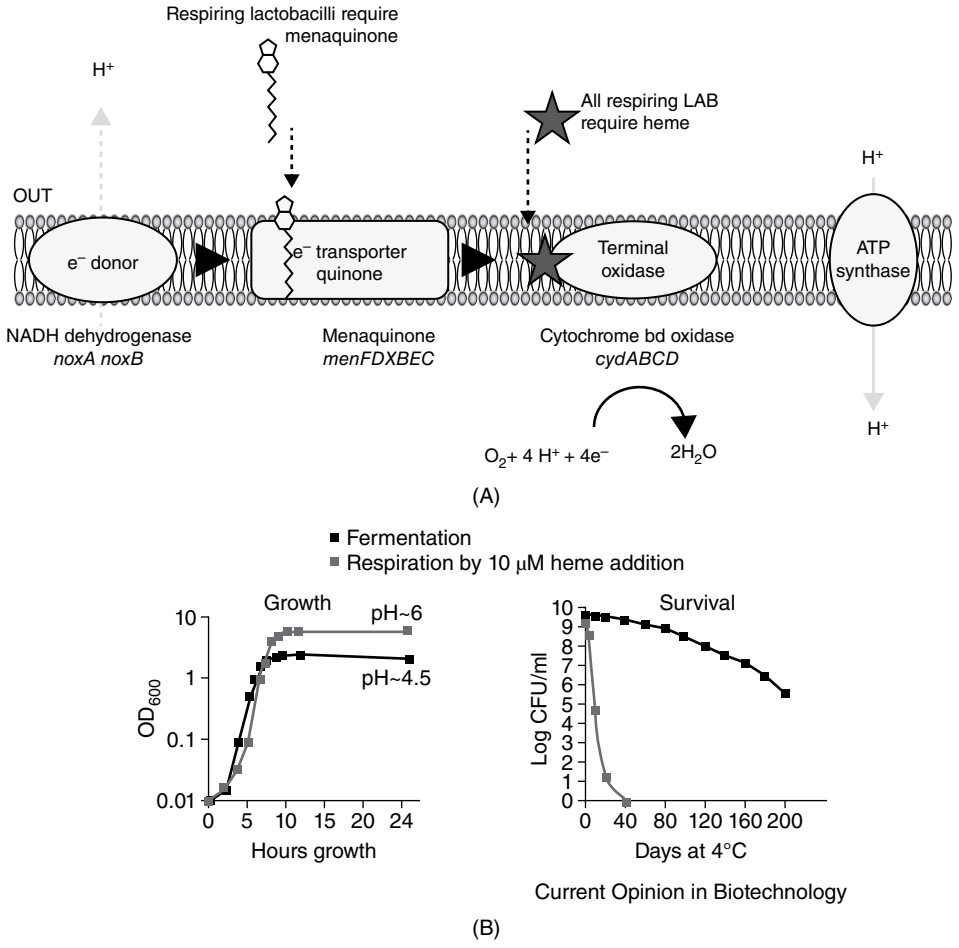


Figure 3.1.1 Respiration in lactic acid bacteria is summarized in A. Shown are the electron transport proteins, genes, and co-factors necessary for aerobic respiration. Growth and survival of *Lactococcus lactis* under fermentative and respiratory conditions and with or without heme are shown in B. From Lechardeur et al., 2011, with permission.

porphyrin groups. The latter is combined with iron to make heme, which is required for activity of cytochrome proteins, as well as the enzyme catalase. Thus, lactococci are heme auxotrophs, and heme (or a heme precursor) must be added to the medium for respiration to occur. For some lactic acid bacteria, a second electron transport factor, menaquinone, is also required.

Subsequent studies showed that respiratory growth follows a fermentative period and occurs primarily during the later stages of growth. Interestingly, expression of the heme transport system is subject to negative regulation, mediated by the catabolite control protein CcpA (Gaudu et al., 2003). The activity of CcpA is high during rapid growth on glucose and genes for other catabolic pathways are repressed (see below). Thus, it appears that CcpA might be the metabolic conduit between fermentation and respiration.

Practical considerations

The relevance of this discovery became even more significant when researchers performed growth experiments with lactococci grown under respiring conditions (i.e., aerobic, with heme added). Not only did cells perform respiratory metabolism, they also grew better (Duwat et al., 2001). That is, they grew for a longer time and reached higher cell densities compared to non-respiring cells. Moreover, during prolonged incubation at 4°C, respiring cells remained viable for a longer time. The enhanced viability was likely because the pH remained high (near 6.0), since less lactic acid was produced via fermentation (Figure 3.1.1B).

The practical significance of these findings should now be evident. Given that one of the main goals of the starter culture industry (Chapter 4) is to maximize biomass while maintaining cell viability, respiratory growth satisfies both goals. Thus, the respiration story certainly captured the attention of the starter culture industry (Pedersen et al., 2005). After all, being able to produce more cells per fermentation run is one of the most effective ways to increase profitability.

These findings on lactococci have been extended to other lactic acid bacteria including species of *Leuconostoc*, *Oenococcus*, and *Lactobacillus* (Brooijmans et al., 2009; Grandvalet, 2017; Pedersen et al., 2012; Ianniello et al., 2016; Zotta et al., 2014). However, several starter culture lactic acid bacteria, including *Streptococcus thermophilus* and *Lactobacillus delbrueckii*, evidently lack the ability to respire (Pedersen et al., 2012).

As had initially been suggested decades earlier, these more recent studies confirmed that molar growth and ATP yields are higher during respiratory growth. Thus, it may well be that the respiratory pathway provides these bacteria an alternative and efficient lifestyle choice. Pedersen suggested these pathways are constitutive, and that the bacteria are merely waiting for heme to appear (Pedersen et al., 2008; Pedersen et al., 2012).

References

Brooijmans, B., B. Smit, F. Santos, J. van Riel, W.M. de Vos, and J. Hugenholtz. 2009. Heme and menaquinone induced electron transport in lactic acid bacteria. Microb. Cell Fact. 8:28.

Duwat, P., S. Sourice, B. Cesselin, G. Lamberet, K. Vido, P. Gaudu, Y. Le Loir, F. Violet, P. Loubière, and A. Gruss. 2001. Respiration capacity of the fermenting bacterium *Lactococcus lactis* and its positive effects on growth and survival. J. Bacteriol. 183:4509–4516.

Gaudu, P., G. Lamberet, S. Poncet, and A. Gruss. 2003. CcpA regulation of aerobic and respiration growth in *Lactococcus lactis*. Mol. Microbiol. 50:183–192.

Gaudu, P., K. Vido, B. Cesselin, S. Kulakauskas, J. Tremblay, L. Rezaiki, G. Lamberet, S. Sourice, P. Duwat, and A. Gruss. 2002. Respiration capacity and consequences in *Lactococcus lactis*. Antonie von Leeuwenhoek 82:263–226.

Grandvalet, C. 2017. *Oenococcus oeni*: queen of the cellar, nightmare of geneticists. Microbiology 163:297–299.

Ianniello, R.G., T. Zotta, A. Matera, F. Genovese, E. Parente, and A. Ricciardi. 2016. Investigation of factors affecting aerobic and respiratory growth in the oxygen-tolerant strain *Lactobacillus casei* N87. PLoS ONE 11:e0164064.

Lechardeur, D., B. Cesselin, A. Fernandez, G. Lamberet, C. Garrigues, M. Pedersen, P. Gaudu, and A. Gruss. 2011. Using heme as an energy boost for lactic acid bacteria. Curr. Opin. Biotechnol. 22:143–149.

Orla-Jensen, S. 1919. The Lactic Acid Bacteria. Host and Son, Copenhagen.

Pedersen, M.P., C. Garrigues, K. Tuphile, C. Brun, K. Vido, M. Bennedsen, H. Møllgaard, P. Gaudu, and A. Gruss. 2008. Impact of aeration and heme activated respiration on *Lactococcus lactis* gene expression: identification of a heme-responsive operon. J. Bacteriol. 190:4903–4911.

Pedersen, M.P., P. Gaudu, D. Lechardeur, M.-A. Petit, and A. Gruss. 2012. Aerobic respiration metabolism in lactic acid bacteria and uses in biotechnology. Annu. Rev. Food Sci. Technol. 3:37–58.
Pedersen, M.B., S.L. Iversen, K.I. Sørensen, and E. Johansen. 2005. The long and winding road from the research laboratory to industrial applications of lactic acid bacteria. FEMS Microbiol. Rev. 29:611–624.
Ritchey, T.W. and H.W. Seely. 1976. Distribution of cytochrome-like respiration in streptococci. J. Gen. Microbiol. 93:195–203.
Sijpesteijn, A.K. 1970. Induction of cytochrome formation and stimulation of oxidative dissimilation by hemin in *Streptococcus lactis* and *Leuconostoc mesenteroides*. Antonie Van Leeuwenhoek 36:335–348.
Zotta, T., A. Ricciardi, R.G. Ianniello, E. Parente, A. Reale, F. Rossi, L. Lacumin, G. Comi, and R. Coppola. 2014. Assessment of aerobic and respiratory growth in the *Lactobacillus casei* group. PLoS ONE 9:e99189.

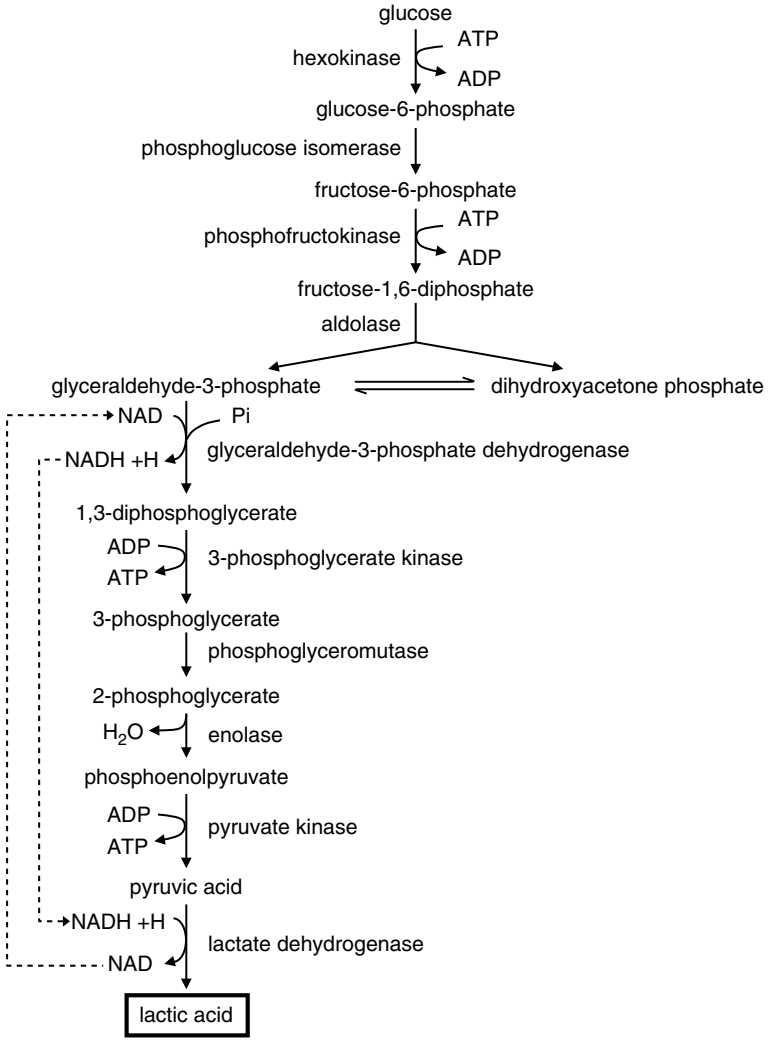


Figure 3.1 The Embden-Meyerhof-Parnas pathway used by homofermentative lactic acid bacteria.

organisms), *Pediococcus acidilactici* and *Lactobacillus sakei* (used in sausage cultures), and *Tetragenococcus* (used in soy sauces).

Heterofermentation

Heterofermentative lactic acid bacteria metabolize sugars via the phosphoketolase pathway (Figure 3.2). In obligate heterofermentative bacteria, aldolase is absent, and instead the enzyme phosphoketolase is present. Product formation depends on substrate and atmospheric conditions. In general, approximately equimolar amounts of lactate, acetate and CO_2 are produced, along with 1–2 moles of ATP per monomer. Oxidation of NADH and

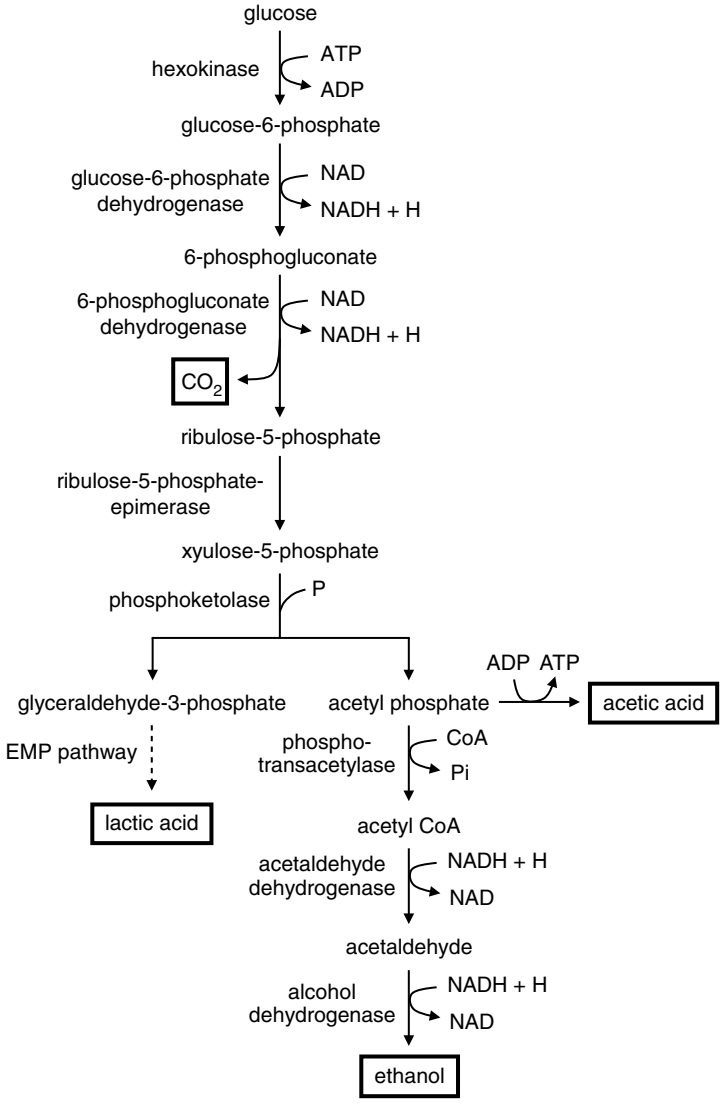


Figure 3.2 The phosphoketolase pathway used by heterofermentative lactic acid bacteria.

maintenance of the [NADH]/[NAD^+] balance occurs via the two reductive reactions catalyzed by acetaldehyde dehydrogenase and alcohol dehydrogenase. Many of the lactic acid bacteria used in food fermentations are heterofermentative, some in the form of starter cultures, but others as part of the autochthonous microbiota. Included are *Leuconostoc mesenteroides* subsp. *cremoris* and *Leuconostoc lactis* (dairy fermentations), *L. mesenteroides* subsp. *mesenteroides* and *Leuconostoc kimchii* fermented vegetables), *Oenococcus oeni* (wine fermentations), and *Lactobacillus sanfranciscensis* (sourdough bread).

As noted above, product yields for both pathways may vary during actual fermentation processes, and will depend on the type and concentration of substrate, on the growth temperature and atmospheric conditions, and the growth phase of the cells. Some of the carbohydrate carbon is incorporated into cell mass. However, it has also been shown that under certain conditions, such as sugar-limitation or aerobiosis, homofermentative lactococci can divert some of the pyruvate away from lactate and toward other alternative end-products, such as acetate, formate or acetaldehyde. This so-called heterolactic fermentation may provide cells with additional ATP or serve as a way to deal with excess pyruvate (Box 3.2).

Box 3.2 Fermentation products from pyruvate

Lactic acid bacteria, as previously noted, are either homofermentative, heterofermentative, or facultative heterofermentative (where both pathways are present). However, even obligate homofermentative strains of *Lactococcus* have the potential to produce acetic acid, ethanol, acetoin, CO_2 and end-products other than lactic acid. These alternative fermentation end-products are formed when pyruvate concentrations are elevated, a situation that occurs only under particular conditions.

In general, this metabolic scenario occurs when the rate of intracellular pyruvate formation exceeds the rate at which pyruvate is reduced to lactate (via lactate dehydrogenase). The pyruvate can form from sugar substrates (see below), but also from citrate and amino acids (Ganzle, 2015). In either case, the excess intracellular pyruvate must be removed because it could otherwise become toxic. Moreover, when excess pyruvate is generated as a result of sugar metabolism, re-oxidizing the NADH formed from glycolysis (upstream in the pathway) becomes necessary to avoid a metabolic bottleneck. Accordingly, other electron acceptors must be used. Several different pathways in lactic acid bacteria appear to serve this function (Figure 3.2.1; Cretenet et al., 2014; Cocaign-Bousquet et al., 1996; Garrigues et al., 1997; Ganzle, 2015). In addition, at least one of these alternative pathways include a substrate level phosphorylation reaction and, therefore, provides the cells with additional ATP.

What are the conditions or environments that result in pyruvate accumulation and induction of alternate pathways? There are several possible situations where these reactions occur. First, glycolysis is subject to several levels of regulation, such that when fermentation substrates are limiting, the glycolytic flux tends to be diminished (Axelsson, 2004). Specifically, when the concentration of fructose-1,6-diphosphate (a glycolytic regulator that forms early during glycolysis) is low, the activity of lactate dehydrogenase (an allosteric enzyme that occurs at the end of glycolysis) is reduced. Thus, pyruvate accumulates.

The glycolytic flux also may be decreased during growth on less preferred carbon sources, such as galactose, again resulting in excess pyruvate. At the same time that

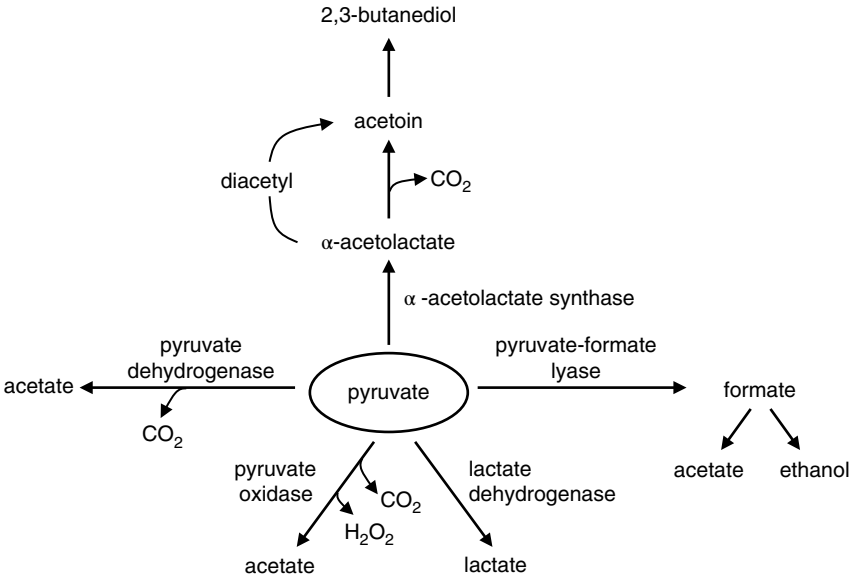


Figure 3.2.1 Fate of pyruvate in lactic acid bacteria. Adapted from Gänzle, 2015 and Teusink and Molenaar, 2017.

lactate dehydrogenase activity is decreased, the enzyme, pyruvate-formate lyase, is activated. This enzyme splits pyruvate to form formate and acetyl CoA. The latter is subsequently reduced to ethanol or phosphorylated to acetyl phosphate (both reactions releasing CoA). Importantly, the acetyl phosphate can be used as part of a substrate level phosphorylation reaction (via acetate kinase), resulting in the formation of an ATP.

Under aerobic environments, however, pyruvate-formate lyase is inactive, and other pathways become active. In the pyruvate dehydrogenase pathway, for example, pyruvate is decarboxylated by pyruvate dehydrogenase, and acetate and CO_2 are formed. NADH that would normally reduce pyruvate is oxidized directly by molecular oxygen when the environment is aerobic, rendering it unavailable for the lactate dehydrogenase reaction.

Finally, excess pyruvate can be diverted to end-products that may have a more functional role in certain fermented dairy products. Specifically, pyruvate can serve as a substrate for α-acetolactate synthase to form α-acetolactate. The α-acetolactate may then be further oxidized to form diacetyl, which has desirable aroma properties.

References

Axelsson, L. 2004. Lactic acid bacteria: classification and physiology. In: Salminen, S., A. von Write, and A. Ouwehand, eds. Lactic Acid Bacteria Microbiological and Functional Aspects, 3rd edn. Marcel Dekker, Inc. New York, pp. 1–66.

Cocaign-Bousquet, M., C. Garrigues, P. Loubière, and N. Lindley. 1996. Physiology of pyruvate metabolism in *Lactococcus lactis*. Antonie Van Leeuwenhoek 70:253–267.

Cretenet, M., G. Le Gall, U. Wegmann, S. Even, C. Shearman, R. Stentz, and S. Jeanson. 2014. Early adaptation to oxygen is key to the industrially important traits of *Lactococcus lactis* ssp. *cremoris* during milk fermentation. BMC Genomics 15:1054.

Gänzle, M.G. 2015. Lactic metabolism revisited: metabolism of lactic acid bacteria in food fermentations and food spoilage. Curr. Opin. Food Sci. 2:106–117.
Garrigues, C., P. Loubière, N. Lindley, and M. Cocaign-Bousquet. 1997. Control of shift from homolactic acid to mixed-acid fermentation in *Lactooccus lactis*: predominant role of the NADH/NAD^+ ratio. J. Bacteriol. 154:1–9.
Teusink, B. and D. Molenaar. 2017. Systems biology of lactic acid bacteria: For food and thought. Curr Opin Syst Biol.

These pathways also are of practical importance in fermented foods, as unexpected end-products, such as acetic acid or CO_2, may cause flavor or appearance defects.

Sugar transport by lactic acid bacteria

Before a sugar substrate can enter a catabolic pathway, it must first get from the outside to the inside of the cell. Thus, the first step in sugar metabolism involves the transport of the substrate across the cytoplasmic membrane. This is not a trivial process. Indeed, transport is challenging for several reasons, not the least of which is that the cell membrane is ordinarily impermeable to polar solutes (e.g., sugars, amino acids, peptides). In the absence of transport systems, these solutes would be unable to transverse the membrane and gain entry into the cell. Second, transport is bioenergetically demanding, and cells must devote a considerable amount of their total energy resources to support active transport. Third, some sugars are phosphorylated during the transport event, which then dictates the catabolic pathway used by that organism. Finally, transport systems may serve a regulatory role, influencing expression and activity of other transport systems. In general, lactic acid bacteria rely on several different systems to transport carbohydrates, depending, in part, on the species and the specific sugar. In the section to follow, the architectural, physiological, biochemical, and molecular properties of the main transport systems in lactic acid bacteria will be reviewed.

The phosphoenolpyruvate-dependent phosphotransferase system

There are three main ways lactic acid bacteria transport sugars. The phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) is used by most mesophilic, homofermentative lactic acid bacteria, including lactococci and pediococci for transporting lactose and glucose. In contrast, other lactic acid bacteria transport sugars via either symport-type or ATP-dependent systems (described below). Another group of transporters are the precursor-product exchange systems that provide an altogether different (and somewhat more efficient) means for transporting sugars (Box 3.3).

The PTS consists of a cascade of several cytoplasmic and membrane-associated proteins whose function is to sequentially transfer a high-energy phosphate group from PEP, the initial donor, to the incoming sugar (Figure 3.3A). The phosphorylation step coincides with the vectorial movement of the sugar substrate across the membrane, resulting in the intracellular accumulation of a sugar-phosphate. While each sugar substrate will require specific PTS components, the two cytoplasmic proteins, Enzyme I and HPr (for histidine-containing protein) are non-specific, meaning that they can serve multiple phosphotransferase systems. It is the membrane-associated PTS components, however, that are substrate-specific. These proteins form an Enzyme II complex, which may consist of individual proteins or domains.

Box 3.3 Energy conservation through efficiencies in transport

Transport of nutrients from the environmental medium across the cytoplasmic membrane and into the cytoplasm is one of the most important of all microbial processes. For many microorganisms, including lactic acid bacteria, nearly 20% of the genome is devoted to transport functions, and cell membranes are literally crammed with a hundred or more transport permeases, translocators, and accessory proteins (Lorca et al., 2005). The function of transporters, of course, is to provide a means for the cell to selectively permit solutes to move, back and forth as the case may be, across a generally impermeable membrane.

For most nutrients (and other transport substrates), however, transport is not cheap, in that energy (e.g., ATP) is usually required to re-locate molecules from one side of a membrane to another. This is because nutrient transport generally occurs against a concentration gradient, meaning that the concentration of the substrate is usually much lower in the outside medium than it is on the inside (i.e., within the cytoplasm). Although passive or facilitated diffusion, where the concentration gradient (higher outside than inside) drives transport occurs occasionally, this is not the normal situation, and instead some sort of active, energy-requiring process is required.

The metabolic cost of transport is especially high for lactic acid bacteria, given the limited and generally inefficient means by which these bacteria make energy. After all, glycolysis generates only two molecules of ATP per molecule of glucose or hexose fermented. If ATP or its equivalent (e.g., phosphoenolpyruvate or the proton motive force) is required to "move" mono- and disaccharides across the cytoplasmic membrane, the net energy gain by the cell may be reduced by as much as 50%. On the other hand, if the driving force for solute transport does not depend on an energy source, then the cell can conserve that un-spent energy or use it for other reactions.

By what means can cells transport solutes without having to spend energy? In many lactic acid bacteria, metabolism of substrate (or precursor) molecules results in a large amount of metabolic products that must be excreted from the cell. In some cases, these products are not further metabolized; in other cases, they may even be toxic if allowed to accumulate intracellularly. In any event, a rather large concentration gradient is formed, where the inside concentration of "product" is much higher than the outside concentration. Efflux of "product" molecules, therefore, is driven by the concentration gradient in a downhill manner. The carrier for efflux in these situations, however, actually has affinity not just for the product, but also for the substrate. Moreover, it operates in opposing directions, such that product excretion (via the downhill concentration gradient) can drive uphill uptake of the precursor substrate (against the concentration gradient). These so-called precursor-product exchange systems, therefore, represent a novel means by which cells can accumulate nutrients and metabolic substrates without having to consume much-needed sources of energy.

Examples of precursor-product exchange systems in lactic acid bacteria

Precursor-product exchange systems are widely distributed in lactic acid bacteria, and are used to transport fermentation substrates, amino acids, and organic acids (Geissler et al., 2016; Konings, 2002; Lorca et al., 2007; Trip et al., 2013). These systems can be

electroneutral, without a net change in the electric charge across the cell membrane, or electrogenic, where a charge is generated across the membrane. For example, a neutral sugar precursor (lactose) exchanged for a neutral sugar product (galactose) is electroneutral, as is the exchange of the amino acid arginine for citrulline (Figure 3.3.1, panels A and C). However, a di-anionic precursor (citrate) exchanged for a mono-anionic product (lactate) is electrogenic, since it results in an increase of the transmembrane electric charge (Figure 3.3.1, panel B).

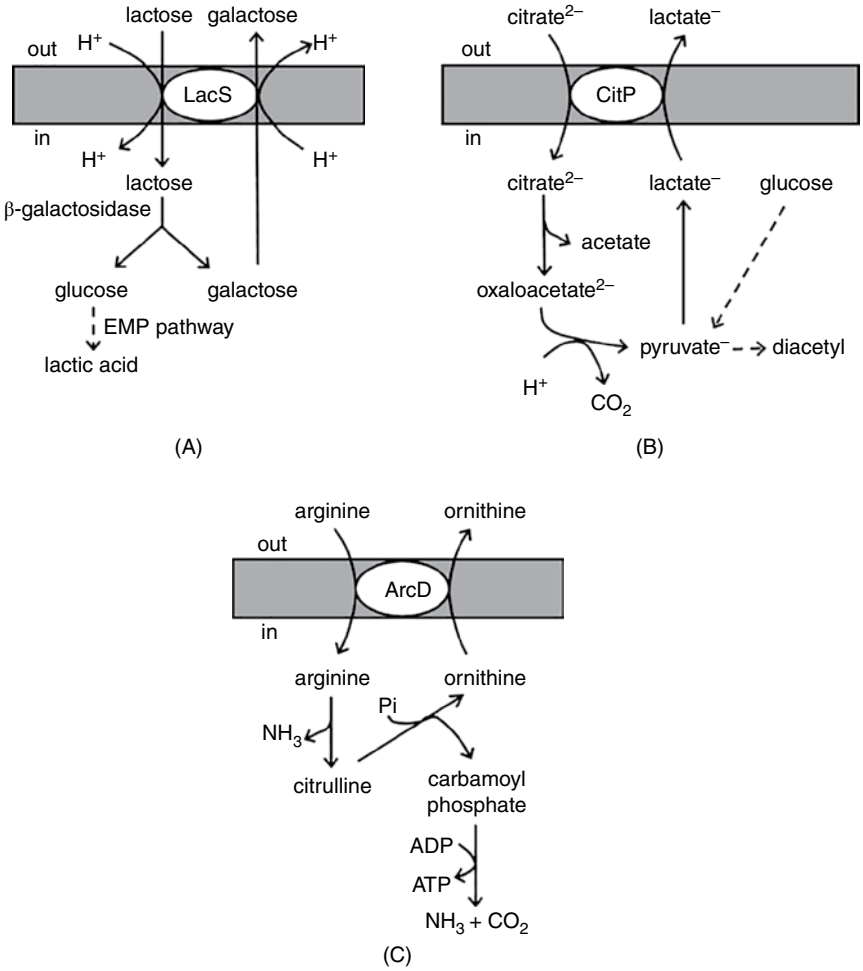


Figure 3.3.1 Three examples of precursor-product exchange systems in lactic acid bacteria are shown. In *Streptococcus thermophilus*, lactose is transported by the LacS system (panel A). Intracellular hydrolysis releases free galactose, which is not metabolized, but is instead secreted. The efflux reaction drives uptake of lactose. In citrate-fermenting lactic acid bacteria, transport occurs via the CitP citrate permease (panel B). Metabolism generates lactic acid, whose efflux drives citrate uptake. This reaction is electrogenic, in that a proton is consumed in the cytoplasm, increasing the PMF. Some lactic acid bacteria metabolize the amino acid arginine via the arginine deiminase pathway (panel C), generating NH_3, CO_2, ATP, and ornithine. The latter is effluxed via the arginine transporter (ArcD), in exchange for arginine. The cell, therefore, gains energy from arginine (one mole of ATP per arginine metabolized), without having to spend transport energy.

References

Konings, W.N. 2002. The cell membrane and the struggle for life of lactic acid bacteria. Antonie van Leeuwenhoek 82:3–27.

Lorca, G.L., R.D. Barabote, V. Zlotopolski, C. Tran, B. Winnen, R.N. Hvorup, A.J. Stonestrom, E. Nguyen, L.-W. Huang, D. Kimpton, and M.H. Saier, Jr. 2007. Transport capabilities of eleven Gram-positive bacteria: comparative genomic analyses. Biochim. Biophys. Acta. 176:1342–1366.

Trip, H., N.L. Mulder, and J.S. Lolkema. 2013. Cloning, expression, and functional characterization of secondary amino acid transporters of *Lactococcus lactis*. J. Bacteriol. 195:340–350.

Geissler, A.J., J. Behr, K. von Kamp, and R.F. Vogel. 2016. Metabolic strategies of beer spoilage lactic acid bacteria in beer. Int. J. Food Microbiol. 216:60–68.

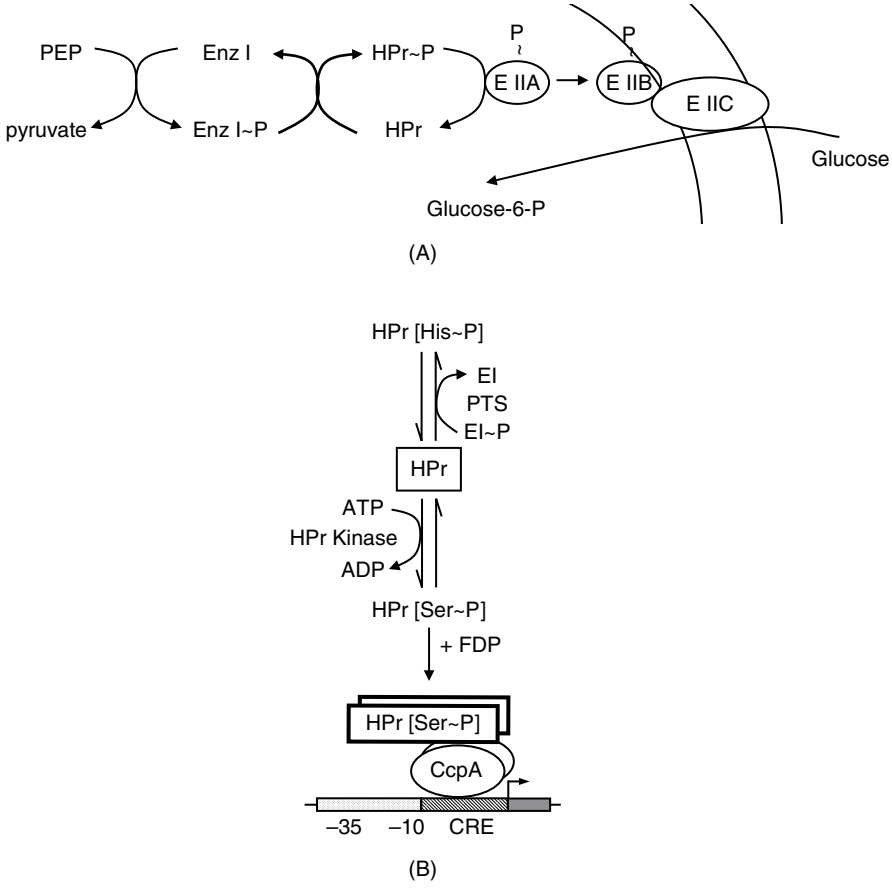


Figure 3.3 The phosphotransferase system (PTS) in Gram-positive bacteria. The PTS cascade is initiated by the cytoplasmic proteins, Enzyme I and HPr (panel A). Phosphorylated HPr (HPr~P) then transfers the phosphoryl group to substrate-specific Enzyme II. The latter consists of several domains, shown here as EIIA, EIIB, and EIIC. Depending on the organism and the substrate, EII complexes may be organized as EIIA and EIIBC or as EIIABC. Regulation of the PTS is mediated, in part, via the phosphorylation state of HPr (panel B). Phosphorylation by HPr kinase results in formation of HPr[Ser~P], which, along with CcpA and fructose diphosphate (FDP), form a dimeric complex that recognizes and binds to CRE sites and prevents transcription of catabolic genes.

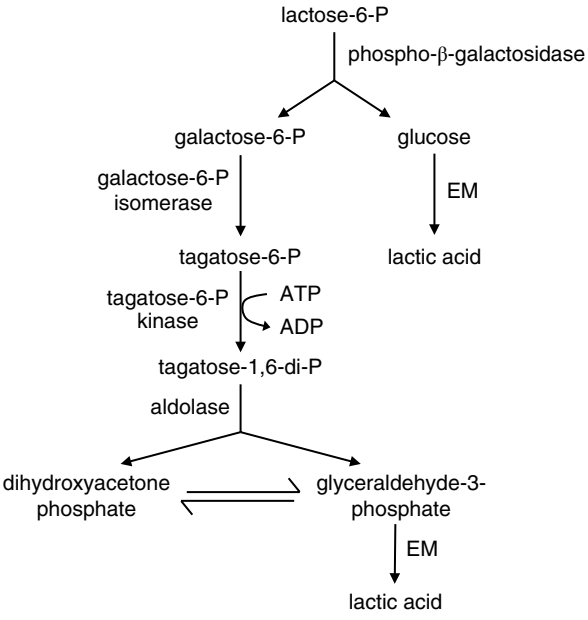


Figure 3.4 Tagatose pathway in lactococci. Galactose-6-phosphate is formed from hydrolysis of lactose-phosphate, the product of the lactose PTS. Isomerization and phosphorylation form tagatose-1,6-diphosphate which is split by an aldolase, yielding the triose phosphates that feed into the EMP pathway.

For example, the lactose PTS Enzyme II complex in *L. lactis* contains three protein domains, Enz IIA^{lac} and Enz $IIBC^{lac}$.

In the case of glucose, the product of the PTS is glucose-6-phosphate, which can then feed directly into the glycolytic pathway. The PTS has the advantage, therefore, of sparing the cell of the ATP that ordinarily would be required to phosphorylate the free sugar. When lactose is the substrate, the product is lactose-phosphate (or more specifically, glucose-β-1,4-galactosyl-6-phosphate). While lactose is readily hydrolyzed by β-galactosidase, which is widespread in the microbial world, lactose-phosphate is not hydrolyzed by this enzyme. Rather, hydrolysis of lactose-phosphate is catalyzed by the enzyme, phospho-β-galactosidase. The products of this reaction are free glucose and galactose-6-phosphate. The glucose is subsequently phosphorylated by hexokinase, forming glucose-6-phosphate that then feeds into the glycolytic pathway, as described earlier. The galactose-6-phosphate, in contrast, is metabolized by the tagatose pathway (Figure 3.4), eventually leading to the formation of the same triose phosphates, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, that form during glycolysis. It is perhaps not unexpected that the structural (and regulatory) genes coding for lactose transport and hydrolysis are located on the same operon as the galactose/tagatose genes (Figure 3.5).

Symport and ABC transport systems in lactic acid bacteria

Although the PTS is widely distributed among lactic acid bacteria, several species rely on other active transport systems to transport sugars. The latter include symport systems, driven by ion gradients, and ATP-binding cassette (ABC) systems fueled by ATP (Figure 3.6). Moreover, it is often the case that an organism uses a PTS for one sugar and a symport or

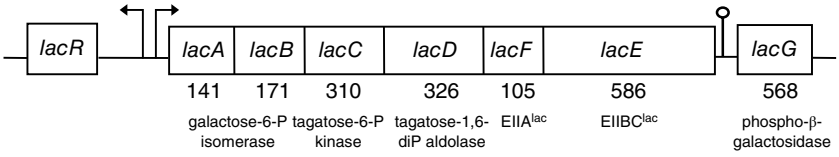


Figure 3.5 The *lac* operon in lactococci. The operon consists of four structural genes (*lacABCD*) coding for enzymes of the tagatose pathway, two genes, *lacFE*, coding for lactose-specific PTS proteins, and the *lacG* gene coding for phospho-β-galactosidase. The *lacR* gene codes for a repressor protein. Promoter sites and directions are shown by the arrows, and potential transcriptional terminators are shown as a hairpin loop. The number of amino acid residues for each protein is given. Adapted from de Vos et al., 1990.

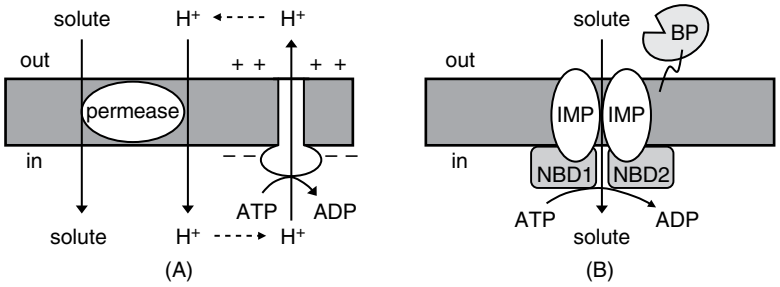


Figure 3.6 Two main types of solute transport in lactic acid bacteria. In Panel A, a proton symport system, fueled by the proton gradient, drives solute uptake. In panel B, transport is mediated by an ABC system. The solute is first captured by a binding protein (BP) which delivers it to the integral membrane proteins (IMP). ATP hydrolysis occurs via nucleotide binding domains (NBD).

ABC system for another. According to the Transport Classification system (www.tcdb.org), symport system transporters belong to a class of secondary carriers within the Major Facilitator Superfamily. Symport systems are widely distributed in nature. They consist of a membrane permease that has binding sites for both the substrate and a coupling ion, usually protons. Transport of the substrate is driven by the ion gradient across the membrane. Indeed, symport transport, as is generally true for other transport systems, is all about driving forces.

The most common ion gradient in bacteria is the proton gradient (called the proton motive force or PMF), although there are also symport systems driven by sodium ion gradients (such as the melibiose system that exists in *Lactobacillus casei*). The PMF is comprised of the sum of two components: (1) the charge or electrical difference ($\Delta\Psi$) across the membrane, where the outside is positive and the inside is negative; and (2) the chemical difference (ΔpH) across the membrane, where the proton concentration is high on the outside and low inside. The positively charged protons then flow "downhill" across this gradient (i.e., toward the inside) in symport with the uphill intracellular accumulation of the solute. Thus, in symport transport, the coupling ion or gradient "drives" transport of the substrate.

In lactic acid bacteria, symport systems exist for several sugars (based on biochemical and genetic evidence), including lactose (in *Lactobacillus brevis, Lb. delbrueckii*, *Lactobacillus acidophilus*), galactose (*L. lactis*), raffinose (*Pediococcus pentosaceus*), melibiose (*L. lactis*), and xylose (*Lactobacillus pentosus*). Of course, the formation of a proton gradient and a PMF is an energy-requiring process. The main means by which lactic acid bacteria generate the PMF is via the proton-translocating ATPase (also called the F_0F_1-ATPase), which requires ATP. Thus, although the PMF can drive transport of sugars and other nutrients, it does not

come without a cost, in the form of ATP. It is noteworthy, however, that lactic acid bacteria have evolved ways to make a PMF by alternative routes that spare the cell of ATP (Box 3.3).

For those organisms that transport lactose by a symport system, the intracellular product is free lactose (i.e., chemically unaltered, in contrast to the lactose PTS, where the product is lactose-phosphate). Intracellular hydrolysis of the intracellular lactose then occurs via β-galactosidase, yielding glucose and galactose. The former is phosphorylated by hexokinase (as described above) to form glucose-6-phosphate, which feeds directly into the EMP pathway. To convert galactose into glucose-6-phosphate requires the presence of the Leloir pathway (Figure 3.7), a three enzyme pathway, whose expression is subject to strong negative regulation in some lactic acid bacteria. In particular, *S. thermophilus* and *Lb. delbruecki* subsp. *bulgaricus* transport and hydrolyze lactose by a PMF and a β-galactosidase, respectively, but ferment only the glucose and not the galactose moiety. Thus, these bacteria are phenotypically galactose-negative.

The observation that most strains of *S. thermophilus* and *Lb. delbruecki* subsp. *bulgaricus* do not ferment galactose has been the subject of considerable research interest. This is because there are important practical consequences of this peculiar phenomenon. Although most strains of these bacteria have the genes encoding for the Leloir pathway enzymes, the genes are poorly transcribed or expressed, due to mutations within the promoter region of the operon. As a result, the intracellular galactose is largely unfermented and is excreted via the lactose transport system (LacS in *S. thermophilus*), a seemingly wasteful process. What makes this process especially interesting, however, is that LacS can catalyze a lactose:galactose exchange reaction, such that galactose efflux drives lactose uptake. Thus, what might initially appear to be an inefficient and wasteful process (i.e., secreting a perfectly good energy source into the medium), is, instead, an efficient, energy-saving means of exchanging a readily fermentable sugar for one that is slowly metabolized, if at all.

The other major group of transport systems used by lactic acid bacteria for accumulating sugars are the ABC transport systems. These systems consist of several proteins or protein domains whose function is to capture specific substrates and then direct their translocation across the membrane through specific channels or pores. Two of these proteins or domains are membrane-spanning (i.e., integrated within the membrane) and serve as

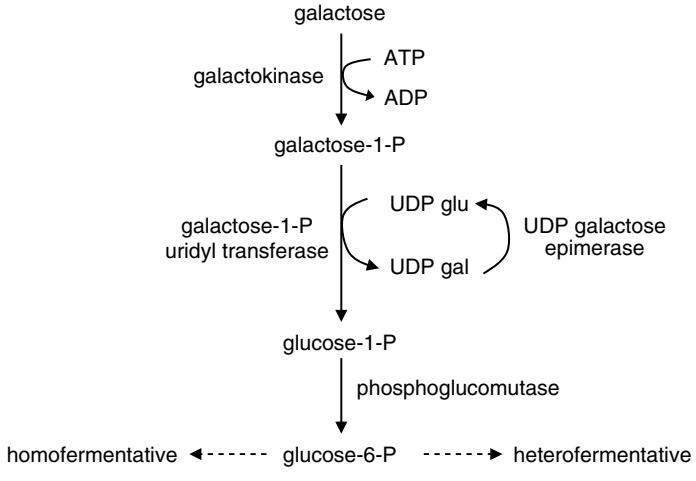


Figure 3.7 The Leloir pathway in lactic acid bacteria.

porters. Two intracellular proteins/domains bind and hydrolyze ATP. An additional protein is extracellular, but is tethered to the cell surface and has substrate-binding activity. The energy released from ATP hydrolysis drives transport. In general, ABC systems are used to transport amino acids, peptides, and osmoprotectants. However, several ABC systems are involved in sugar transport, including the maltose ABC systems in *Lb. plantarum* and the oligosaccharide transport system in *Lb. acidophilus*.

Regulation of transport systems

As noted above, regulation of sugar metabolism in lactic acid bacteria is intimately connected to the transport machinery. In *L. lactis*, for example, lactose fermentation is regulated biochemically and genetically at the level of transcription through a signal transduction cascade mediated via the lactose transport system. Biochemical regulation occurs in two major ways. First, several glycolytic enzymes are allosteric, and their activities are subject to the intracellular concentration of specific glycolytic metabolites. In principle, the pathway is regulated such that when substrates are abundant, glycolysis is fully engaged and when substrates are in short supply, glycolysis is reduced. Thus, in milk fermentations where plenty of lactose is available, lactose transport and metabolism will result in formation of high intracellular concentrations of fructose-1,6-diphosphate (FDP). High levels of FDP activate pyruvate kinase and lactate dehydrogenase. Activation of these enzymes keeps glycolysis moving along at a fast rate, provided there is sufficient substrate. In contrast, when lactose is limiting, rapid flux is not so critical. Thus, in the absence of FDP and presence of phosphate, pyruvate kinase activity decreases, causing a metabolic bottleneck in glycolysis, right at the step at which PEP would otherwise be used to drive ATP formation. This cessation of glycolysis eventually results in accumulation of PEP. As noted from the discussion above, PEP is also used by the PTS, so storing up PEP during lean times, readies the cell for PTS-mediated transport when suitable sugars ultimately become available.

A second biochemical mechanism for regulating lactose metabolism more directly involves the PTS transport proteins, and the cytoplasmic component, HPr, in particular. As outlined above, the PTS cascade starts when HPr is phosphorylated by Enzyme I. Ordinarily, phosphorylation by Enzyme I occurs at a specific amino acid residue of HPr, namely at histidine-15 [His-15]. However, HPr can also be phosphorylated at a second site, specifically at a serine residue [Ser-46]. This phosphorylation reaction is catalyzed by an ATP-dependent HPr kinase. Importantly, when HPr is in the latter phosphorylation state (i.e., HPr [Ser-46-P]), phosphorylation at the His-15 site does not occur. Thus, HPr cannot be suitably "charged" and the PTS cannot function. At the same time, HPr [Ser-46-P] is still biologically active, as it can bind directly to the interior side of other sugar permeases and prevent transport of those sugars, a process known as inducer exclusion.

It is interesting to note that HPr kinase is activated by fructose-1,6-diphosphate, the glycolytic precursor that reaches high concentrations only when the substrate is plentiful. That is, HPr is evidently converted to HPr [Ser-46-P] just when one would expect HPr to be in the free or non-phosphorylated state (i.e., in the active transport mode). Thus, it seems counter-intuitive that the cells would slow down transport when substrates are so readily available. In other words, why would the cell down-regulate the activity of the transport system in times of plenty?

One possible answer is that the cell is simply modulating or curbing its appetite by not transporting more substrate than it can reasonably consume. This hypothesis is supported by the observation that HPr not only exerts biochemical control on transport, but that HPr

[Ser-46-P] also regulates transcription of sugar transport genes. Specifically, HPr [Ser-46-P] interacts with a DNA-binding, trans-acting protein called CcpA (See Box 3.1 and Figure 3.3B). The HPr [Ser-46-P]-CcpA complex binds to 14 base pair DNA regions called Catabolite Responsive Elements (CRE). These CRE sites are located just upstream of the transcription start sites of certain catabolic genes. When these CRE regions are occupied by the HPr [Ser-46-P]-CcpA complex, transcription by RNA polymerase is blocked or reduced and mRNA is not transcribed. This process, whereby transcription of specific catabolic genes, including other PTS genes, is blocked in the presence of a preferred sugar, is called catabolite repression. Thus, catabolic repression provides a molecular mechanism by which the cell can quickly adapt to the availability of sugars in the environment and to use those sugars in a hierarchal manner.

Finally, sugar metabolism in lactic acid bacteria can also be genetically regulated directly via repressor proteins. For example, in *L. lactis*, the lactose PTS operon is negatively regulated by LacR, a repressor protein encoded by the *lacR* gene. In the presence of lactose, *lacR* expression is itself repressed, and transcription of the *lac* operon is induced. However, when lactose is absent or when cells are grown on glucose, LacR is expressed and transcription of the *lac* genes are repressed. Although a CRE site is also located near the transcriptional start site of the *lac* operon, suggesting that the *lac* genes are subject to CcpA-mediated repression, it seems that LacR may have primary responsibility for regulating sugar metabolism.

Sugar metabolism by *Saccharomyces cerevisiae*

Metabolism of carbohydrates by *S. cerevisiae* and related yeasts is not dramatically different from the lactic acid bacteria. These yeasts are facultative anaerobes that are able to utilize a wide range of carbohydrates. In *S. cerevisiae*, as in the homofermentative lactic acid bacteria, fermentable sugars are metabolized via the glycolytic pathway to pyruvate, yielding two moles of ATP per hexose. In contrast to the lactic fermentation where pyruvate is directly reduced to lactate, *S. cerevisiae* instead decarboxylates pyruvate to form acetaldehyde, which is then reduced to ethanol. Thus, the end-products of sugar fermentation by *S. cerevisiae* are ethanol and CO_2.

There is one other important difference between how *S. cerevisiae* and lactic acid bacteria metabolize sugars. While most lactic acid bacteria are obligate fermentors and have limited means for respiratory metabolism, *S. cerevisiae* has an intact citric acid cycle and a functional electron transport system. Thus, *S. cerevisiae* and related yeasts can readily grow and respire under aerobic conditions.

Glucose transport is the rate-limiting step in glycolysis by *S. cerevisiae*. Remarkably, *S. cerevisiae* has as many as 18 functional glucose transporters. The transporters are referred to as hexose transporters or HXT proteins, because they transport other hexoses. Equally surprising, perhaps, is the fact that glucose transport by these hexose transport systems occurs by facilitated diffusion. These are energy-independent systems, driven entirely by the concentration gradient. Some of these transporters are constitutive or inducible, some have low or high affinity for their substrates, and others function not as transporters, per se, but rather as molecular sensors (see below). Thus, the seemingly excessive redundancy inferred by such a large number of transporters instead provides these organisms with the versatility necessary to grow in environments having a wide range of glucose concentrations.

This metabolic flexibility has practical significance. During beer or wine fermentations, for example, the initial fermentable carbohydrate concentration is high, but then decreases substantially as the fermentation proceeds. Having transport systems that can function at

high, low, and in-between substrate concentrations is essential in order for *Saccharomyces* to ferment all of the substrate. Complete fermentation of sugars ensures that these products can reach full attenuation or dryness, qualities important in beer and wine manufacture.

Regulation of sugar metabolism in *S. cerevisiae* is similar, but only in principle, to that in lactic acid bacteria. With regard to the actual mechanisms and the extent to which other catabolic pathways are affected, the regulatory features of sugar metabolism in *S. cerevisiae* are quite different. In general, catabolite repression occurs when two or more fermentable sugars are present in the medium and the cell must decide the order in which these sugars are to be fermented. Since glucose is usually the preferred sugar, genes that encode for metabolism of other sugars are subject to catabolite repression. In lactic acid bacteria, catabolite repression is mediated via the phosphorylation state of HPr, in concert with CcpA (see above). In *S. cerevisiae*, catabolite repression (more frequently referred to as glucose repression) assumes a broader function. Glucose not only represses transcription of genes for sugar utilization, but also turns off genes coding for pathways involved in several other important processes, including the citric acid cycle, the glyoxylate cycle, mitochondrial oxidation, and glycerol utilization.

How does glucose mediate this global response in *S. cerevisiae*? It now appears that glucose generates a specific signal that is transmitted via protein cascades and that ultimately results in protein-promoter interactions. Several such cascades actually exist, accounting for situations where glucose is present at high or low concentrations. The main pathway involves the repressor protein, Mig1 (Figure 3.8). This is a DNA-binding protein, produced when glucose is present. In the presence of glucose, Mig1 is localized in the nucleus, where it (in conjunction with accessory proteins) binds to promoter regions, blocking transcription of the downstream genes. When glucose is absent, however, Mig1 becomes phosphorylated by Snf1, a protein kinase, and is translocated out of the nucleus, resulting in de-repression of the regulated genes (i.e., those genes are then expressed). A second protein cascade pathway is also involved in glucose induction. In this system, the membrane proteins, Snf3 and Rgt2,

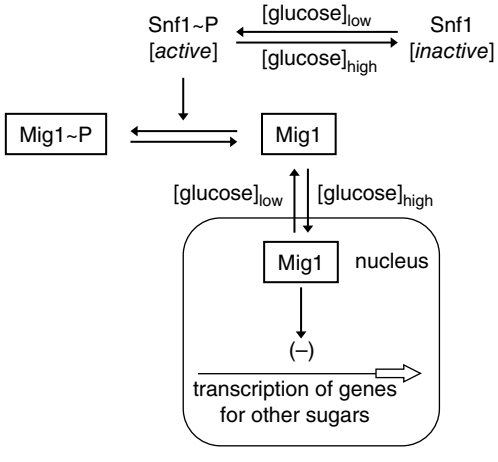


Figure 3.8 Catabolite repression in *Saccharomyces*. The key regulatory protein is Mig1. When glucose is low, Snf1~P phosphorylates Mig1, trapping it in the cytoplasm, where it is inactive, allowing expression of catabolic genes. In the presence of glucose, Mig1 is not phosphorylated and remains in the nucleus, where it represses catabolic gene expression. Adapted from Gancedo, J.M. 1998. Microbiol. Mol. Biol. Rev. 62:334–361.

sense the presence and level of glucose and then generate a signal that either activates or inactivates specific glucose transporters (whose activities depend on the glucose concentration).

OTHER METABOLIC SYSTEMS

Certainly, sugar metabolism, and the lactic and ethanolic fermentations, in particular, form the basis for the manufacture of most fermented foods and beverages. However, other metabolic activities are also important. As noted below, protein metabolism serves the needs of the cells, but also provide flavor and texture attributes in cheese. In other dairy products, such as buttermilk and sour cream, lactic acid bacteria perform fermentations that yield diacetyl, a compound that imparts the characteristic flavor of these products. In Swiss-type cheeses, the propionic acid fermentation results in formation of several metabolic endproducts that likewise give these cheese their unique features. Finally, while fungi do not ferment substrates in the classical sense (see above), they nonetheless convert proteins and fats into an array of products having strong aromatic and flavor properties.

Exopolysaccharide synthesis in lactic acid bacteria

Lactic acid bacteria not only hydrolyze and metabolize carbohydrates, many species are also very good at reverse reactions. Specifically, nearly every genera involved in food fermentations contain species that are able to synthesize exopolysaccharides (EPS). The latter are made from monosaccharides as the starting material, and involve many enzymes and corresponding genes. These EPS are important for many reasons (described in more detail in Chapter 5), but especially for modifying the rheological behavior and water holding capacity of yogurt and other cultured dairy foods.

Protein metabolism by lactic acid bacteria

While many bacteria are able to grow on ammonia or other sources of inorganic nitrogen, lactic acid bacteria are unable to assimilate inorganic nitrogen and, instead must rely on preformed amino acids to satisfy their amino acid requirements. Since most foods contain only a small pool of free amino acids, this means that lactic acid bacteria must first be able to degrade proteins and large peptides and then also be able to transport the free amino acids and small peptides released during proteolysis. The enzymes and transport systems necessary to perform these functions constitute the proteolytic system. This system has been very well studied, especially for the lactic acid bacteria involved in dairy fermentations. For these bacteria, the milk protein, casein, serves as the primary substrate. Casein metabolism by lactic acid bacteria is not only important nutritionally for the organisms, but its degradation during cheese manufacture has major implications for flavor and texture development (Chapter 6). However, in vegetable, bread, and other fermentations, protein metabolism is somewhat less important.

The casein utilization system in lactic acid bacteria involves three main steps. First, casein is hydrolyzed by extracellular proteinases to form peptides. Next, the peptides are transported into cells via peptide transport systems. Finally, the peptides are hydrolyzed by intracellular peptidases to form free amino acids (Figure 3.9). Each of these steps is described below.

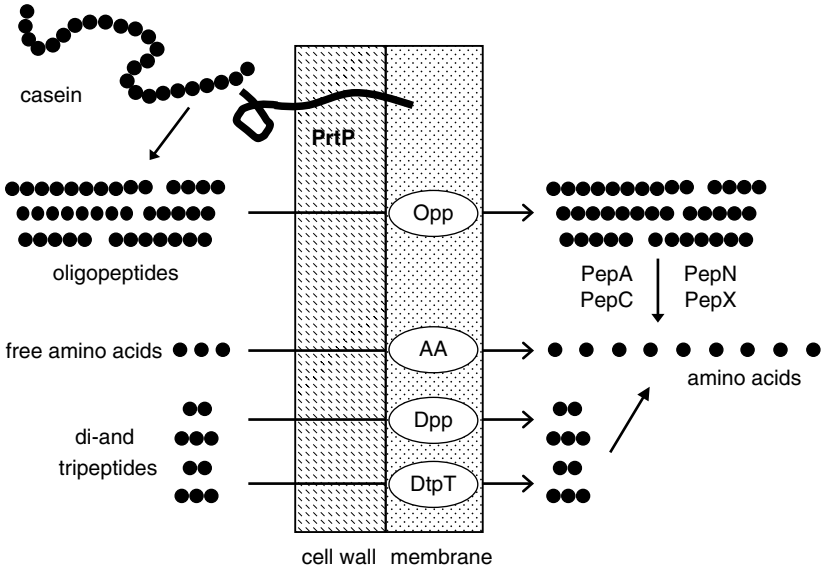


Figure 3.9 Proteolytic system in lactococci. Milk casein is hydrolyzed by a cell envelope-associated proteinase (PrtP). The oligopeptides are transported into the cell by the oligopeptide transport system (Opp). Intracellular oligopeptides are then hydrolyzed by one of several cytoplasmic peptidases (e.g., PepA, PepC, PepN, and PepX) to form amino acids. Extracellular di- and tripeptides and free amino acids in milk are transported by di- and tripeptide transporters (DtpT, DtpP) and amino acid (AA) transporters. Intracellular di- and tripeptides are then hydrolyzed to amino acids.

1. **The proteinase system** Hydrolysis of casein by lactic acid bacteria occurs via a cell envelope-associated serine proteinase called PrtP. This enzyme is actually synthesized as a large (>200 kDa) inactive pre-pro-proteinase. The "pre" portion contains a leader sequence, whose function is to direct the protein across the cytoplasmic membrane. The "pro" sequence presumably stabilizes the protein during its synthesis. After both of these regions are removed, the now mature proteinase remains anchored to the cell envelope. With casein as a substrate, more than 100 products are formed by PrtP. The majority are large oligopeptides (up to 30 amino acid residues), with most of these being between 4 and 10 residues.
2. **Peptide transport systems** Despite their rather large size and bulkiness, the oligopeptides generated by PrtP can be transported directly into the cell without further extracellular hydrolysis. In addition, although there are a myriad of peptides formed by PrtP, relatively few transporters deliver these peptides into the cell, and even fewer are actually essential for growth in milk. For example, two lactococcal peptide transporters, DtpT and DtpP are used to transport di- and tripeptides, respectively. However, given that di- and tripeptides are generally not released during casein proteolysis, the role of these transporters in nitrogen nutrition is probably not very critical. Indeed, lactococci having mutations in the *dtpT* and *dtpP* genes grow fine in milk. In contrast, lactococci and other lactic acid bacteria instead rely on an oligopeptide transport system (Opp) to satisfy their amino acid requirements. The Opp system transports about 10 to 14 different peptides of varying size (between 4 and 11 amino acid residues). Unlike *dtpT* and *dtpP* mutants, strains unable to express genes coding for the Opp system do not grow in milk.

3. **Peptidases** In the final step of protein metabolism, peptides accumulated in the cytoplasm by the Opp system are hydrolyzed by intracellular peptidases. There are more than 20 different peptidases produced by lactococci and lactobacilli that ultimately generate the pool of amino acids necessary for biosynthesis and cell growth. Included are endopeptidases (that cleave internal peptide bonds) and exopeptidases (that cleave at terminal peptide bonds). The latter group consists entirely of aminopeptidases – enzymes that hydrolyze peptide bonds starting from the amino end of the peptide. The role of carboxypeptidases in lactic acid bacteria is less clear as they have not been reported. Ultimately, for lactic acid bacteria to fully utilize peptides accumulated by the Opp system requires the combined action of intracelluar endopeptidases, aminopeptidases, dipeptidases and tripeptidases.

The citrate fermentation

Citrate, a six carbon, tricarboxylic acid, is a common constituent of plants and is also found in milk at varying concentrations, depending on the season and the diet of the animal. Several species of lactic acid bacteria can ferment citrate and produce the aroma and flavor compound diacetyl. While diacetyl has a pleasant, delicate buttery flavor in cultured dairy products, it can also be formed in beer, where buttery flavors are undesirable.

In those lactic acid bacteria that have citrate-fermenting ability, citrate is first transported into the cell by a pH-dependent, PMF-mediated citrate permease (CitP). The intracellular citrate is then split by citrate lyase to form acetate and oxaloacetate (Figure 3.10). The acetate is released directly back into the medium, whereas the oxaloacetate is decarboxylated to

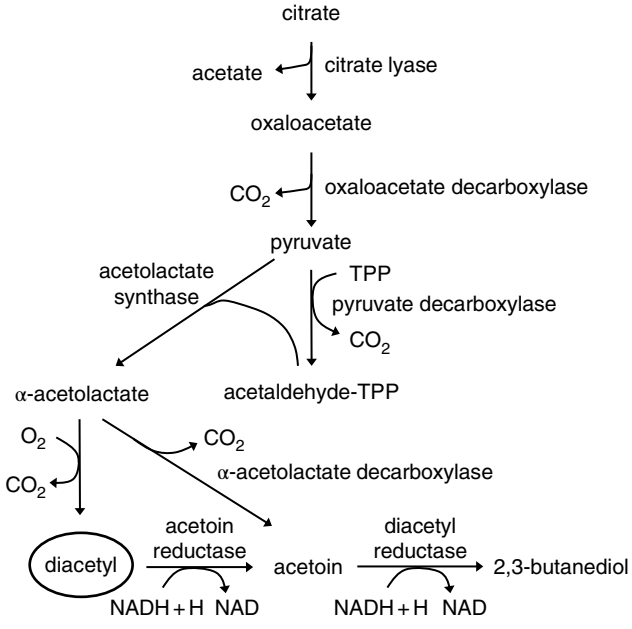


Figure 3.10 Citrate fermentation pathway in lactic acid bacteria. The dashed line indicates the non-enzymatic, oxidative decarboxylation reaction.

form pyruvate and CO_2. As described earlier in this chapter, pyruvate generated during glycolysis is ordinarily reduced by lactate dehydrogenase. However, the pyruvate liberated from citrate may increase beyond the reducing capacity of lactate dehydrogenase and the supply of NADH. This may result in a glycolytic bottleneck. Therefore, the excess pyruvate is removed by a decarboxylation reaction catalyzed by thiamine pyrophosphate (TPP)-dependent pyruvate decarboxylase. The product, acetaldehyde-TPP, then condenses with another molecule of pyruvate, via α-acetolactate synthase, forming α-acetolactate. The latter is unstable in the presence of oxygen and is non-enzymatically decarboxylated to form diacetyl.

Careful examination of the citrate-to-diacetyl pathway reveals that it does not generate ATP and would appear to be of no metabolic advantage to the organism. In fact, as noted above, citrate transport via CitP is PMF-driven and therefore costs the cell energy. Despite these observations, there is a way in which the cell can derive energy during the citrate fermentation. First, recall that the PMF is comprised of two components – the charge gradient ($\Delta\Psi$) across the membrane and the chemical or pH gradient (ΔpH) across the membrane. The greater the difference in the concentration of positively charged protons on the outside relative to the inside, the greater is the PMF. One way to form such a proton gradient would be to pump protons from inside to outside via the ATPase, which of course requires ATP. Another way to form this gradient would be to somehow consume cytoplasmic protons, which would have the same net effect, i.e., fewer protons inside relative to outside.

Returning to the citrate pathway, note that during the intracellular oxaloacetate decarboxylation reaction, a cytoplasmic proton is consumed. This causes the cytoplasmic pH and the ΔpH component of the PMF to increase. There is also a second related mechanism at work. Specifically, when both citrate and a fermentable sugar are present, the efflux of intracellular lactate can drive uptake of extracellular citrate. Since lactate is monovalent and citrate (at physiological pH) is divalent, the citrate permease can act as an electrogenic precursor-product exchanger (i.e., making the inside more negative and the outside more positive), resulting in a net increase in the $\Delta\Psi$ or electrical component of the PMF. Collectively, therefore, citrate fermentation results in an increase in the metabolic energy available to the cell.

The propionic acid fermentation

The propionic acid fermentation is performed by *Propionibacterium freudenreichii* subsp. *shermanii* and related species. In fermented foods, the relevance of this pathway is limited to the manufacture of Swiss-type cheeses. As will be described in more detail in Chapter 5, the propionic acid pathway must be carefully controlled to obtain cheeses having the appropriate appearance and flavor attributes.

Although lactose can be fermented by most dairy strains of propionibacteria, little to no lactose is available during the time at which these bacteria are given the opportunity to grow (i.e., several weeks after the primary lactose fermentation is complete). However, these bacteria are able to use lactate, generated by the lactic starter culture, as an energy source. Lactate fermentation occurs via the propionate pathway, which yields propionate, acetate, and CO_2. The stoichiometry is rather consistent – 3 moles of lactate fermented gives 2 moles of propionate, one mole of acetate, and mole of CO_2. The cell nets one mole of ATP per lactate. In cheese, however, the actual amount of end-products may vary as a result of condensation reactions, co-metabolism with amino acids, or strain variation. The propionic acid pathway is quite complex and requires several metal-containing enzymes and vitamin cofactors (Chapter 5).

Metabolism by fungi

As noted above, the metabolic activities of *Penicillium*, *Aspergillus*, *Rhizopus*, and other fungi are quite unlike that of bacteria and yeasts. The latter have a mostly fermentative metabolism, growing on simple sugars and producing only a few different end-products. In contrast, fungal metabolism is characterized by secretion of numerous proteolytic, amylolytic, and lipolytic enzymes. The end products of these enzymatic reactions – amino acids, simple sugars, and fatty acids, can then serve as substrates for the fungi themselves, other microorganisms, and further metabolic reactions.

The metabolic pathways used by fungi often result in an array of unique products. For example, when *Penicillium roqueforti*, the blue mold organism, grows in cheese, substantial proteolysis occurs through elaboration of several extracellular proteinases, endopeptidases and exopeptidases. The resulting amino acids are subsequently metabolized via deaminases and decarboxylases releasing amines, ammonia, and other possible flavor compounds. Additional end-products that are characteristic of blue cheese flavors are also generated from lipid metabolism. About 20% of triglycerides in milk are initially hydrolyzed by *P. roqueforti*-produced lipases, releasing free fatty acids, including short chain, volatile fatty acids, such as butyric and caproic acids. Subsequent metabolism of free fatty acids can then occur via β-oxidation pathways, yielding a variety of methylketones. The latter compounds are responsible for the characteristic flavor and aroma of blue cheese.

Growth of the Brie cheese mold, *Penicillium camemberti*, results in a similar sequence of metabolic events. Proteinases and lipases diffuse through the cheese (since the mold grows only at the surface), generating amino and free fatty acids. Subsequent metabolism of the amino acids results in formation of ammonia, methanethiol and other sulfur compounds, presumably derived from sulfur-containing amino acids. Lipolysis of triglycerides and fatty acid metabolism by *P. camemberti* are just as important in Brie-type cheeses as in blue-veined cheese, and methylketones are abundant. In the cheese environment, both *P. roqueforti* and *P. camemberti* can use lactic acid as a carbon source. The consumption of lactic acid along with the formation of ammonia, amines, and other alkaline end-products, may cause the pH of these cheeses to increase to near-neutral levels. Since low pH is one of the main barriers that prevent spoilage and pathogenic bacteria from growing in cheese, these metabolic events have important food safety and quality implications (addressed in Chapter 5).

METABOLIC ENGINEERING AND CELL COMMUNICATION

As described earlier in this chapter and emphasized throughout this text, the key to successful fermentations is to exert control. The difference between fermentation and spoilage ultimately boils down to controlled and predictable growth and metabolism versus uncontrolled and unpredictable growth and metabolism. Therefore, one way to improve a given fermentation process would be to impose either greater control on that process or alternatively simply engineer the preferred metabolic route directly into the organism of interest. The latter strategy, referred to as metabolic engineering, provides for a more precise and consistent metabolic result. For example, if increased diacetyl production by a lactic acid bacterium is the goal, rather than manipulate substrate or oxygen levels, the pathway can be genetically manipulated, such that carbon flow is directed to diacetyl rather than lactate (e.g., by inactivating lactate dehydrogenase).

The affordability of genome sequencing, along with plug-and-play computational tools, now makes it possible to screen genomes, not only for a particular enzyme, but for entire pathways or clusters of pathways. Moreover, a variety of molecular tools now exist that can be used to inactivate some pathways and activate others. Depending on the nature or means by which the modification was introduced, the organism may or may not be considered a genetically modified organism (i.e., GMO). The use of such strains in the fermented foods industry will be discussed in Chapter 4.

Finally, microbiologists have long appreciated that many food and beverage fermentations involve a community of organisms, often working together in concert. Thus, systems biology approaches (where entire communities are studied) have been adopted to understand and exploit the full potential of fermentative bacteria in foods and beverages. Recently, the means by which fermentation organisms can actually communicate has been described (Box 3.4). Interestingly, microbial communication in fermented foods has practical as well as academic relevance.

Box 3.4 Communication in fermented foods

Lactic acid bacteria, like other microorganisms, have evolved the ability to communicate. This remarkable discovery, first reported for marine organisms in the late 1970s (Waters and Bassler, 2005) has implications in clinical, environmental, and of course, food microbiology (Rul and Monnet, 2015). Indeed, cell communication is critical for the formation of biofilms, expression of enzymes and bacteriocins, during growth in mixed communities, and other activities that commonly occur during the manufacture of fermented foods (Rul and Monnet, 2015).

In principle, cell communication among unicellular organisms serves several important functions, much as communication does in multicellular organisms. One of the main functions is to alert fellow microbial members that their population has reached some critical density. At that point, expression of particular genes or pathways may be induced. Specifically, these represent genes that would ordinarily not be expressed if the population was below a critical threshold. Given that this process depends on cell density (i.e., reaching a "quorum"), the name for this form of microbial communication is called quorum sensing (QS). Importantly, the sensing part of this process is conveyed via various small molecules (described below).

For pathogenic bacteria, genes controlled by QS pathways include those encoding for virulence or resistance to antibiotics and other antimicrobial agents. Quorum sensing is also important for bacterial symbionts, where the process was first discovered. In this case, *Vibrio fischeri* and other marine bacteria induce bioluminescence and thereby protect their eukaryotic partners (e.g., squid) from predation. In turn, the latter provide QS bacteria a nutrient-rich environment (Waters and Bassler, 2005). Other physiological processes, including spore germination and motility, are also influenced by QS.

How communication occurs

Communication in microbial communities is mediated by chemical molecules. These small, diffusible molecules are synthesized and secreted by individual producer cells. Once the concentration is sufficiently high, they are detected by other cells, who

respond by inducing particular genes. The specific nature of these molecules varies, depending on the producers. In general, four main types of QS molecules are now known (Figure 3.4.1). In Gram negative bacteria, molecules having acyl-homoserine lactone (AHL) structural backbones serve as autoinducers and are the most common form of QS chemical signals (Papenfort and Bassler, 2016). These AHL molecules are actually the signaling part of multi- (either two or three) component systems comprised of autoinducer synthases and autoinducer receptor/transcriptional regulators. The well-characterized LuxIR system, for example, consists of LuxI, the synthase that makes AHL. The latter is then secreted into the extracellular medium. When the concentration is sufficiently high, AHL diffuses back into resident cells and binds to LuxR, activating transcription of more AHL and the enzyme lucifinerase (which benefits the host, as noted above). Thus, even if only a small number of cells produce AHL, once a critical concentration is reached, the entire population ultimately responds.

Quorum sensing systems in Gram-positive bacteria are similar in principle, but different with respect to specific components. In particular, QS in Gram-positive bacteria rely on peptides (or pheromones) as the autoinducing signals, and a two component signal transduction system comprised of a sensor kinase and response regulator for transmitting the signals into functional products. The autoinducing peptides (AIP) are not diffusible as are AHLs, but instead must be processed and then exported into the medium. Neither do they diffuse back into the cell, thus the signal must be mediated via membrane bound sensor kinase proteins. In this case, extracellular AIP binds to the sensor kinase which then initiates a phosphorylation cascade, starting with phosphorylation of the response regulator. The latter protein, in its phosphorylated state, induces expression of various functional genes, as well as more AIP. Alternatively, some Gram-positive bacteria can transport inducing peptides via an oligopeptide transporter, and once inside, they induce gene expression via transcriptional regulation (Monnet et al., 2016).

A third QS system, present in both Gram-negative and Gram-positive bacteria, relies on synthesis of furanosyl borate diester, also called autoinducer 2 (AI-2). Finally, yeasts appear to produce several alcohols, including a large 15-carbon molecule called farnesol, that have QS activity (Hornby et al., 2001).

As noted above, QS systems have been suggested to function in foods, including fermented foods, such as sourdough and yogurt (Gobbetti and Di Cagno, 2013). There are, however, inherent experimental challenges, given that most foods already contain many substances that could interfere or quench autoinducing activities. Nonetheless, it makes ecological sense that food fermentation bacteria would rely on QS to regulate various activities. Not only are these bacteria competing within a particular niche, they may also be reliant on symbiotic partners to provide necessary nutrients or to create better conditions for growth.

Perhaps the most well studied example of an AI system is the set of gene that encode for production of nisin and other bacteriocins (Kleerebezem, 2014). These peptide antimicrobials are produced by many strains of lactic acid bacteria and are inhibitory against related genera and species. In addition to the structural gene (nisA), synthesis of nisin in *Lactococcus lactis* is dependent on several other genes, including genes encoding for proteins necessary for post-translational modification and processing and translocation of nisin from the cytoplasm to the extracellular medium. Expression of

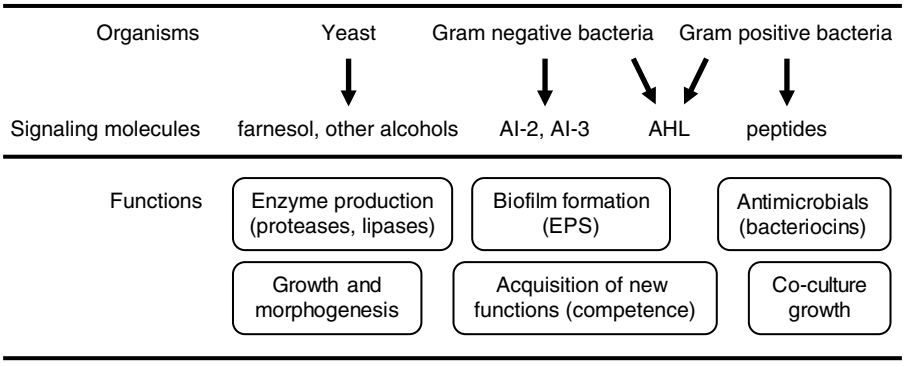


Figure 3.4.1 The role of communication in food fermentations. Shown are the main groups of inducing molecules and their potential functions in foods. Adapted from Rul and Monnet, 2015, with permission.

these genes is also dependent on NisR and NisK. These proteins are part of the nisin QS system, with NisK acting as the integral membrane, auto-phosphorylating sensor kinase and NisR as the cytoplasmic response regulator. When the latter is phosphorylated, it induces transcription of *nis* gene cluster.

In a vegetable model system, expression of bacteriocin production by *Lactobacillus plantarum* C2, a plant isolate, was regulated by QS but only on solid medium and not liquid broth. The authors suggested that proximity to competitors, as would occur in food conditions, is necessary to induce bacteriocin production, which would ultimately enhance colonization of a fermented vegetable environment (Rizzello et al., 2014).

Biofilm formation is another example of a QS phenomenon that occurs in fermented food environments. Recently, Priha et al. (2014) reported on efforts to exploit QS inhibitors or quenchers to prevent colonization and biofilm formation on brewery equipment. They first identified several plant extracts that reduced ACH signaling by brewery spoilage bacteria, and one extract was subsequently shown to reduce biofilm formation.

References

Gobbetti, M. and R. Di Cagno. 2013. Bacterial Communication in Foods. Springer, Berlin.

Hornby, J.M., E.C. Jensen, A.D. Lisec, J.J. Tasto, B. Jahnke, R. Shoemaker, P. Dussault, and K.W. Nickerson. 2001. Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. Appl. Environ. Microbiol. 67:2982–2992.

Kleerebezem, M. 2004. Quorum sensing control of antibiotic production; nisin and subtilin autoregulate their own biosynthesis. Peptides 25:1405–1414.

Monnet, V., V. Juillard, and R. Gardan. 2016. Peptide conversations in Gram-positive bacteria. Crit. Rev. Microbiol. 42:339–351.

Papenfort, K., and B.L. Bassler. 2016. Quorum sensing signal-response systems in Gram-negative bacteria. Nature Rev. Microbiol. 14:576–588.

Priha, O., V. Virkajärvi, R. Juvonen, R. Puupponen-Pimiä, L. Nohynek, S. Alakurtti, M. Pirttimaa, and E. Storgards. 2014. Quorum sensing signalling and biofilm formation of brewery-derived bacteria, and inhibition of signalling by natural compounds. Curr. Microbiol. 69:617–627.

Rizzello, C.G., P. Filannino, R. Di Cagno, M. Calasso, and M. Gobbetti. 2014. Quorum-sensing regulation of constitutive plantaricin by *Lactobacillus plantarum* strains under a model system for vegetables and fruits. Appl. Environ. Microbiol. 80:777–787.

Rul, F. and V. Monnet. 2015. How microbes communicate in food: a review of signaling molecules and their impact on food quality. Curr. Opin. Food Sci. 2:100–105.
Waters, C.M., and B.L. Bassler. 2005. Quorum sensing: cell-to-cell communication in bacteria. Annu. Rev. Cell Dev. Biol. 21:319–346.

BIBLIOGRAPHY

Bosma, E.F., J. Forster, and A.T. Nielsen. 2017. Lactobacilli and pediococci as versatile cell factories – Evaluation of strain properties and genetic tools. Biotechnol. Adv. 35:419–442.
Conrad, M., J. Schothorst, H.N Kankipati, G.V. Zeebroeck, M. Rubio-Texeira, and J. M. Thevelein. 2014. Nutrient sensing and signaling in the yeast *Saccharomyces cerevisiae.* FEMS Microbiol. Rev. 38:254–299.
Gänzle, M.G., 2015. Lactic metabolism revisited: metabolism of lactic acid bacteria in food fermentations and food spoilage. Curr. Opin. Food Sci. 2:106–117.
Holzapfel, W.H. and B.J.B. Wood, eds. 2014. Lactic Acid Bacteria: Biodiversity and Taxonomy, 1st edn. John Wiley, Oxford.
Jones, A.L. 2012. The future of taxonomy. Adv. Appl. Microbiol. 80:23–35.
Kowalczyk, M., B. Mayo, M. Fernández, and T. Aleksandrzak-Piekarczyk. 2016. Updates on metabolism in lactic acid bacteria in light of "omic" technologies. In: Biotechnology of Lactic Acid Bacteria: Novel Applications. F. Mozzi, R.R. Raya, and G. M. Vignolo, eds. John Wiley, Oxford.
Liu, M., J.R. Bayjanov, B. Renckens, A. Nauta, and R.J. Siezen. 2010. The proteolytic system of lactic acid bacteria revisited: a genomic comparison. BMC Genomics.11:36.
Pedersen, M.B., P. Gaudu, D. Lechardeur, M.A. Petit, and A. Gruss. 2012. Aerobic respiration metabolism in lactic acid bacteria and uses in biotechnology. Annu. Rev. Food Sci. Technol. 3:37–58.
Pfeiler, E.A. and T.R. Klaenhammer. 2007. The genomics of lactic acid bacteria. Trends Microbiol. 15:546–553.
Sauer, M., H. Russmayer, R. Grabherr, C.K. Peterbauer, and H. Marx. 2017. The efficient clade: lactic acid bacteria for industrial chemical production. Trends Biotechnol. 35:756–769.
Sneath, P.H.A. 1957. Some thoughts on bacterial classification. J. Gen. Microbiol. 17:184–200.
Teusink, B. and D. Molenaar. 2017. Systems biology of lactic acid bacteria: For food and thought. Curr Opin Syst Biol. 6:7–13.
Zaunmüller, T., M. Eichert, H. Richter, and G. Unden. 2006. Variations in the energy metabolism of biotechnologically relevant heterofermentative lactic acid bacteria during growth on sugars and organic acids. Appl. Microbiol. Biotechnol. 72:421–429.

4 Starter cultures

> Nowadays it must be clear to every zymotechnologist who has made himself familiar with the results of recent investigation, that wherever fermentation organisms are made use of, the aim must be the same, namely, to give up the old traditional method which depended upon mere chance. In this entire field a new era has now commenced.
>
> Emil Chr. Hansen, from Practical Studies in Fermentation, published in 1896

INTRODUCTION

One of the main attributes of fermented foods is their uniqueness. Unlike other processed foods, the successful manufacture of fermented foods and beverages relies on biologically active organisms. Indeed, with few exceptions, it is not possible to produce these foods without microorganisms. Nonetheless, it is possible to produce non-fermented counterparts of sour cream or summer sausage simply by adding food-grade acids to the raw material. Likewise, pickles and olives are often made by addition of brines to fresh cucumbers and olives, and even bread and soy sauce can be made by chemical means.

Despite the technical feasibility of producing these products without fermentation, the non-fermented versions generally lack many of the desired organoleptic qualities that would ordinarily be present, and that consumers expect, in the fermented products. This is in part because chemical acidulants or leavening agents generally perform a single function, such as lowering the pH or generating carbon dioxide. In contrast, in traditional fermented foods, microorganisms perform many functions. They are responsible for producing a wide assortment of aromatic and flavorful metabolic end-products, they consume some nutrients and produce others, and they cause important rheological and textural changes. Replicating these events by other means is simply not possible.

If microorganisms are indeed necessary for converting raw materials into fermented food products that have specific characteristics and properties, then the very next question to ask is "what is the best way to ensure that the appropriate organisms are present in the starting material?" In other words, how are fermentations started? There are essentially three ways to start or initiate food fermentations. For certain, the oldest method simply relies on the proliferation of indigenous microorganisms present in the raw material. Raw

Microbiology and Technology of Fermented Foods, Second Edition. Robert W. Hutkins.

milk and meat, for example, usually harbor the very bacteria necessary to convert these materials into cheese and sausage. Grapes and grape crushing equipment, likewise, often contain the yeasts responsible for fermenting sugars into ethanol and for transformation of juice into wine.

Although these fermentations are sometime said to be "spontaneous", this is an unfortunate misnomer. Spontaneous fermentations infer the existence of an outdated biological theory in which living organisms somehow emerged from non-living matter. Obviously, this terminology is intended to refer to fermentations begun or induced in the absence of any exogenous microorganisms and instead rely on the autochthonous or naturally-occurring microbiota. For these natural fermentations to be successful, however, requires not only that the "correct" microorganisms be present, but also that suitable conditions for their growth are established. Exclusion of oxygen or addition of salt, for example are among the external factors that may be necessary to promote particular fermentations. Even when these requirements are satisfied, however, there is no guarantee that the product will meet the relevant quality expectations, be safe to consume, or even be consistently produced. Still, many foods are produced by natural fermentation, including some sausages (Chapter 7), fermented vegetables (Chapter 8), and wines (Chapter 11). In addition, coffee and cocoa beans also undergo a natural fermentation that occurs shortly after the beans are harvested and that serves an essential processing function (Chapter 15).

Another way to initiate fermentations is via a method commonly known as backslopping. This approach is based on returning a portion of a successfully fermented product to fresh raw material. Backslopping is probably nearly as ancient as the natural fermentation practice described above. Moreover, the backslopping technique works for many fermented foods and beverages, including beer, cheese and cultured dairy products, bread, and vinegar. Indeed, backslopping methods are still practiced today in many small-scale and traditional production facilities, as well as in developing countries. Likewise, many home-made products often rely on backslopping. The principle, regardless of product or scale, is essentially the same. Specifically, any successfully fermented product should contain the relevant type and number of microorganisms, and, given a fresh opportunity, these organisms will perform much the same as they had in the previous fermentation. Despite the detractors of the backslopping technique, it can be argued that this practice actually selects for hardy and well-acclimated organisms with many of the desired traits necessary for successful production.

Starting a fermentation by backslopping must have not only worked fairly regularly, but in reality, this practice was, for many centuries, the only game in town. After all, it wasn't until the 19th century that Pasteur demonstrated that fermentations were caused by microorganisms. Before this discovery the very idea of a starter culture could not even be conceived. Soon, Lister, Koch, Orla-Jensen, and other early microbiologists developed pure culture techniques that made it possible to isolate and identify the organisms responsible for food fermentations and spoilage. Koch's postulates regarding the germ-disease connection could then be applied to fermentation science. Thus, an organism could be isolated from soured or fermented milk, purified, and then re-introduced into fresh milk causing the expected fermentation. The organism could then be re-isolated from the newly fermented product. The implication of this discovery, that pure cultures could be obtained and used to start fermentations, did not go unnoticed. Indeed, these observations resulted in a third way to produce fermented foods, namely via the use of a starter culture containing the relevant microorganisms for that particular product. Starter cultures now dominate fermented food and beverage industries.

ROLE OF STARTER CULTURES

It is often suggested by advocates of traditional manufacturing methods that natural fermentations, whether initiated by the endogenous microbiota or by backslopping, yield products that have unique or singular quality attributes. Naturally-fermented wines, for example, are often claimed to be superior to wines made using a starter culture. A so-called microbial terroir, the argument goes, exists within the vineyard and winery that influences the final product in a way that a starter culture cannot duplicate. Even if a fermentation were to occasionally fail, the argument continues, it would certainly be worth a small risk in order to end up with a truly exceptional product.

Perhaps this approach would be fine on a small scale basis, given the inherent flexibility in terms of time and quality expectations. In contrast, however, modern large-scale, high throughput industrial production of fermented foods and beverages demands consistent product quality and predictable production schedules, as well as stringent quality control to ensure food safety. Failed fermentations are not an option.

Simply defined, starter cultures consist of specific microorganisms or groups of microorganisms that are introduced directly into food materials to overwhelm the existing microbiota and bring about desired changes in the finished product. In general, relevant changes may include enhanced or novel functionality, extended shelf-life and preservation, reduced food safety risks, improved nutritional or health value, enhanced sensory qualities, and greater economic value. Although some fermented foods can be made without a starter culture, as noted above, the addition of concentrated and viable microorganisms, in the form of a starter culture, ensures (usually) that products are manufactured on a timely and repeatable schedule, with consistent and predictable product qualities. For modern, large volume fermentations, there is also a volume factor that must be considered. In other words, there is no easy way to produce the amount of culture necessary for a large-scale cheese manufacturer, to cite just one example, without the use of concentrated starter cultures (see below). Thus, for all practical purposes, with a few notable exceptions, starter cultures are now considered an essential component of nearly all commercially-produced fermented foods.

CULTURE HISTORY

The history of fermentation starter cultures is essentially the history of microbiology. As noted above, microbiology was only established as a scientific discipline in the 1860s and 1970s, most notably by Louis Pasteur. He, along with Lister, Koch, Ehrlich, and other early microbiologists were concerned about the role of microorganisms as a cause of infectious disease. However, many of the questions addressed by these scientists also dealt with foods, including fermented foods and beverages. In fact, it's fair to say that many members of the microbiology community at the turn of the twentieth century were essentially food microbiologists, working on very applied sorts of problems.

Not only were fermented foods, like all foods, prone to spoilage, but quality and safety issues were a constant challenge. As the scale of emerging beer, wine, bread, and dairy industries increased in size and volume, the pressure to develop manufacturing practices that could deliver more consistent product also increased. The discovery that specific bacteria and yeast were responsible for initiating (as well as spoiling) food fermentations led to the realization that it would be possible to control and improve fermentation processes. Although

the brewing, baking, and fermented dairy industries were quick to apply this new knowledge and to adopt starter culture technologies, other fermented food industries did not adopt starter cultures until relatively recently. And some still rely on natural fermentations.

One of the first such efforts to purify a starter culture was initiated in 1883 at the Carlsberg Brewery in Copenhagen, Denmark. There, Emil Christian Hansen used a dilution method to isolate pure cultures of brewing yeast, derived from a mixed culture that occasionally produced poor quality beer. Subsequently, he was able to identify which strain produced the best (or worst) beers. Hansen also was the first to isolate the two types of brewing yeast, the top (or ale) yeast and the bottom (or lager yeast). One such strain, which he named "Unterhefe Nr. 1" (or bottom yeast number 1), became the first pure starter culture to be used in beer making. Eventually, all of the beer produced by the Carlsberg Brewery was made using the Hansen strain, which also became the prototypical lager type strain.

Although Pasteur had also proven that wine fermentations, like those of beer, were performed by yeast, there was little interest at the time in using pure yeast cultures for wine-making. That satisfactory wine could be made using selected strains was demonstrated in 1890. However, it wasn't until many years later, in the 1960s, that wine yeasts became available as starter cultures for wine fermentations.

At around the same time and place, another Hansen, Christian D.A. Hansen, was working on the extraction of enzymes from bovine stomach tissue. This work led to the isolation of the enzyme chymosin, an essential ingredient used in cheese manufacture to coagulate milk. Prior to this time, chymosin had been prepared and used as a crude paste (essentially ground-up calf stomachs). Using the Hansen process, chymosin could be partially purified in a stable, liquid form, and the milk coagulation activity standardized. A company (Chr. Hansen) was formed and factories dedicated to chymosin production were built in Copenhagen in 1874 and in New York in 1878. Later, Hansen, who was trained as a pharmacist, developed procedures to produce natural coloring agents for cheese and other dairy products. By the end of the century, the Chr. Hansen company began producing dairy starter cultures, thus establishing a full-service business that continues even today to be a world-wide supplier of starters cultures and other products for the dairy, meat, brewing, baking, and wine industries.

The baker's yeast culture industry followed a similar path. Two immigrant brothers from Europe, Charles and Max Fleischmann, began a yeast factory in Cincinnati, Ohio in 1868. They produced a compressed yeast cake for use by commercial as well as home baking markets. The availability of this yeast dramatically changed bread manufacture. In part, this was because bread produced using this yeast culture was more consistent and in many cases superior to breads made using brewing yeasts, which was the common form at the time. More than 40 years later, in 1923, a baker's yeast production facility was built in Montreal, Canada, by Fred Lallemand, another European immigrant.

With the exception of brewer's and baker's yeasts, nearly all of these early starter cultures consisted of organisms that were used by the dairy industry. In particular, there was considerable interest in flavor-producing cultures that could be used for butter manufacture. At the turn of the twentieth century, most of the butter produced in the United States and Europe was cultured butter, meaning it was made from partially soured cream (in contrast to sweet cream or non-fermented butter which now dominates the US market). The cream was soured either by a natural fermentation (i.e., in the absence of an added culture) or by addition of previously soured cream or buttermilk. Butter made in this manner has an appealing flavor and aroma and longer shelf-life due to the acids and other metabolic products produced by fermentation. These methods, however, often resulted in inconsistent product quality. By the

1880s, researchers in Europe and the United States showed that specific strains of lactic acid bacteria could be grown in pure culture, and then used to ripen cream.

TECHNOLOGICAL CHALLENGES

The first dairy starter cultures were liquid cultures, prepared by growing pure strains or mixtures of strains in heat-sterilized milk. The main problem with these cultures was that they could easily become over-acidified and lose viability. In an effort to maintain a more neutral pH, calcium carbonate was often added as a buffer. Still, liquid cultures had a relatively short shelf life, which eventually led to the development of dehydrated cultures. The latter were produced simply by passing liquid cultures through cheese cloth, followed by air drying at 15°C to 18°C. Although these cultures were more stable, they also required several transfers in milk to revive the culture and return it to an active state.

Lyophilization technology was developed in the early part of the twentieth century and freeze dried dairy cultures became available by the 1920s. However, cell viability remained a problem, and even these products required growth in intermediate or "mother" cultures to activate the cells. Eventually, frozen cultures were introduced in the 1960s, and these cultures soon became the most popular form for dairy cultures. When frozen rapidly (usually with liquid nitrogen) and maintained at low temperature (< −20°C), these cultures remained viable and active. They did, however, require that the end user maintain these cultures at the appropriate conditions. Although frozen cultures are still widely used for dairy, meat, and other fermentations, significant improvements in freeze drying technologies have made lyophilized cultures the dominant form of starter culture for many food applications.

Culture R & D

The technologies described above were largely developed by researchers located at starter culture companies, as well as at universities and research institutes. Most of the culture supply houses in the US and Europe started out as family-owned enterprises and then grew to become rather large companies with significant research and production capabilities. By the 1980s, large pharmaceutical-based corporations, who were already well versed in fermentation technology with strong Research and Development departments, acquired several of these companies. This trend has continued well into the twenty-first century. However, some smaller companies have remained, selling mostly specialized products.

As noted above, starter cultures are not the only product the industry sells. In addition to a wide portfolio of strains for many different functions, the industry also sells several ancillary products. In particular, in the cheese industry, cultures are often propagated in bulk culture fermenters or tanks that require specialized culture media. The industry also sells enzymes, coloring agents, and other ingredients used in the manufacture of fermented foods. For many products, but especially for cheese and cultured dairy products, technical support is also important. This is because consistent culture performance is required for large high volume, high throughput food companies (and small ones too), and starter culture companies may be expected to provide nearly round-the-clock technical service and support to their customers. If a fermentation is delayed, slow, or simply deviates from the normal, the culture (and ultimately, the culture supplier) will often be blamed. Interestingly, in the entire food processing industry, cultures are about the only food ingredient supplied in a biologically active, yet somewhat sensitive form. Although enzymes also fall into this category, they are generally more easily standardized and stabilized.

A discussion of the history of starter culture science and technology would be incomplete without noting one of the main driving forces for much of the basic and applied research on lactic starter cultures. For sure, much of the early research focused on metabolism, physiology, classification, and applications of these bacteria. However, it was the bacteriophage problem that had perhaps the most important practical implications. The observation that bacteriophage (or simply phage), viruses whose hosts are bacteria, could infect and then kill starter culture bacteria was first made in the 1930s. Since that time, but especially since the 1970s (coinciding with the advent of molecular biology), several research groups in the United States and from around the world have devoted significant attention to understanding and controlling bacteriophage. This research has led to countless other important and fundamental discoveries about lactic acid bacteria. Bacteriophage problems and how they are controlled will be discussed later in this chapter.

STARTER CULTURE MICROORGANISMS

In Chapter 2, many of the microorganisms that are important in fermented foods were described. However, not all of these organisms are produced or marketed as starter cultures. Some fermented products do not lend themselves to starter culture applications, and for others, the market is simply too small or too specialized. For example, sauerkraut and pickle fermentations are initiated by the natural microbiota, and commercial manufacturers can produce these products without a starter culture (and associated expenses). Nonetheless, there are advantages even for these products, such that cultures and manufacturing procedures now exist for controlled starter culture-mediated pickle fermentations. In other cases, the manufacturer may prefer to maintain and propagate their own proprietary cultures. Many breweries, for example, have the necessary laboratory facilities and personnel to support internal culture programs. The production of fungal-fermented foods (described in Chapter 4) also frequently relies on house strains.

When starter culture technology were first developed (and for many years thereafter), the actual organisms contained within most culture preparations were generally not well defined. That is, the specific strains or species present in the culture were often not identified. Rather, a culture was used simply because it worked, meaning that it produced a good product with consistent properties. These undefined cultures, however, are now rarely used in industrial fermentations. Instead, the organisms present in modern commercial starter culture preparations are usually very well defined, often to a strain level. They are also carefully selected based on the precise phenotypic criteria relevant for the particular product (Table 4.1).

Bacterial starter cultures

The most important group of bacteria used as starter organisms are the lactic acid bacteria (Table 4.2). In fact, only a few non-lactic acid bacteria are commercially available as starter cultures. As described in Chapter 2, the lactic acid bacteria (LAB) consist of a cluster of Gram-positive cocci and rods that share several physiological and biochemical traits. They metabolize sugars via either homofermentative or heterofermentative metabolism, and they grow over a wide temperature range. Likewise, they vary with respect to salt tolerance, osmotolerance, aerobiosis, and other environmental conditions, accounting, in part, for the diversity of habitats with which they are associated.

Table 4.1 Desirable properties of starter cultures.

Culture	Property
Dairy cultures	Controlled lactic acid production rates
	Short lag phase
	Phage resistance
	Ease of manufacture
	Stability and consistency
	Produce desired flavor and texture
	Preservation tolerance
	Lack of off-flavors
Meat cultures	Fast acidification
	Produce desired flavor
	Antimicrobial activity
Beer cultures	Rapid fermentation
	Produce desired flavor
	Preservation tolerance and stability
	Flocculation
	Lack of off-flavors
	Proper attenuation
	Growth at wide temperature range
	Tolerant to osmotic, temperature, and handling stresses
Wine cultures	Osmotolerant
	Ethanol tolerant
	Flocculation, sedimentation
	Growth at low temperature
	Produce consistent flavor
	Malolactic fermentation
Bread cultures	Freeze tolerant
	Produce desired flavor
	Produce adequate leavening

Most of the LAB available as starter cultures belong to one of six genera. The dairy LAB represent the main group and include species of *Lactococcus, Streptococcus, Leuconostoc*, and *Lactobacillus*. The dairy starters are generally grouped as mesophiles or thermophiles, depending on the product application, but even this designation has become somewhat blurry. Thus, strains of *Streptococcus thermophilus,* a thermophile, are sometimes used in products associated with mesophilic starter cultures (e.g., for cheddar cheese), and mesophilic *Lactococcus lactis* susbsp. *lactis* are occasionally incorporated in thermophilic cultures (e.g., for mozzarella and pizza cheese).

Dairy cultures perform three main functions: (1) they ferment lactose and acidify the milk; (2) they generate flavor or flavor precursors; and (3) they modify the texture and rheological properties of the product. Lactic acid bacteria used as starter cultures for other fermented food products may have similar functions, although acidification is usually by far the most important. In sourdough bread, for example, starter culture strains of *Lactobacillus sanfansiscens* ferment maltose and lower the dough pH via production of lactic acid, but the culture also produces acetic acid, CO_2, and other flavor and aroma compounds.

Table 4.2 Lactic acid bacteria used as starter cultures.

Organism	Application
Lactococcus lactis subsp. *lactis*	Cheese, cultured dairy products
Lactococcus lactis subsp. *cremoris*	Cheese, cultured dairy products
Lactococcus lactis subsp. *lactis* biovar. *diacetylactis*	Cheese, cultured dairy products
Lactobacillus helveticus	Cheese, cultured dairy products
Lactobacillus delbrueckii subsp. *bulgaricus*	Cheese, yogurt
Lactobacillus sanfranciscensis	Sourdough bread
Lactobacillus casei	Cheese, cultured dairy products
Lactobacillus sakei	Sausage
Lactobacillus plantarum	Sausage, fermented vegetables
Lactobacillus curvatus	Sausage
Streptococcus thermophilus	Cheese, yogurt
Pediococcus acidilactici	Sausage, fermented vegetables
Pediococcus pentosaceus	Sausage
Tetragenococcus halophilus	Soy sauce
Oenococcus oeni	Wine
Leuconostoc lactis	Cheese, cultured dairy products
Leuconostoc mesenteroides	Cheese, cultured dairy products, fermented vegetables

Starter cultures used for sausage fermentations often contain strains of *Pediococcus.* Their main job is to produce lactic acid and reduce the pH to a level inhibitory to undesirable competitors. Commercial strains may also produce bacteriocins that inhibit pathogens and enhance preservation (see below). For other applications, the lactic starter culture may serve a purpose quite removed from those described above. A good example of a unique, but critical fermentation is that performed by *Oenococcus oeni*. The function of *O. oeni*, an organism used during the wine fermentation, is to convert malic acid to lactic acid. This reaction results in a slight increase in pH and is necessary to soften or de-acidify overly acidic wines.

Although LAB are the most important and most widely-used group of starter culture bacteria, other non-lactic acid bacteria also are included in various starter cultures or more likely provided as adjuncts (Table 4.3). In the cheese industry, *Propionibacterium freudenreichii* subsp. *shermanii* is used to manufacture Emmenthaler and other Swiss-type cheeses and *Brevibacterium linens* is used in the manufacture of so-called surface-ripened cheeses, such as Limburger, Muenster, and brick cheeses. In Europe (and occasionally in the United States), manufacturers of dry fermented sausages add species of *Kocuria*, *Staphylococcus*, or *Micrococcus* to the meat mixtures. These organisms produce the enzyme nitrate reductase which reduces the curing salt, sodium (or potassium) nitrate, to the nitrite form, and in so doing, enhances development of flavor and color attributes. Finally, the manufacture of vinegar involves oxidation of ethanol by *Acetobacter aceti* and related species.

Yeast starter cultures

Bread manufacturers are one of the largest users of yeast starter cultures. Several different forms of yeast starter cultures are available, ranging from the moist yeast cakes, used exclusively by the baking industry, to the active dry yeast packages sold at retail to consumers. Yeasts used for the bread fermentation (i.e., baker's yeast) are classified as *Saccharomyces*

Table 4.3 Other organisms used as starter cultures.

Organisms	Products (application)
Bacteria	
Brevibacterium linens	Cheese (surface pigment)
Propionibacterium freudenreichii subsp. *shermanii*	Cheese (eye formation in Swiss)
Staphylococcus carnosus	Meat (acid, flavor, color)
Mold	
Penicillium camemberti	Cheese (surface ripening)
Penicillium roqueforti	Cheese (interior ripening)
Penicillium chrysogenum	Sausage (surface ripening)
Aspergillus oryzae	Soy sauce, miso (enzyme secretion)
Rhizopus microspores subsp. *oligosporus*	Tempeh (produce mycelia)
Yeast	
Saccharomyces cerevisiae	Bread (carbon dioxide production)
Saccharomyces cerevisiae	Ale beers (ethanol and carbonation)
Saccharomyces pastorianus	Lager beers (ethanol and carbonation)
Saccharomyces cerevisiae	Wine (ethanol)

cerevisae, and are selected, in large part, based on their ability to produce large amounts of carbon dioxide in rapid fashion. Wine, beer, distilled spirits, and nearly all other alcoholic beverages are also made using yeast cultures (Table 4.3).

Although many of the ethanol-producing yeasts are also classified as *S. cerevisae*, they differ markedly from those strains used for bread manufacture. As noted above, many of the wines produced in Europe are made via natural fermentations, relying on the naturally-occurring yeasts present on the grape surface and winery equipment. The trend for most wine manufacturers, however, has been to use starter culture yeasts. As with other starter cultures, strain selection is based primarily on the desired flavor and sensory attributes of the final product. However, other production traits, including the ability to flocculate, to grow at high sugar concentrations, and to produce adequate ethanol levels, are also relevant. The brewing industry is another major user of yeast starter cultures, although some large breweries maintain their own proprietary cultures. As for wine, strain selection has been based on characteristics relevant to the needs of the particular brewer, and includes factors such as flocculation, flavor development, and ethanol production rates.

Fungal starter cultures

Although the market for fungal starter cultures is modest, many manufacturers of fungi-fermented foods often prefer to use starter cultures rather than to propagate their own cultures. Thus, fungal starter cultures are available for several types of cheeses, including both the blue-veined types (e.g., Roquefort and Gorgonzola), as well as the white surface mold-ripened cheeses (e.g., Brie and Camembert). The blue mold cultures consist of spore suspensions of *Penicillium roqueforti* and white mold cheese cultures contain *Penicillium camemberti*. Fungal cultures are also used in the production of many of the Asian fermented foods. The main examples include tempeh, made using *Rhizopus microsporus* subsp. *oligosporus*, and miso and soy sauce, made using *Aspergillus sojae* and *Aspergillus oryzae.*

There is also a small but important market for fungal starter cultures used to produce European-style sausages and hams.

STRAIN IDENTIFICATION

Although it may seem obvious that the precise identities of the organisms present in a starter culture should be known, strain identification is not a trivial matter. Microbial systematics is an important discipline in the microbiological sciences and is especially relevant in fermented foods and the starter culture industry. In addition, microbial systematics is an evolving science that has been significantly influenced by advances in genetics, genomics, and various molecular techniques. Thus, the movement or reassignment of a given organism from one species to another occurs fairly regularly. In recent years, for example, strains of lactic acid bacteria, brewer's yeasts, and acetic acid-producing bacteria used in vinegar production have all been reclassified into different species or assigned new names altogether.

Admittedly, the numerous changes that have occurred in microbial classification and nomenclature can be challenging for starter culture manufacturers. However, for several reasons, it is still essential that the microbial contents of a starter culture be accurately described and that species identification be based on the best available taxonomical information (Box 4.1). First, culture suppliers need to include the correct species names on their products, since "generally recognized as safe" (GRAS) status is affirmed only for specific organisms. A cheese culture claiming to contain species of *S. thermophilus*, but actually containing closely related strains of *Enterococcus,* would be mis-labeled. Second, accurate identification is necessary for the simple reason that culture propagation and production processes require knowledge of the organism's nutritional and maintenance needs, which are often species-dependent.

Box 4.1 Who's who in microbiology?

Early days. More than a century ago, in the very beginning of microbiology, Pasteur, Lister, and their contemporaries could observe an organism microscopically, note its appearance, describe a few physiological properties, and then assign a name to it (Krieg, 1988). Thus, classification was initially based on a limited number of morphologic and phenotypic properties or traits. This situation changed in the twentieth century as more biochemical, physiological, and genetic characteristics became known and as objective methods for assigning particular organisms to specific groups were adopted. Soon, microbial taxonomy and classification became established disciplines in microbiology.

Lactic acid bacteria are defined. Although classification and nomenclature are important for all biologists, how an organism is named and the taxonomical group into which it belongs are especially relevant for starter culture microbiologists. For many years, up until the 1970s, identification of lactic acid bacteria relied mainly on ecological, biochemical, and physiological traits. The first comprehensive description of these bacteria was provided in 1919 by Orla-Jensen, and 20 years later the Sherman scheme was published (Orla-Jensen, 1919 and Sherman,

Table 4.1.1 Sherman scheme for classification of *Streptococcus*[1].

Division	Representative species[2]	Growth at:		Growth in presence of:			Survival at 60°C, 30 minutes	NH_3 from peptone
		10°C	45°C	6.5% NaCl	Methylene blue (0.1%)	pH 9.6		
Pyogenic	*Streptococcus pyogenes*	–	–	–	–	–	–	+
Viridans	*Streptococcus thermophilus*	–	+	–	–	–	+	–
Lactic	*Lactococcus lactis* subsp. *lactis*	+	–	–	–	–	+	+
Entero-coccus	*Enterococcus faecium*	+	+	+	+	+	+	+

[1] Adapted from Sherman, 1937.
[2] Names reflect current nomenclature.

1937). So important were these classification schemes that the 1937 Sherman paper was the very first article in the very first issue of the prestigious *Bacteriological Reviews* journal series (now called *Microbiology and Molecular Biology Reviews*). The Sherman scheme, which still remains useful, systematically placed streptococci into four distinct groups: pyogenic, viridan, lactic, and enterococci (Table 4.1.1).

Naming names. These early papers also reflect the changing nature of bacterial taxonomy and nomenclature. In the Orla-Jensen treatise, for example, a description is given of the dairy lactic acid bacterium that was originally called *Bacterium lactis* (by Lister in 1878), then changed, successively to *Streptococcus lactis*, and eventually to *Lactococcus lactis* (Schleifer et al., 1985). Interestingly, Orla-Jensen noted 70 years earlier that "it would be tempting to employ the name *Lactococcus*" for these bacteria (the genus name first used by the famous Dutch microbiologist, Martinus Beijerinck).

Although the initial classification schemes for lactic acid bacteria were based on phenotypic properties, serological reactions have also been used to differentiate streptococci. The Lancefield reactions (named after Rebecca Lancefield) were based on the antigenic properties of cell wall-associated components, resulting in more than thirteen distinct groups. The dairy streptococci (now *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*) were found to possess the group N antigen and became referred to as the Group N streptococci. The other major dairy streptococci, *Streptococcus thermophilus*, was not antigenic and could not be serologically grouped. Currently, the Lancefield groupings are mostly used for classifying pathogenic streptococci, including *Streptococcus pyogenes* and *Streptococcus pneumonia* (Group A) and *Streptococcus agalactiae* (Group B). Ultimately, however, one taxonomist suggested that "serology is best forgotten when working with dairy streptococci" (Garvie, 1984).

Identifying lactic acid bacteria. As noted above, phenotypic traits are often used as a first screen to classify lactic acid bacteria. The most informative distinguishing characteristics include: (1) temperature and pH ranges of growth; (2) tolerance to sodium chloride, methylene blue, and bile salts; (3) production of ammonia from arginine; and (4) carbohydrate fermentation patterns. Some of these are physiologically relevant in fermented foods (e.g., salt tolerance and growth at low or high temperature), whereas others are simply diagnostic (e.g., inhibition by methylene blue).

Based on these criteria, as well as microscopic morphology, it is rather easy to perform the relevant tests and to obtain a presumptive identification of a given lactic acid bacterium. Phenotypic kits, based primarily on sugar fermentation profiles, are commonly used to identify lactic acid bacteria to the species level. Other identification schemes that rely on membrane fatty acid composition, enzyme structure similarities, and other properties also exist, although they are used less often.

Molecular fingerprints. Despite the value of traditional identification schemes, there is no doubt that the methods described above lack the power and precision of genome-based techniques. In fact, advances in nucleic acid-based bacterial fingerprinting methods and sequencing methods have led, not only to new identification tools, but also to renewed interest in bacterial evolution and ecology. The ability to distinguish between strains of the same species is important not only for identification purposes, but also because it provides a basis for understanding the phylogenetic and evolutionary relationships between starter culture organisms. Although morphological, biochemical, and other phenotypic characteristics remain useful for genus and species identification, molecular approaches that rely on nucleotide sequences have proven to be more reliable, more reproducible, and more robust.

Among the techniques still in use for fingerprinting and genotyping are pulsed field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), and ribotyping. Despite being labor-intense, they were very reliable and discriminatory. Nonetheless, they have been mostly supplanted by DNA sequencing methods, including 16S ribosomal RNA sequence analysis, multilocus sequence typing (MLST), and more recently, whole genome sequencing. Sequence-based methods, whether based on 16s or whole genomes have become fast and relatively cheap, and can be out-sourced to private labs. A DNA extract or even a colony on a plate can be shipped by overnight delivery on Monday, and the sequence or a 16S rRNA-based identification can be emailed to the customer's inbox as early as the next day.

Chemical fingerprints. Another method that has become popular in the past decade relies on mass spectrometry (MS) to identify organisms to the species level. In brief, MS methods involve: (1) an ionizing source to convert microbial macromolecules to ions in the gas phase; (2) an analyzer that separates the resulting ions based on their charge-to-charge ratio; and a detector that measures those ions (Sauer and Kliem, 2010). For microbiological applications, ionization is usual via electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI), and the most common analyzers are time of flight (TOF) mass analyzers. The mass fingerprint obtained by MS methods can then be compared to a database that contains fingerprint spectra from referenced organisms (Welker, 2011).

Whether derived from molecular or chemical methods, strain identifications are important for obvious scientific reasons, but there are also commercial reasons. Many strains used as cultures or probiotics possess unique characteristics, and such strains may be proprietary or protected by patents. The reader should also be aware that there is a certain degree of subjective decision-making involved in the classification process, despite efforts by taxonomists to be totally objective. Thus, the value of a given classification system depends entirely on its acceptance by the microbiology community.

References

Amor, K.B., E.E. Vaughan, and W.M. de Vos. 2007. Advanced molecular tools for the identification of lactic acid bacteria. J. Nutr. 137:741S–747S.

Garvie, E.I. 1984. Taxonomy and identification of bacteria important in cheese and fermented dairy products. In: F.L. Davies and B.D. Law, eds. Advances in the Microbiology and Biochemistry of Cheese and Fermented Milk. Elsevier, London, pp. 35–66.

Krieg, N.R., 1988. Bacterial classification: an overview. Can. J. Microbiol. 34:536–540.

Orla-Jensen, S. 1919. The Lactic Acid Bacteria. Andr. Fred Host, Copenhagen.

Schleifer, K.H., J. Kraus, C. Dvorak, R. Kilpper-Bälz, M.D. Collins, and W. Fischer. 1985. Transfer of *Streptococcus lactis* and related streptococci to the genus *Lactococcus* gen. nov. System. Appl. Microbiol. 6:183–195.

Sauer, S. and M. Kliem. 2010. Mass spectrometry tools for the classification and identification of bacteria. Nature Rev. Microbiol. 8:74–82.

Sherman, J.M. 1937. The streptococci. Bacteriol. Rev. 1:3–97.

Welker, M. 2011. Proteomics for routine identification of microorganisms. Proteomics 11:3143–3153.

Finally, should the product manufacturer prefer to include species information on the label of a fermented product, the species name should be correct. Currently, this would be voluntary, since such information is not required. For example, yogurt and other cultured dairy products that contain probiotic bacteria often include species information, since some consumers may actually look for and recognize the names of particular species. For such products, the name of the organism (and perhaps even the strain) is especially important because probiotic activity depends on the actual species or strain. Unfortunately, species declarations on consumer products are often incorrect or are outdated. Adding to the public confusion is the use, presumably for marketing purposes, of trivial or fanciful names that give the illusion of an official species.

STARTER CULTURE MATH

One of main reasons for why manufacturers use starter cultures is to ensure a consistent and predictable fermentation. However, a starter culture also addresses a more fundamental challenge, namely how to produce enough cells to accommodate the inoculum demands of large-scale fermentations. For most fermented foods, the first requirement of a starter culture is that it initiates a fermentation promptly and rapidly. Although exceptions exists (e.g., in the case of the carbon dioxide-evolving fermentation that occurs late in the Swiss cheese process), it is usually necessary that the fermentation commence shortly after the culture is

added. And while a short lag phase may be tolerated, a long lag phase is generally a bad sign. This could indicate that culture numbers are low or that viability has been impaired. Thus, for a starter culture to function effectively, it must contain a large number of viable microorganisms.

As the mass or volume of the starting material increases, either larger starter culture volumes, greater starter culture cell concentrations, or possibly both are required. For example, if a 1% starter culture inoculum is ordinarily used for a given product, then 1 kg of starter culture would be necessary to inoculate 100 kg of the food material. This modest-sized inoculum could easily be produced from a colony or stock culture, simply by one or two successive transfers through an intermediate culture (e.g., 0.1 ml or one colony into 10 ml, followed by 10 ml into 1 L).

When pure cultures, rather than backslopping techniques, were first introduced a century ago in the dairy industry, this approach of making intermediate and mother cultures was invariably how cultures were routinely prepared and used. As the size of the industry increased, however, such that larger and larger starter culture volumes were required, it was no longer feasible for cheese manufacturers to prepare cultures in this manner. In other words, a cheese plant receiving 1 million L of milk per day would need 10,000 L of culture, plus all of the intermediate cultures (and incubations) necessary to reach this volume.

CULTURE DELIVERY: BULK VERSUS DIRECT-TO-VAT

To address this situation, two general types of cultures were developed. These are still manufactured and sold to the fermented food and beverage industries. The first type, referred to in the dairy industry as bulk cultures, are used to inoculate a bulk tank. The bulk starter culture is essentially the equivalent of several intermediate cultures that traditionally have been required to build up the culture. After a suitable incubation period in the appropriate culture medium, the fully-grown bulk culture (akin to a mother culture) is then used to inoculate the raw material. The starter culture organisms comprising the bulk culture will remain viable for many hours, provided they are protected against acid damage, oxygen, hydrogen peroxide, or other inhibitory end-products. In the cheese and fermented dairy products industry, bulk cultures are routinely used to inoculate production vats throughout a manufacturing day. Maintaining culture viability is still an important issue, however, as discussed below.

A bulk culture is not warranted for many fermented foods simply because raw material volumes are more modest in size. That is, the amount of culture necessary to inoculate the fermentation substrate can easily be met using the culture as supplied directly from the manufacturer. For example, baker's yeasts may be supplied as yeast cream or yeast cakes, which can be added directly to the dough ingredients just prior to mixing. Similarly, meat starter cultures, whether in frozen can form or lyophilized packets, are added directly to the meat mixture. Even dairy cultures that are designed to be inoculated directly into the food material are now widely available, thus eliminating the need to prepare bulk cultures.

In the dairy industry, these cultures are referred to as direct-to-vat set cultures. They have many advantages that have made them very popular. They eliminate the labor, hardware, and capital costs associated with the construction, preparation, and maintenance of bulk starter culture systems. They also eliminate leftover or wasted bulk culture. Although they were initially produced as frozen concentrates packaged in cans, they are now available as pourable pellets or lyophilized powders, making it easy to dispense the exact amount necessary

for inoculation. Lyophilized cultures also have the additional advantage of not requiring freezer capacity. Perhaps most importantly, eliminating the bulk culture fermentation also means that bacteriophage have one less opportunity to infect the culture and cause trouble.

When direct-to-vat set cultures are used to inoculate large volumes, the culture must be highly concentrated to deliver a sufficient inoculum into the raw material. Freezing, centrifugation, filtration, lyophilization, and other concentration steps may indirectly reduce culture viability, ultimately leading to slow-starting fermentations. Improvements in bioprocessing and concentration technologies have minimized most of these problems. Still, direct-to-vat cultures are generally more expensive than bulk cultures, and, despite their convenience, may not be economical for all operations.

CULTURE COMPOSITION

The original cultures used by the dairy industry were comprised of a mixture of strains. They typically contained several strains, species, or even genera. It was entirely possible that the identities of the organisms in a mixed culture were not known, nor were the individual species characterized microbiologically or biochemically. Even the proportion of different organisms in a mixed culture would not necessarily have been constant from one product lot to another.

Nonetheless, these mixed undefined cultures did have a proven history, and they are still occasionally used as starter cultures for many dairy and other food applications. Moreover, depending on the product, mixed cultures may have functional advantages. For example, the heterogeneity of these cultures may provide greater complexity of flavor or aroma. These mixed strain starter cultures are especially common in the Netherlands for the manufacture of Gouda, Edam, and related cheese varieties. The fermentation part of the cheese making process can be quite long, and it is not unusual for a given strain to be infected and subsequently killed by bacteriophage that inhabit the cheese plant environment (or that are present within the culture itself). However, given the diversity of lactic acid bacteria present in these mixed cultures, there are likely strains that are resistant to that particular bacteriophage and that can then complete the fermentation. In fact, it is well established that frequent exposure to different bacteriophages provides a natural and effective mechanism for ensuring that phage-resistant strains will be present in repeatedly propagated mixed cultures.

Despite their proven track record, mixed cultures are not without problems. In particular, product quality may be inconsistent and fermentation rates may vary widely, affecting production schedules. For small-scale operations, where time is somewhat flexible and where quality variations may be more tolerable, these issues are not so serious. For large production facilities, however, where precise schedules are critical and consistent product quality is expected, mixed cultures have become less common. Starter cultures comprised of defined strains, with more precise biological and biochemical properties, are now prevalent. These cultures, referred to as defined cultures, simply refer to cultures that contain microbiologically characterized strains.

DEFINED CULTURES

Defined starter cultures can be comprised of a single individual strain or as a blend of two or more strains. The origin of the defined strains that are used commercially varies. Some were simply present in traditional mixed cultures and others have been isolated from natural

sources. For example, in the case of dairy starters, milk production habitats and cheese serve as good sources; in the case of wine starters, grapes and wine-making equipment are good locations to find suitable yeasts.

Before they could be considered for use as cultures, defined strains must first be identified and characterized for relevant metabolic and physiological properties, phage-resistance (in the case of dairy strains), and other desirable traits. For fungal starters, safety is an important issue and the inability of a putative culture strain to produce mycotoxins is an essential criterion. In addition, when multiple strains are assembled into a culture blend, all strains must be compatible. That is, one strain must not produce inhibitory agents (e.g., bacteriocins, hydrogen peroxide, or killer factors) that would affect growth of other organisms or that would cause one strain to dominate over others.

Ever since the defined strain concept for dairy starters was introduced in the 1970s, there has been debate about whether single, paired, or multiple strain blends are preferred. Multiple strains are necessary and required for some applications, such as yogurt cultures, which requires the presence of two different organisms, *S. thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. Sour cream cultures, likewise, contain acid-producing lactococci and flavor-producing *Leuconostoc* sp. Many sausage cultures similarly contain species of *Lactobacillus* and *Pediococcus.* However, even for products requiring only a single organism (e.g., *Lactococcus lactis* used for cheddar cheese manufacture), paired or multiple strains are often desired.

In dairy cultures, the rationale for the inclusion of multiple defined strains is based mainly on bacteriophage concerns. Multiple strain starters are blended such that they contain strains with dissimilar phage sensitivity patterns. That is, each strain is resistant to different phage types, so that if one strain is infected, the other strains can complete the fermentation. This approach is reminiscent of the natural mixed strain cultures described above, except that the defined strain blends would be expected to behave in a more predictable and consistent manner. Identifying which strains have become sensitive to the indigenous phage in that particular plant requires regular monitoring of phage levels in the cheese whey, and in-plant tests have been developed for this purpose. More sophisticated phage enumeration assays can also be conducted at culture supplier laboratories. When phage titers reach some critical level or a particular strain begins to show sensitivity, that strain is removed from the blend and replaced with a new phage-resistant strain or derivative. Multiple strain cultures can also be rotated in a particular order to achieve an even greater level of security.

MANUFACTURE OF STARTER CULTURES

Starter cultures are now mass-produced in modern, large-volume fermentors. The manufacturing process actually begins with a single colony isolate or stock culture, which is then grown in a small volume prior to inoculation into the production fermentor (Figure 4.1A). The fermentors, centrifuges, and other down-stream steps operate under aseptic conditions, not unlike those used in the pharmaceutical industry for production of antibiotics, vaccines, or other biomedical products (Figure 4.1B). They almost always operate in a batch mode, because continuous cell production systems have not yet been adopted by the industry. The size of the fermentors may vary, from as little as 100 liters to 10,000 liters. However, most of the basic operational parameters and control features are essentially the same for both small and large batches.

Culture media and operating conditions

The choice of media varies, depending on the organisms being grown and the nutrient requirements necessary for optimum cell production. For example, dairy starter cultures are frequently produced using milk- or whey-based media, whereas molasses or corn syrup can be used as the basal medium for other lactic cultures. Specialized nutrients are also often added to the medium. Water-soluble B vitamins may be required for optimum growth of lactococci and lactobacilli. Some species of *Streptococcus*, *Leuconostoc*, and *Lactobacillus* may also require specific amino acids. Other compounds, such as the surfactant Tween 80, are added to the growth medium to promote membrane stability of lactobacilli and other LAB during subsequent frozen storage and lyophilization.

To achieve high cell density and maximize biomass production and cell viability, it is important that inhibitory metabolic end-products be removed or neutralized. In particular, lactic acid can decrease the medium pH to a level low enough to inhibit cell growth. Although the optimum pH for cell growth depends on the specific organism (e.g., lactobacilli prefer a slightly more acidic medium pH than lactococci), lactic acid bacteria generally grow best

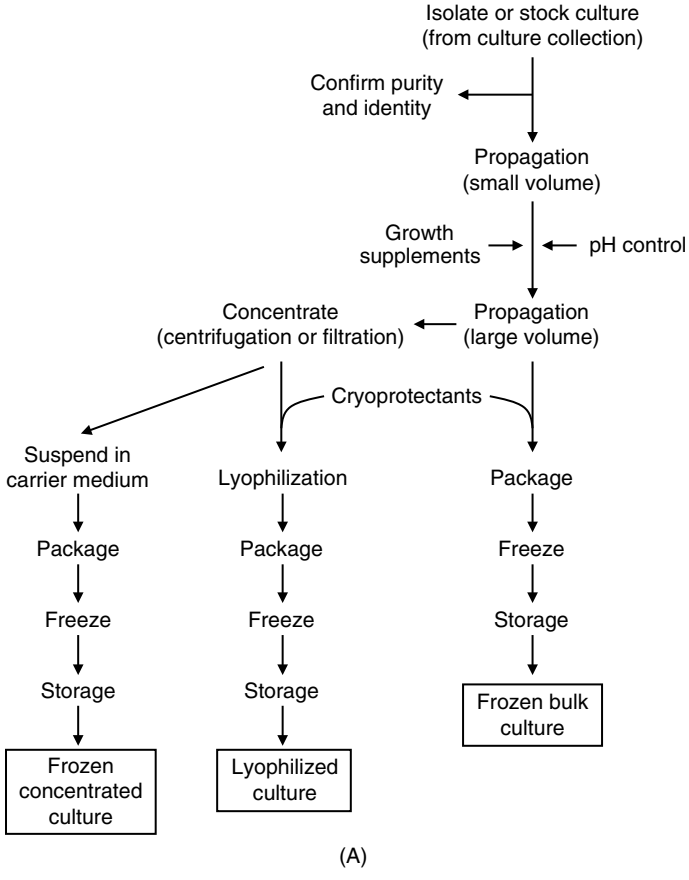
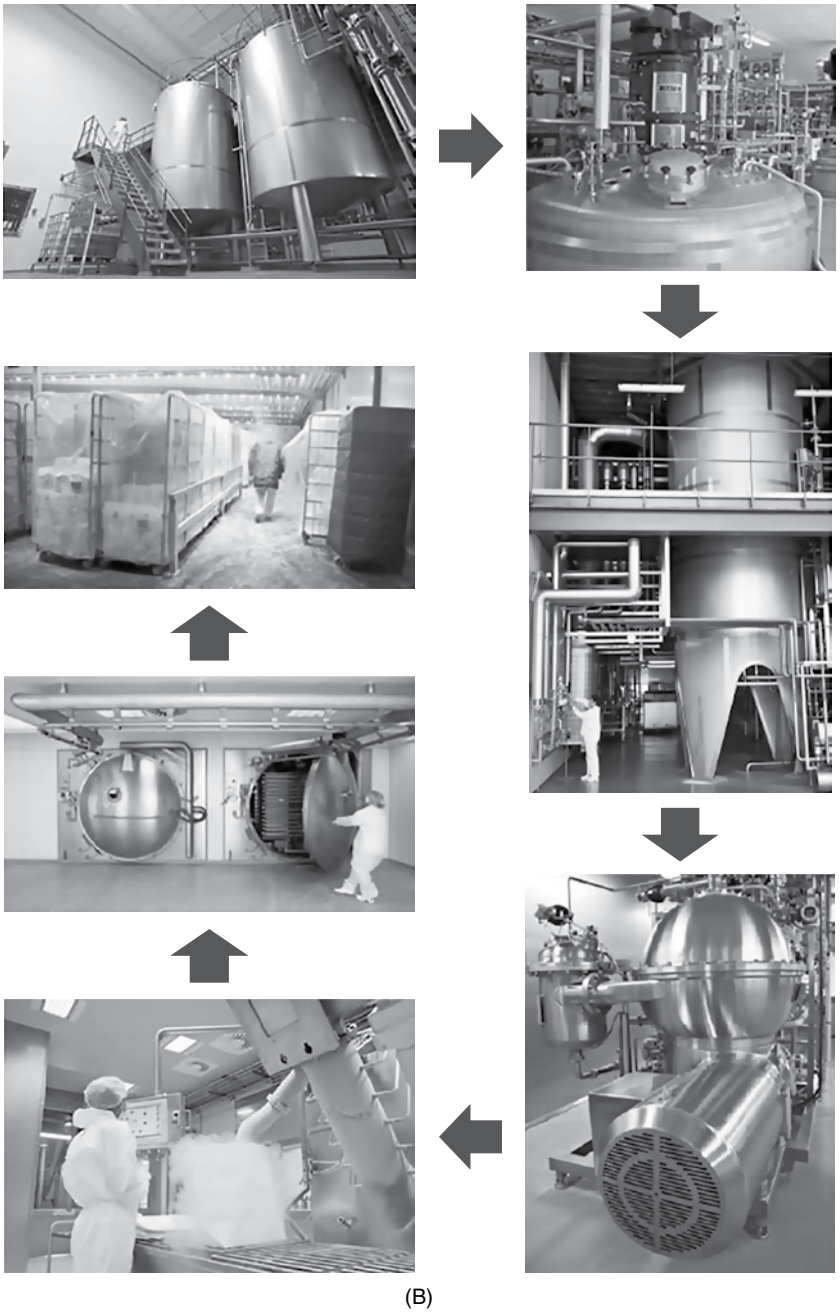


Figure 4.1 Production flow chart (A) of industrial starter cultures and associated manufacturing equipment (B), from (clockwise) mixing tanks, top and main section of fermentor, continuous centrifuge, freeze pelleting, freeze dryer, and frozen storage. Figures courtesy of Chr. Hansen.



(B)

Figure 4.1 (*Continued*)

within a pH range of 5 to 6. Therefore, neutralization of the accumulated acid via addition of alkali, usually gaseous NH_3, NH_4OH, Na_2CO_3, or KOH, is essential. Similarly, hydrogen peroxide can also accumulate to inhibitory levels during culture production as a result of peroxide-forming reactions. Hydrogen peroxide formation can be reduced or removed by

minimizing incorporation of oxygen and by adding catalase directly to the fermentor during cell production.

Provided that optimum growth conditions are provided, as described above, maximum cell densities of 10^9 to 10^{10} cells per ml are typically obtained. In general, lactic acid bacteria are harvested either at late log phase or early stationary phase. However, the optimum harvest time depends on the specific organism. At this point, the cells can be: (1) packaged directly as frozen liquid cultures; (2) concentrated and then frozen; or (3) lyophilized (freeze dried).

Frozen liquid cultures are dispensed into metal or plastic containers with volumes typically ranging from 100 ml to 500 ml, then rapidly frozen in liquid nitrogen (< −196°C). This had long been the most common form for lactic acid bacterial cultures, although it is no longer as common. Concentrated frozen cultures, in contrast, became popular since the higher cell densities led to their use in direct-to-vat applications. For both types of frozen cultures, it is critically important that the cultures remain frozen (< −45°C) and that temperature fluctuations be avoided during transport and during storage at the manufacturing site. This is because freeze-thaw cycles result in the formation of ice crystals, which can then puncture and kill cells, reducing cell number and viability when the cells are thawed and used.

To produce concentrated frozen cultures, cells are first grown to high cell densities (as described above) and then concentrated either by continuous centrifugation or cross-flow membrane filtration. The cells may then be washed to remove the spent medium and resuspended in a suspension solution containing stabilizing agents (see below). The concentrated cells are then packaged into cans (usually 300 g to 500 g) at cell densities as high as 10^{11} to 10^{12} cells per gram and are rapidly frozen. Thus, the concentrated culture contains about the equivalent amount of cells that would normally be present in a fully grown bulk culture. Typically, a single 500 g can is sufficient to inoculate 5000 kg of milk.

Another cool way to freeze concentrated cells is via pellet technology. This is done by adding concentrated cells drop-wise directly into liquid nitrogen. The cells freeze instantaneously in the form of pellets, which can be collected and dispensed into paperboard containers or foil or plastic pouches. Provided they are maintained in a frozen state, the pellets are pourable and are easy to weigh out dispense.

Finally, it is possible to concentrate and preserve cells by one of several different dehydration processes. Spray drying, for example, can be very effective for some biological materials; however, it is inapplicable for lactic acid bacteria, due to substantial loss in cell number and viability. In contrast, lyophilization, or freeze drying, has long been known to be a more gentle process for dehydrating and preserving starter culture lactic acid bacteria. Although some cell inactivation or injury may still occur, lyophilized cells are generally more stable than cells concentrated by other means.

The lyophilization process involves removal of water from a frozen material by applying sufficient vacuum (< 1.0 Torr) such that sublimation of ice occurs. Cells are first grown to high cell density, harvested, and concentrated, as described above. The cells slurries can be frozen in trays or in pellet form (see above), and then subjected to the freeze drying process (usually about 5 Pascal to 20 Pascal for 18 to 30 hours). Freeze dried cultures can contain from 10^9 to 10^{12} cells/gram. The cells are usually packaged in foil pouches or other oxygen-impermeable material. Although lyophilized cells are best maintained at −20°C, they show good stability even at refrigeration temperature. Lyophilized cultures are now as popular as frozen direct-to-vat set starter cultures, especially for yogurt and other cultured milk products.

On occasion, initial growth of both frozen and lyophilized cells may be sluggish, with a prolonged lag phase. To maintain cell viability, various cryoprotectant agents are usually added to protect the cells against freeze and stress damage. Depending on the cell species, cryoprotectant agents include glycerol, lactose, sucrose, trehalose, ascorbate, and glutamate. Moreover, some microorganisms simply do not lyophilize well, and suffer a more serious loss in cell number and viability. For example, there are several reports indicating that dairy lactobacilli, including *L. delbrueckii* subsp. *bulgaricus*, *L. helveticus,* and *L. acidophilus*, may be particularly sensitive to lyophilization, and that special efforts must be made to ensure that these organisms remain viable (Box 4.2). This is important because one or more of these organisms are included in commercial yogurt and other dairy cultures, which are increasingly produced in lyophilized forms.

Box 4.2 Warming up to lyophilization

It is already a considerable challenge for culture companies to identify new starter cultures strains that possess the desired genetic and metabolic properties necessary to produce high quality fermented foods and beverages on a consistent basis. In addition, it is essential that any new or improved strain be amenable to whatever conditions are used to grow, concentrate, and preserve the final culture. This means that the strains must be tolerant to high cell density growth, centrifugation or filtration, freezing, drying, lyophilization, oxidative stress, and long-term storage. Thus, if an organism, no matter how good it is for making cheese or yogurt or wine, lacks tolerance or resistance to these production treatments, it will likely not make it as a starter culture.

Lyophilization microbiology. Lyophilization is one of the most convenient and preferred methods for culture preservation (Fonseca et al., 2015). Although lyophilization is one of the more gentle means for long-term preservation of starter cultures, the initial freezing and subsequent sublimation steps can take a toll on microorganisms (Foerst and Santivarangkna, 2014). Freezing is detrimental to cells for several reasons. First, as water turns to ice, water activity decreases and the intracellular solutes become concentrated, causing osmotic stress. Importantly, ice crystals that form during freezing can damage membranes and puncture cells.

Within the lactic acid bacteria, dairy lactobacilli can be especially sensitive to the freezing and lyophilization process (Shao et al., 2014). In part, this may be due to geometry – large rods have more surface area than small spherical cells, and are more prone to crystal-induced damage. The drying or sublimation step also influences survival and viability, because cells are prone to collapse as vacuum is applied and as the temperature increases in the freeze drying chamber. Conditions should be selected to prevent reaching this so-called collapse temperature in order to prevent structural damage to the cells (Foerst and Santivarangkna, 2014).

Growth conditions. Several factors must be considered to achieve high survival rates and to enhance cell viability during freezing and lyophilization. The first is how the cells are grown. When exposed to a decrease in temperature, many bacteria, including lactic acid bacteria, alter their membrane lipid composition by synthesizing

and incorporating specific fatty acids into the membrane (Hansen et al., 2015; Louesdon et al., 2014b; Meneghel et al., 2017). This change in membrane composition provides fluidity at low temperature and helps to minimize membrane damage. Therefore, lower, sub-optimal growth temperatures (e.g., 25°C) may enhance resistance to subsequent freezing conditions (Shao et al., 2014).

Cryoprotectants. Another means by which cells protect themselves against freezing and dehydration is by accumulating cryoprotectant molecules within their cytoplasm. These compounds can reach high intracellular concentrations (above 1 M) and function, in part, by preventing water loss and eventual plasmolysis. Among the common protectant molecules used by lactic acid bacteria are various sugars and amines, including betaine, carnitine, and proline (Alonso, 2016; Louesdon et al., 2014a). Cyroprotectants can be transported into the cell or synthesized *de novo* from precursor molecules. Including these substances in the growth medium allows the bacteria to stock up so that when freezing occurs, they will be loaded with protectant molecules. Although glycerol is also known to enhance cryotolerance, it is not clear that this compound is actively accumulated in lactic acid bacteria.

Sub-lethal stress. Exposure to low temperature may induce expression of genes involved in the general stress response system in lactic acid bacteria. The genetic response to cold shock conditions is mediated by specific sigma factors that regulate transcription of genes whose protein products are necessary for defending the cell against stress damage or death. The function of these so-called stress proteins varies, but, in general, involves repair or degradation of misfolded proteins and stabilization of macromolecules and membranes. Thus, greater cell survival and stability rates might be achieved during lyophilization by prior induction of the stress-response system.

This approach might be counter-intuitive in at least one respect. It is well established, for example, that rapid freezing is better than slow freezing, since the latter generates large ice crystal that are very damaging to cells. However, lactic acid bacteria, adapted to low temperature either by slow cooling or by exposure to a pre-freezing stress, may have greater survival rates compared to rapidly frozen cells, presumably because the stress-response system had been induced in the adapted cells.

Freeze drying medium. After cells are grown and harvested, they are usually resuspended in a suitable medium prior to freezing and lyophilization. For dairy applications, the basal medium is typically milk-based. However, because the suspension medium will remain a component of the final cell mixture, it should not only be optimized to maintain viability during freeze drying, but the medium should also contain components that enhance survival and preservation while the cells are in the dehydrated state. Among the compounds that appear to be most effective are various sugars and sugar alcohols, including lactose, mannose, glucose, trehalose, and sorbitol, as well as amino acids and phenolic antioxidants (Carvalho et al., 2003). The latter may be especially important, because exposure to oxygen or air is known to reduce viability of dehydrated cells.

Storage and rehydration. Although lyophilized cells are generally considered to be quite stable, they are sensitive to environmental abuses that may occur during storage. High relative humidity, warm storage temperatures, and temperature fluctuations significantly reduce survival rates and cell viability (Montel Mendoza et al., 2013). Aerobiosis, as noted above, may cause detrimental oxidation reactions.

The conditions by which lyophilized cells are rehydrated can also have a significant influence on the viability of the culture. In general, the best suspension medium approximates the medium in which the cells were originally dehydrated. Suspension media that are hypotonic or that are too warm or cold should be avoided (Carvalho et al., 2004).

Cryotolerant mutants. As described above, there is both a genetic as well as a physiological basis for cryotolerance. These observations suggest that it may be possible to identify mutants that have enhanced cryotolerance. For example, spontaneous mutants were obtained by passing *Lactobacillus delbrueckii* subsp. *bulgaricus* cells through thirty growth and freeze-thaw cycles (Monnet et al., 2003). Although it is not clear what the nature of the mutation(s) may have been that conferred enhanced crytolerance, this approach appears to be a useful means to generate naturally cryotolerant strains.

References

Alonso, S. 2016. Novel preservation techniques for microbial cultures. In: Novel Food Fermentation Technologies. K.S. Ojha and B.K. Tiwari, eds. Springer International Publishing, Berlin.

Carvalho, A.S., J. Silva, P. Ho, P. Teixeira, F.X. Malcata, and P. Gibbs. 2003. Protective effect of sorbitol and monosodium glutamate during storage of freeze-dried lactic acid bacteria. Le Lait. 83:203–210.

Carvalho, A.S., J. Silva, P. Ho, P. Teixeira, F.X. Malcata, and P. Gibbs. 2004. Relevant factors for the preparation of freeze-dried lactic acid bacteria. Int. Dairy J. 14:835–846.

Fonseca, F., S. Cenard, and S. Passot. 2015. Freeze-drying of lactic acid bacteria. Methods in Molecular Biology. 1257:477–488.

Foerst, P. and C. Santivarangkna. 2014. Advances in starter culture technology: focus on drying processes. In: Advances in Fermented Foods and Beverages: Improving Quality, Technologies and Health Benefits. W. Holzapfel, ed. Woodhead Publishing, Cambridge, pp. 249–270.

Hansen M.L., M.A. Petersen, J. Risbo, M. Hümmer, and A. Clausen. 2015. Implications of modifying membrane fatty acid composition on membrane oxidation, integrity, and storage viability of freeze-dried probiotic, *Lactobacillus acidophilus* La-5 Biotechnol. Prog. 31:799–807.

Louesdon, S., S. Charlot-Rougé, V. Juillard, R. Tourdot-Maréchal, C. Béal. 2014a. Osmotic stress affects the stability of freeze-dried *Lactobacillus buchneri* R1102 as a result of intracellular betaine accumulation and membrane characteristics. J. Appl. Microbiol. 117:196–207.

Louesdon, S., S. Charlot-Rougé, R. Tourdot-Maréchal, M. Bouix, and C. Béal. 2014b. Membrane fatty acid composition and fluidity are involved in the resistance to freezing of *Lactobacillus buchneri* R1102 and *Bifidobacterium longum* R0175. Microbial Biotechnol. 8:311–318.

Meneghel, J., S. Passot, S. Dupont, and F. Fonseca. 2017. Biophysical characterization of the *Lactobacillus delbrueckii* subsp. *bulgaricus* membrane during cold and osmotic stress and its relevance for cryopreservation. Appl. Microbiol. Biotechnol. 101: 1427–1441.

Monnet, C., C. Béal, and G. Corrieu. 2003. Improvement of the resistance of *Lactobacillus delbrueckii* ssp. *bulgaricus* to freezing by natural selection. J. Dairy Sci. 86:3048–3053.

Montel Mendoza, G., S.E. Pasteris, M.C. Otero, and M.E Nader-Macias. 2013. Survival and beneficial properties of lactic acid bacteria from raniculture subjected to freeze-drying and storage. J. Appl. Microbiol. 116:157–166.

Shao, Y.Y., S.R. Gao, H.L. Guo, and H.P. Zhang. 2014. Influence of culture conditions and preconditioning on survival of *Lactobacillus delbrueckii* subspecies *bulgaricus* ND02 during lyophilization. J. Dairy Sci. 97:1270–1280.

Evaluating culture performance

As noted before, fermented foods are the only processed foods whose manufacture requires a biologically-active ingredient, namely the starter culture. Simply stated, it is the job of the starter culture to carry out the desired fermentation, promptly and consistently, and to generate the expected flavor and texture properties relevant to the specific food product. The particular requirements for a given strain, however, depend entirely on the application for that culture. As customers have come to expect particular specifications for their final products, the specific traits and properties expressed by culture organisms have also become quite demanding. Thus, strains of lactic acid bacteria used as dairy starter cultures are now selected based not just on lactic acid production rates, but also on flavor and texture properties, salt sensitivity, compatibility with other strains, and phage resistance.

A good example of the selection criteria applied for starter cultures occurs for yogurt cultures. Selected strains of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* used as yogurt starter cultures should have, at minimum, rapid acid development rates and phage resistance, but other factors may be just as important. That is, how much acetaldehyde the culture produces, whether or not exopolysaccharides are synthesized, and to what extent the cultures contributes to post-fermentation acidity are all factors that influence strain selection. Moreover, strains of these bacteria that would make them suitable as thermophilic starter cultures for yogurt manufacture does not necessarily mean they will be useful as thermophilic starters for mozzarella or Swiss cheese manufacture, since the requirements for the latter products are different from those of yogurt. Likewise, traits desirable for brewing strains of *Saccharomyces cerevisiae* (e.g., flocculation and ethanol tolerance) are quite different from those important for baker's yeasts strains of *S. cerevisiae* (where gassing is more important).

Several phenotype-based tests are routinely performed to assess functional properties of starter cultures. Fermentation performance for dairy cultures, for example, can be easily determined by inoculating heat-treated milk with a standardized inoculum, incubating the material at an appropriate temperature, and then measuring the decrease in pH as a function of time. For some cultures, lag times may be important and can also be estimated from growth curve data. Similar tests are performed for meat starter cultures, whose main function is also to produce lactic acid.

Another critical test used specifically for dairy cultures involves the determination of bacteriophage resistance. Laboratory-based methods have been developed for this purpose that mimic cheese making. Briefly, cells are inoculated into culture tubes of milk, a representative bank or factory phage mixture is added, and the tubes are incubated according to a typical cheese production time-temperature profile. If the pH at the end of the incubation cycle has reached the expected target value, then it is assumed that the culture was not affected by the added phage. The entire process is then repeated multiple times, adding a portion of the whey from the previous cycle. The absence of a sufficient pH decrease indicates that the culture has become sensitive to the added bacteriophage and should be removed from use.

Yeast cultures used for wine, beer, and bread should also ferment rapidly, have good sensory properties, and be stable during storage. Depending on the product, these yeasts must also possess other specific properties. For example, a critical physiological characteristic of wine and beer yeasts is their ability to flocculate or sediment at the end of the fermentation. Sedimentation of cells enhances their separation from the wine or beer and reduces autolysis and subsequent release of intracellular constituents.

Other performance characteristics of yeast starters also depend on the specific product. Lager beer yeasts, for example, should be able to grow at low temperature and produce flavorful end-products. The ability of brewing yeasts to ferment all available sugars (at least those that are fermentable), a property known as attenuation, is a critical property. In wine manufacture, wine yeasts are also often selected based on growth at low temperatures (10°C to 14°C), as well as resistance to sulfiting agents. For some applications, wine yeast should also have high ethanol tolerance and osmotolerance.

Finally, the main requirements for baker's yeasts, in addition to producing good-flavored bread, are to have a short lag phase and to produce carbon dioxide rapidly in the dough (gassing rate). For some applications, such as frozen doughs, the yeasts should also have good cryotolerance, the ability to withstand freezing conditions, so that the frozen dough will rise after thawing.

Compatibility issues

One other requirement for multiple-strain starter cultures is to ensure that all of the organisms are biologically compatible. For example, some lactic acid bacteria produce bacteriocins, peroxides, or other antimicrobial substances that could inhibit other organisms. Even individual strains of a single species may inhibit other strains of the same species. If a multiple strain *L. lactis* culture was assembled without regard to compatibility issues, it would be entirely possible that one strain may produce a bacteriocin that was inhibitory to the other *L. lactis* strains in that culture. In much the same manner, some wine and beer yeasts produce inhibitory substances called "killer" toxins that inhibit other yeasts. Compatibility also extends to the growth characteristics of the organisms in a given culture. Organisms that produce acids and lower the pH quickly may prevent growth of their flavor-producing culture partners, a situation encountered in sour cream and cultured buttermilk.

HOW STARTER CULTURES ARE USED

There are two general ways that starter cultures are used. As described above, frozen or lyophilized starter cultures can simply be inoculated directly into the food substrate (i.e., direct-to-vat). This is the normal means by which active baker's yeast cultures, fermented meat starter cultures, and many dairy cultures are added to the starting food material. In the case of frozen cultures, the cans are first thawed in cold (and usually chlorinated) water immediately prior to use.

Bulk cultures

In contrast to the direct-to-vat type cultures, some fermented foods are inoculated with bulk cultures (Figure 4.2). Bulk cultures are intended to increase the size of the starter culture (in terms of total cell number and volume) by several orders of magnitude (2000 L to 4000 L is about an average size tank).

In general, bulk culture media are not that different from the fermentation media used to mass produce starter culture cells. They contain a basal medium consisting of a fermentable carbohydrate (usually lactose, but glucose or sucrose can also be used) and a nitrogen source (usually hydrolyzed proteins from milk or whey). Culture media are also supplemented with vitamins, minerals, and other nutrients. Yeast extract and corn steep liquor are good sources.

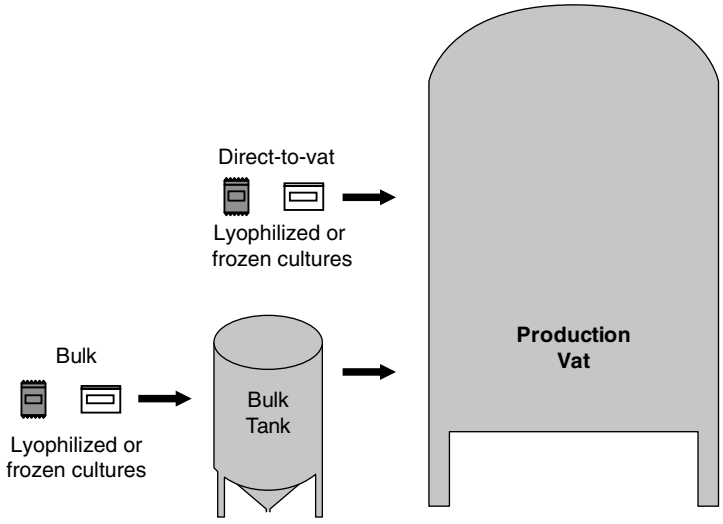


Figure 4.2 Using bulk and direct-to-vat cultures.

Carbonate, citrate, or phosphate salts may be added to provide buffering. All ingredients used in the bulk culture medium must be food grade.

The dairy industry is by far the main user of bulk cultures (although the liquid cream yeasts used by large bread manufacturers could be considered a bulk culture). When bulk dairy cultures are used, additional steps are necessary to ensure the organisms have reached high cell densities and that they are active and viable. In particular, growth of lactic acid bacteria in milk or whey is accompanied by a marked decrease in pH. In the absence of buffering, the medium pH will decrease to 4.5 or below. These cells will eventually lose viability and behave sluggishly when inoculated into the starting material. Therefore, preventing acid injury or damage during the production and propagation of starter cultures is essential.

There are two general ways to minimize or prevent acid damage to lactic starter cultures. One approach relies on the addition of alkaline solutions into the bulk culture tank to neutralize the acid produced during fermentative growth. Neutralizing agents may include sodium or ammonium hydroxide or ammonia gas. In actual practice, these external pH control systems consist of a tank fitted with a pH electrode, a pH monitoring device, and a microprocessor. When the pH has decreased below a critical pre-set threshold, a pump adds neutralizing agent to the tank until an upper pH limit is reached. As cells grow and produce acid, the pH never falls below the set threshold. The optimum pH range depends on the specific organisms; most external control systems for mesophilic dairy starter bacteria maintain a pH between 5.8 and 6.2. These systems yield bulk starter cultures with cell densities as much as ten times greater than uncontrolled cultures, and with enhanced viability.

An alternative approach to the external pH control systems is to incorporate buffer salts directly into the bulk culture medium. In theory, the acids produced by the culture would be immediately neutralized by the buffer salts. These so-called self-buffering or internal pH control systems have one major advantage – they do not require the purchase of the expensive external pH control hardware. Internal pH control media are formulated such that the medium pH is maintained above 5.0.

Both external and internal pH control media provides similar advantages. Since higher cell densities are achieved, and cell viability is enhanced, less culture is needed. In addition, because the cells are maintained in a viable state, they can be used for a longer period of time.

BACTERIOPHAGE AND DAIRY FERMENTATIONS

Bacteriophage, and specifically, the problems they cause during cheese and dairy fermentations, has been the driving force for much of the decades-long research on starter cultures (Box 4.3). Bacteriophage capable of infecting the culture bacteria will likely be present in any open environment where lactic fermentations occur. Their ability to multiply and spread rapidly in a cheese or yogurt factory may lead to decimation of the starter culture.

Box 4.3 Bacteriophages of lactic acid bacteria

Viruses are the most abundant organisms on the planet (Aziz et al., 2015). Most of these viruses are bacteriophages, defined simply as viruses that attack bacteria. Not surprisingly, perhaps, bacteriophages are found in every ecosystem where there are bacteria. Thus, bacteriophage are present in relatively high levels in ocean and marine environments, soil, and human habitats. As described below, bacteriophage are also vitally important in fermented foods, and their biology and means of replication have been widely studied.

The physical structure, biology, taxonomy, and life cycle of bacteriophage are unlike those of the bacteria, yeasts, and molds. In fact, bacteriophage look and behave like nothing else in the biological world. In essence, bacteriophage are simply packages of DNA or RNA contained within a protective head that is attached to tail-like structures. The latter enable the phage to adhere to and ultimately parasitize host cells. The ability of bacteriophage to exchange genetic information and rearrange their genomes contributes to their dissemination throughout the environments in which they reside.

Despite their apparent simplicity, their importance in food microbiology cannot be overstated. Bacteriophage are particularly important in food safety microbiology because their genomes may harbor genes encoding for toxins and other virulence factors. When temperate phages (see below) harboring virulence genes infect and lysogenize specific host organisms, the virulence genes can then be transferred to and expressed by the host cells. Several foodborne pathogens, including *Escherichia coli* O157:H7, *Vibrio cholera, Clostridium botulinum* have acquired virulence genes via these phage mediated or so-called "lysogenic conversion" events (Brabban et al., 2005; Kelly et al., 2009).

In the manufacture of fermented foods, especially those made using lactic starter cultures, the presence of bacteriophage can lead to production delays, quality defects, and other detrimental effects (Garneau and Moineau, 2011). The economic consequences of bacteriophage infection in a cheese or yogurt manufacturing facility can be significant. Thus, understanding the ecology, mode of replication, and transmission of

bacteriophage are essential for monitoring and minimizing the incidence of bacteriophage problems and for developing effective strategies for their control.

Bacteriophage classification

Because bacteriophage are parasites and cannot grow outside of a host, they have no physiological or biochemical activities that can be used as a basis for systematic classification. That is, bacteriophage lack most of the phenotypic traits used to describe bacteria. They are neither aerobic nor anaerobic, they have no temperature or moisture optima, per se (aside from attachment kinetics or host-dependent functions), and metabolic pathways do not exist. Thus, for many years, classification of phages that infect lactic acid bacteria was largely based on other criteria, including morphology, structural composition, serology, host range, and DNA homology. These characteristics are still commonly used to classify lactic bacteriophages (Table 4.3.1), although genome and proteome data now provide additional tools that are often faster and less labor-intensive (Mahony et al., 2014).

Table 4.3.1 Taxonomy of lactic acid bacteriophage.

Host	Phage family	Phage species	Number of sequenced members
Lactococcus lactis	Siphovidae	936	51
		P335	15
		c2	2
		1358	1
		Q54	1
		P087	1
		1706	1
		949	2
	Podoviridae	P034	1
		KSY1	1
Streptococcus thermophilus	Siphovidae	*cos*	6
		pac	6
		5093-like	1
Leuconostoc mesenteroides	Siphovidae	Group Ia and b	2
Leuconostoc paramesenteroides	Siphovidae	Group IIa–d	2
Lactobacillus brevis	Myovidae	Unnamed	1
Lactobacillus casei	Siphovidae	Unnamed	1
Lactobacillus delbrueckii	Siphovidae	Unnamed	6
Lactobacillus fermentum	Siphovidae	Unnamed	2
Lactobacillus gasseri	Siphovidae	Unnamed	1
	Myovidae	Unnamed	1

(*Continued*)

Table 4.3.1 (Continued)

Host	Phage family	Phage species	Number of sequenced members
Lactobacillus helveticus	Myovidae	Unnamed	1
Lactobacillus paracasei	Siphovidae	Unnamed	2
	Myovidae	Unnamed	1
Lactobacillus plantarum	Siphovidae	Unnamed	5
	Myovidae	Unnamed	1
Lactobacillus rhamnosus	Siphovidae	Unnamed	1
Lactobacillus sanfranciscensis	Siphovidae	Unnamed	1

Adapted from Mahony and van Sinderen, 2014. See original source for references.

According to the most recent taxonomy (Mahoney and van Sinderen, 2014), lactic bacteriophage are classified in the order, Caudovirales. Within this order, Lactococcal phages belong to one of three families, Podoviridae, Myoviridae, and Siphoviridae. The latter family includes the most common of the ten recognized groups or phage species that infect lactococci. Bacteriophage that infect *Streptococcus thermophilus* are also in the Caudovirales order; most are of the cos- and pac-type (Quiberoni et al., 2010).

Although genomic-based methods are now widely used for classifying phages, morphology remains a proven tool. Accordingly, there are several relevant morphotypes, based on the size, shape, and structure of heads, tails, and collars (Figure 4.3.1). Included, for example, are phages having prolate- or isometric-shaped heads. Phage tails can be long or short, contractile or non-contractile. Within the lactococcal phages, most share structural similarity (i.e., long contractile tails) to three specific lactococcal phages: c2, 936, and P335. Therefore, it is common to refer to other phages as being represented by one of these three types. In particular, phages of the 936 type (long non-contractile tail and isometric protein head) are considered to be the most common type infecting industrial fermentations that rely on lactococci (Mahony et al., 2012).

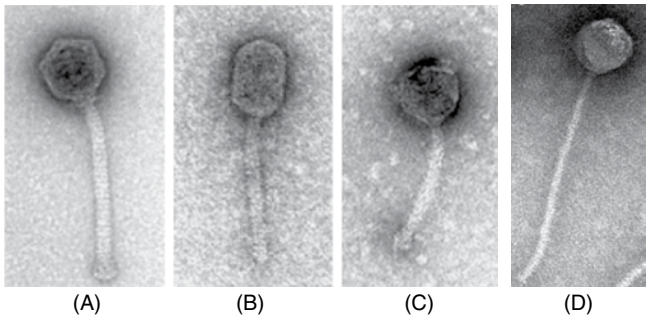


Figure 4.3.1 Electron photomicrographs of *Lactococcus lactis* phage P680 (A; 936 phage species), P684 (B; c2 phage species), and P643 (C; P335 phage species). In D, *Streptococcus thermophilus* phage 128 (a *cos*-type) is shown. From Achigar et al., 2017 and Atamer et al., 2009, with permission.

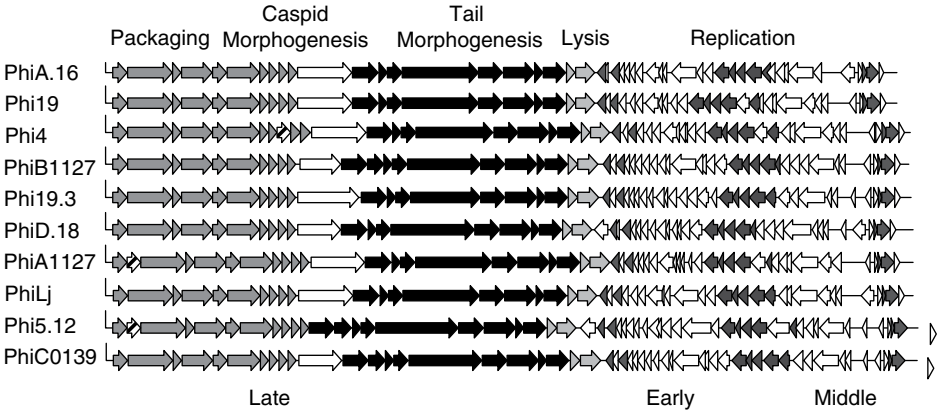


Figure 4.3.2 Alignment of ten lactococcal bacteriophage genomes from the 936 group. Functions and expression patterns are indicated. Adapted from Murphy et al., 2016, with permission.

Bacteriophage genomics has now become one of the most powerful means for characterizing and classifying lactococcal and related phages. Sequence analyses of bacteriophage genomes (literally, 100s from *Lactococcus*, *Lactobacillus* and *Streptococcus thermophilus*) have revealed that gene clusters are arranged in modular fashion (Figure 4.3.2). A good example comes from a recent comparative study on 39 phages from the 936 group (Murphy et al., 2016). These genomes consists of modules that code for particular functions, such as packaging, structure, lysis, and replication. In addition, these modules are transcribed temporally, either during the early, middle, or late stages of the phage replication cycle. Phage classification, then, can be based on similarities in the genetic organization of different phages. Moreover, it is now recognized that the ability of lytic bacteriophage to undergo frequent recombination is likely due to the modular nature of the phage genome. Importantly, recombination by phage may result in the acquisition of new genetic information, including genes that enable the phage to counter host defense systems (Rakonjac et al., 2005).

Bacteriophage biology

Not all bacteriophage infect and lyse their host cells. Some bacteriophage have two routes of infection or "lifestyle" choices: lytic or lysogenic. Lytic phages infect a host, replicate inside that host cell, and eventually lyse the cell, releasing new infectious phage particles. In contrast, a lysogenic infection occurs when so-called temperate bacteriophage infect the host cell, but rather than initiating a lytic cycle, they instead integrate their genome within the host chromosome. The phage then exists as a dormant prophage, and is replicated along with the host genome during cell growth. Importantly, lysogenic cells may be immune to subsequent infection. Although the precise mechanisms dictating whether a temperate phage enters a lytic or lysogenic phase are still emerging, it is known that lytic and lysogenic genes are subject to transcriptional regulation. Specifically, repressors of these two pathways (i.e., lysis or lysogeny) exist that prevent transcription of the relevant genes. It has also well

established that prophage can be induced, such that the phage is excised from the chromosome and is converted from a lysogenic state to a lytic phase.

The observation that prophage induction may occur during cheese manufacture suggests that prophage may be an important source of new phage within fermentation environments. This is of obvious importance to control phage in these environments. However, autolysis of starter (and non-starter) lactic acid bacteria via prophage induction represents a novel means for release of cytoplasmic enzymes that may contribute to cheese ripening (Lortal and Chapot-Chartier, 2005; Visweswaran et al., 2017).

References

Achigar, R., A.H. Magadán, D.M. Tremblay, M.J. Pianzzola, and S. Moineau. 2017. Phage-host interactions in *Streptococcus thermophilus*: Genome analysis of phages isolated in Uruguay and ectopic spacer acquisition in CRISPR array. Sci Rep. 7:43438.

Atamer, Z., J. Dietrich, M. Müller-Merbach, H. Neve, K.J. Heller, and J. Hinrichs. 2009. Screening for and characterization *of Lactococcus lactis* bacteriophages with high thermal resistance. Int. Dairy J. 19:228–235.

Aziz, R.K., B. Dwivedi, S. Akhter, M. Breitbart, and R.A. Edwards. 2015. Multidimensional metrics for estimating phage abundance, distribution, gene density, and sequence coverage in metagenomes. Front. Microbiol. 6:381.

Brabban, A.D., E. Hite, and T.R. Callaway. 2005. Evolution of foodborne pathogens via temperate bacteriophage-mediated gene transfer. Foodborne Pathog. Dis. 2:287–303.

Garneau, J.E. and S. Moineau. 2011. Bacteriophages of lactic acid bacteria and their impact on milk fermentations. Microb. Cell Factories 10, Suppl.1:S20.

Kelly, B.G., A. Vespermann, and D.J. Bolton. 2009. The role of horizontal gene transfer in the evolution of selected foodborne bacterial pathogens. Food Chem. Toxicol. 47:951–968.

Lortal, S. and M.-P. Chapot-Chartier. 2005. Role, mechanisms and control of lactic acid bacteria lysis in cheese. Int. Dairy J. 15:857–871.

Mahony, J., F. Bottacini, D. van Sinderen, and G.F. Fitzgerald. 2014. Progress in lactic acid bacterial phage research. Microb. Cell Fact. 13 (Suppl 1):S1.

Mahony, J., J. Murphy, and D. van Sinderen. 2012. Lactococcal 936-type phages and dairy fermentation problems: from detection to evolution and prevention. Front. Microbiol. 3:335.

Mahony, J. and D. van Sinderen. 2014. Current taxonomy of phages infecting lactic acid bacteria. Front Microbiol 5:7.

Murphy, J., F. Bottacini, J. Mahony, P. Kelleher, H. Neve, A. Zomer, A. Nauta, and D. Van Sinderen. 2016. Comparative genomics and functional analysis of the 936 group of lactococcal Siphoviridae phages. Sci. Rep. 6:21345.

Quiberoni, A., S. Moineau, G.M. Rousseau, J. Reinheimer, and H.-W. Ackermann. 2010. *Streptococcus thermophilus* bacteriophages. Int. Dairy J. 20:657–664.

Visweswaran, G.R.R., D. Kurek, M. Szeliga, F.R. Pastrana, O.P. Kuipers, J. Kok, and G. Buist. 2017. Expression of prophage-encoded endolysins contributes to autolysis of Lactococcus lactis. Appl. Microbiol. Biotechnol. 101:1099–1110.

During the production of bulk cultures, bacteriophage problems have become relatively rare for several reasons. First, the culture media is ordinarily prepared by heating to pasteurization temperatures high enough to inactivate bacteriophage. Also, most modern manufacturing facilities employ aseptic conditions within the culture preparation areas, often including separate rooms and air handling systems to prevent entry of airborne phage. Finally, closed, sterilizable tanks are almost always usually used for culture preparation.

In contrast, cheese manufacturing conditions are not always so restrictive and opportunities for phage infection commonly exist. For example, milk used for cheese manufacture may be unpasteurized or receive only a relatively modest heat treatment. Moreover, the cheese making process is often conducted in an open manner. Thus, the milk and curds are readily accessible to air- or whey-borne phage. Furthermore, in high-throughput cheese production factories, where vats are filled and re-filled several times per day, there is ample time for phage that may initially be present at low levels to propagate and reach populations high enough to cause slow or arrested fermentations and poor quality product.

Ultimately, the lactic starter culture and the phage that infect them are in a sort of race - can the culture do its job (i.e., bring about a successful fermentation) before the phage causes culture inhibition? In general, for cheddar-type cheeses, it only takes about 3 to 3.5 hours for the fermentation to be completed (the so-called set-to-salt time). However, given that a single phage can infect a host cell and replicate within 30 minutes, releasing fifty or more new phage particles into the environment, phage problems can occur quickly. The lytic cycle repeats itself (Figure 4.3) such that it may not take long for the phage population to exceed

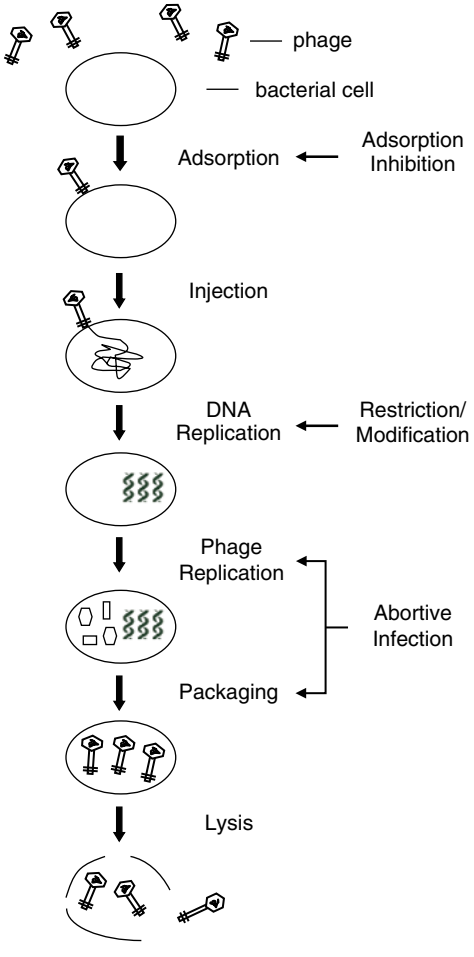


Figure 4.3 Lytic cycle of lactic bacteriophage and steps at which phage resistance mechanisms function.

Table 4.4 Population dynamics of a simulated phage infection[1].

	Phage absent		Phage present		
Time	**Cells/ml**	**pH**	**Cells/ml**	**Phage/ml**	**pH**
0:00	1×10^6	6.5	1×10^6	1	6.6
0:30	2×10^6	6.4	2×10^6–1	50	6.5
1:00	4×10^6	6.3	4×10^6–50	2500	6.3
1:30	8×10^6	6.1	8×10^6–2500	125,000	6.1
2:00	1.6×10^7	5.9	1.6×10^7–125,000	6.25×10^6	6.0
2:30	3.2×10^7	5.7	2.5×10^7–6.25×10^6 = 1.9×10^7	3.1×10^8	5.8
3:00	6.4×10^7	5.5	1.9×10^7–3.1×10^8 = 0	3.1×10^8	5.6
3:30	1.3×10^8	5.2			

[1] Note the following assumptions:

Initial culture level = 10^6 cells/ml of milk
Initial phage level = 1 phage/ml of milk
Culture generation time = 0.5 hours
Phage latent period = 0.5 hours
Desired pH after 3.5 hours = 5.2
Average phage burst size = 50

that of the culture (Table 4.4). In this example, an *L. lactis* culture containing 10^8 cells per ml is inoculated (1%) into a cheese vat, either in the absence or presence of a single phage. Assuming typical generation times and phage latent periods and burst sizes, growth of the culture is significantly impaired. Although this scenario in which the fermentation is actually arrested prematurely by infectious bacteriophage may be uncommon, there are frequent occasions when phage infections either cause production delays or downgrading of cheese quality. Both are costly to the manufacturer.

For many years, lactic starter cultures comprised of mesophilic lactococci have been the "workhorse" cultures. They are used in the manufacture of the many cheeses and cultured dairy products, including cheddar and cheddar-like cheeses, mold-ripened cheeses, Dutch-type cheeses, cottage cheese, cultured buttermilk, and sour cream. Thus, for the most part, bacteriophage that attacked lactococci, especially *L. lactis* susbsp. *lactis*, were really the only concern. Reports of phage problems occurring for products made using other lactic acid bacterial cultures were relatively rare.

This situation changed in the 1990s, when the incidence of phage infections against thermophilic cultures (consisting of *S. thermophilus* and either *L. bulgaricus* or *Lactobacillus* helveticus) increased significantly. It is no coincidence that there had been a huge increase in the production of mozzarella cheese, yogurt, and other products that rely on these thermophilic starter cultures. The emergence of thermophilic bacteriophage underscores the problem faced by the starter culture industry and its customers. Namely, wherever and whenever lactic acid bacteria are used on a large scale, phage that infect those organisms will undoubtedly appear as potential adversaries.

Bacteriophage control strategies

In response to the bacteriophage problem, the dairy starter culture industry, as well as the cheese and cultured dairy products industries, have adopted several strategies (Table 4.5). First, and perhaps most importantly, high standards of hygiene must be applied. In fact,

Table 4.5 Phage control strategies.

Method	Purpose or function
Sanitation	Kill and remove phages in plant environment
Plant design	Keep phages out of production area, prevent cross contamination
Phage resistant cultures	Use strains that grow and perform well even in presence of phages
Culture rotation	Prevent proliferation of phages by limiting access to suitable host

sanitation and asepsis, appropriate plant design, and phage exclusion programs are the first lines of defense against phage. In the absence of a sanitation program, other efforts to control phage (no matter how sophisticated) are unlikely to succeed. Bacteriophage control programs should include:

1. **Sanitizing agents** Several chemical agents used in the dairy industry inactivate bacteriophage. Chlorine and hypochlorite solutions, the primary sanitizing agents used in dairy plants to control microorganisms, are also effective against bacteriophage. Other phage biocidal agents include peracetic acid, ethanol, and quaternary ammonium compounds.
2. **Exclusion** Areas where starter cultures are handled and grown should be isolated from the rest of the processing facility. Because bacteriophage are frequently transmitted via air or airborne droplets, contained areas should be maintained under positive pressure. Filtered air may also be warranted within the starter culture room. It is also important to locate the actual production facilities down stream from the starter preparation area to ensure that waste flow (i.e., whey) does not contaminate the culture area. The latter point is critical, because whey is considered to be the major reservoir of phage within a dairy plant, and the primary vehicle by which phage dissemination occurs. However, even for products that do not generate whey (such as yogurt), phage problems occur.
3. **Phage-resistant cultures** A third approach to reduce infection by bacteriophage takes advantage of the innate ability of some starter culture bacteria to defend against phage attack. Several types of natural phage-resistance mechanisms are now known to exist. Most function by blocking or impeding specific steps during the lytic cycle (Figure 4.3). Examples include inhibition of phage adsorption, restriction of phage DNA, and abortive infection. More recently, phage researchers made the important discovery that some lactic acid bacteria can actually develop a sort of immunity against bacteriophage. These so-called CRISPR systems are described in Box 4.4.

Ultimately, it is possible to isolate strains that possess one or more of these systems and to use them in industrial fermentations. This approach, based on the use of phage-resistant strains, has formed the basis of most dairy starter culture systems. These cultures can be used either on a continuous basis (the same culture every day) or rotated such that strains with the same phage sensitivity pattern are not used for consecutive fermentations. Rotation programs can also be performed even with single or paired strain starter. In either case, it is important to monitor the phage levels in the whey or milk on a regular basis.

When phage titers reach a particular threshold, signifying a strain has become phage-sensitive, that strain is removed from the mixture and replaced by a resistant strain. Since phage proliferation requires susceptible host strains, when those strains are removed, the background level and accumulation of phages will be reduced such that normal fermentation

Box 4.4 The CRISPR revolution

Who would have thought that one of most important scientific discoveries of the twenty-first century would describe a phage defense system used by yogurt and cheese bacteria? Indeed, that is the story of CRISPR and *Streptococcus thermophilus*, first reported in *Science* magazine in 2007 (Barrangou et al., 2007). It is also noteworthy that among the authors of this paper were several microbiologists from Danisco Inc. (now DuPont), one of the major starter culture companies. Soon thereafter, literally dozens of CRISPR applications, ranging from improved cultures to treatments for genetic diseases, were developed, and CRISPR was regularly featured on the cover of leading scientific journals (Figure 4.4.1). In 2015, CRISPR (owing, in part, to its gene editing function) was named *Science* magazine's "Breakthrough of the Year". *The CRISP Journal,* devoted entirely to CRISPR science, was launched in 2018.

What is CRISPR, how does it work, and why was it such an important discovery, worthy of publications in the most prestigious journals? Indeed, it's relevant to note that CRISPR has become as "famous" as PCR, in that its acronym is so well known that it is rarely spelled out. It refers nonetheless to a specialized genetic region in the bacterial chromosome called ***c***lustered ***r***egularly ***i***nterspaced ***s***hort ***p***alindromic ***r***epeats. It is best understood by examining its major genetic and structural features. These include the repeat region, separated by spacers; an AT-rich leader section; and the *cas* genes (Figure 4.4.2; Sorek et al., 2008).

The repeat section consists of multiple 21–48 base pairs (bp) sequences. These sequences are partially palindromic, meaning that the DNA sequence on one strand segment matches the sequence on the complimentary strand. These repeats sequences are interrupted by the spacer sections, 26–72 bp sequences located in-between the repeats. As described below, these spacers contain sequences homologous to phage or other foreign sources of DNA. Finally, the Cas (or *C*RISPR-*as*sociated) section encodes for a variety of enzymes and other proteins.

The genomic sequences that comprise the CRISPR loci had actually been identified in *E. coli* and other organisms as early as the late 1980s (Ishino et al., 1987). However,

Figure 4.4.1 Covering CRISPR. From Molecular Cell, 2013, Science 2014, and Nature, 2016 with permission.

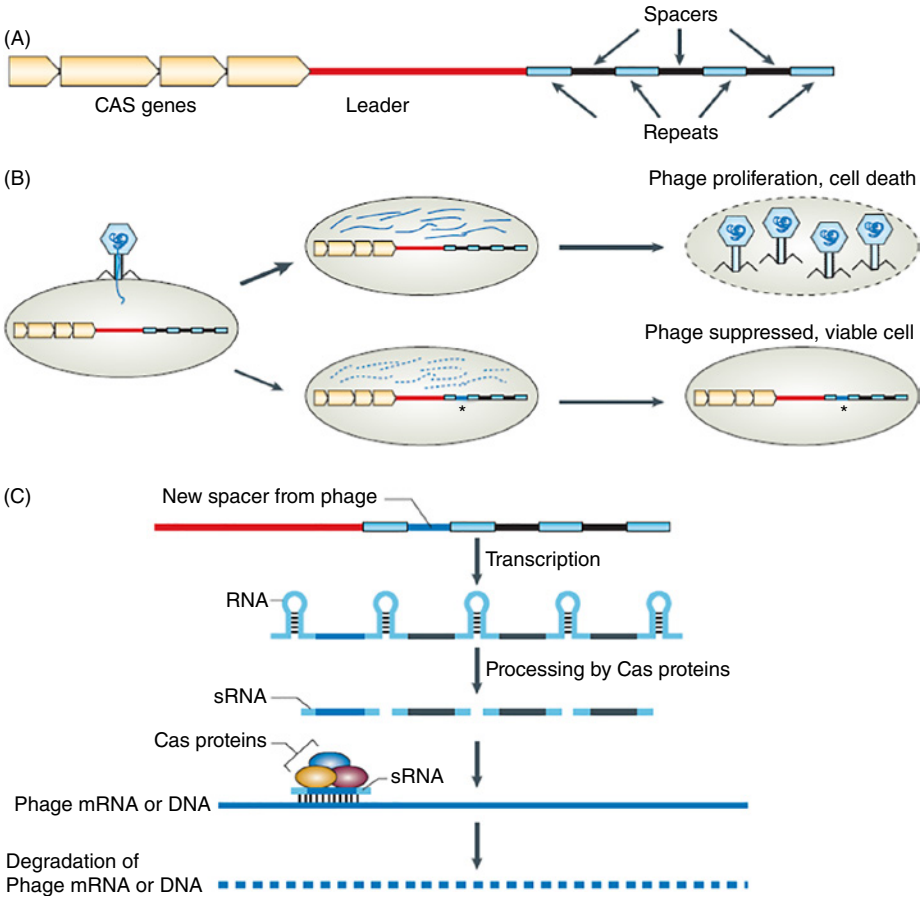


Figure 4.4.2 CRISPR structure and function. The structure of the spacers, repeat, and CAS regions are shown in A. Following an attack by a phage, most cells are inactivated, but a few survive, acquiring phage-derived spacers (indicated by an asterisk), B. Simplified model for CRISPR action is shown in C, where the repeat-spacer array is transcribed into a long RNA, that is first processed by Cas proteins to produce small RNAs that are ultimately degraded. From Sorek et al., 2008, with permission. See the original text for more details.

it would take another 10 years before researchers were able to establish the function of CRISPR, namely that it confers phage resistance for the host cells. Indeed, what makes CRISPR so remarkable and distinct from other phage defense systems (such as those based on restriction/modification and abortive infection), is that CRISPR provides a form of immunity. In higher organisms, exposure to a foreign agent induces immunological memory such that the host organism is able to "remember" that agent. When exposed a second time, the organism launches a full response. In principle, the CRISPR system is very similar. The mechanisms for how this occurs are described below.

Although each of the CRISPR regions are essential, it's the spacer region that ultimately allows the cell to "remember" previous exposures to virulent or invasive phage. When a population of phage first infects target bacteria, presumably killing

many, some of the surviving cells will package regions of phage DNA into the host genome, specifically in the CRISPR spacer region. The spacer region is located at the leader end of the CRISPR region, and the excision and integration steps require two of the cas proteins (cas1 and cas2), mentioned above. It is worth noting that any foreign DNA, whether from phage or plasmids, can be similarly integrated into the spacer.

The relevance of sequences homologous to phage DNA within the CRISPR spacer regions was not lost on the researchers' thinking. Indeed, the presence of these sequences suggested a form of acquired or adaptive immunity, or what one researcher called a "rapid and robust adaptation" to phage (Marraffini, 2015). Indeed, when the cell encounters that same or a highly related phage a second time, transcription of the CRISPR system and the action of Cas enzymes yield small spacer mRNAs (crRNA) corresponding to the DNA of the invading phage. Those crRNAs then interfere with the phage DNA and guide the Cas endonuclease to degrade the newly arrived phage DNA.

There are several important implications of the CRISPR system. First, it occurs naturally. Exposing *S. thermophilus* (or other CRISPR-bearing strains) to phage will generate CRISPR-mediated resistance, without the use of recombinant DNA or genetic engineering techniques. Thus, phage resistant strains developed by CRISPR approaches are food-grade.

Second, cells can accommodate spacer regions from multiple phages. Repeated exposure to different phages increases the breadth of resistance. Thus, phage resistance in commercial strains can be broad-based, an important property considering the diversity of phages that exist in fermentation environments (Grens, 2015).

Third, CRISPR systems are stable, given their integration into the chromosome. Other phage defense systems found in lactic acid bacteria are often plasmid-encoded and can be lost during propagation.

Not so fast, phage <u>not</u> have left the building. Despite the advantages described above, CRISPR does not mean the end of phage problems in the dairy fermentation and starter culture industries. The battle between phage and host is never-ending, and mutation rates among phage populations guarantee that phage species will eventually emerge that can overcome any particular host defense, including CRISPR. For example, phages that have lost spacer DNA (i.e., via mutation or deletion) are not be affected by the CRISPR-mediated defense (Deveau et al., 2008). More recently, anti-CRISPR proteins have been identified (in *Pseudomonas*, not in starter culture strains) that interfere with Cas activity (Bondy-Denomy et al., 2015).

Cutting genomes down to size. Finally, the editing function of the CRISPR Cas enzymes (especially Cas9) has led to many important applications in gene therapy and biotechnology (accounting for its breakthrough status). The editing function also has implications in dairy cultures, as researchers have shown that it is now possible, using CRISPR-Cas, to delete extraneous regions and to "define minimal bacterial genomes" (Selle et al., 2015). These "streamlined" genomes could theoretically be more efficient in industrial applications (Barrangou and Doudna, 2016).

References

Barrangou, R., C. Fremaux, H. Deveau, M. Richards, P. Boyaval, S. Moineau, D. A. Romero, and P. Horvath. 2007. CRISPR provides acquired resistance against viruses in prokaryotes. Science 315:1709–1712.

Barrangou, R. and J.A. Doudna. 2016. Applications of CRISPR technologies in research and beyond. Nature Biotechnol. 34:933–941.

Bondy-Denomy, J, B. Garcia, S. Strum, M. Du, M.F. Rollins, Y. Hidalgo-Reyes, B. Wiedenheft, K.L. Maxwell, and A.R. Davidson. 2015. Multiple mechanisms for CRISPR-Cas inhibition by anti-CRISPR proteins. Nature 526:136–139.

Deveau, H., R. Barrangou, J.E. Garneau, J. Labonte, C. Fremaux, P. Boyaval, D.A. Romero, P. Horvath, and S. Moineau. 2008. Phage response to CRISPR-encoded resistance in *Streptococcus thermophilus*. J. Bacteriol. 190:1390–1400.

Grens, K. 2015. There's CRISPR in your yogurt. Scientist 29, 1.

Ishino, Y., H. Shinagawa, K. Makino, M. Amemura, and A. Nakata. 1987. Nucleotide sequence of the *iap* gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product. J. Bacteriol. 169:5429–5433.

Salmond, G.P. and P.C. Fineran. 2015. A century of the phage: past, present and future. Nat. Rev. Microbiol. 13:777–786.

Marraffini, L.A. 2015. CRISPR-Cas immunity in prokaryotes. Nature 526:55–61.

Selle, K., T.R. Klaenhammer, and R. Barrangou. 2015. CRISPR-based screening of genomic island excision events in bacteria. Proc. Natl. Acad. Sci. USA 112:8076–8081.

Sorek, R., V. Kunin, and P. Hugenholtz. 2008. CRISPR – a widespread system that provides acquired resistance against phages in bacteria and archaea. Nature Rev. Microbiol. 6:181–186.

rates can be achieved. This practice, however, is constrained by the limited availability of phage-unrelated or phage-resistant strains. In theory, it should be possible to isolate new strains from nature that are both phage-resistant and that have good cheese making properties, a strategy that is now standard industry practice (Box 4.5).

Box 4.5 Evolving ideas for improving cultures

For thousands of years, fermented foods and beverages were produced without the benefit of starter cultures. Indeed, it wasn't until the turn of the twentieth century that techniques for isolating and growing pure cultures were developed. Such methods were necessary to mass-produce yeasts cultures for the brewing industry or lactic acid bacteria for dairy fermentations. Of course, only strains that yielded final products having the desired flavor, texture, and other attributes would be considered as a potential starter cultures. Thus, from the earliest days of the starter culture industry, screening and selection of candidate strains was an essential activity. Even today, so-called strain "bioprospecting" remains a viable approach for industrial strain discovery (Ainsworth et al., 2014; Cavanagh et al., 2015; Johansen et al., 2015; Pedersen et al., 2005).

Traditional approaches. In general, these methods involve collecting samples from raw food materials, food environments, and naturally fermented foods, as well as archived culture collections. The isolated strains are then identified and characterized

and phenotypic properties of interest are assessed. Wild organisms from naturally fermented foods have become an especially popular source from which new strains having novel functional properties can be obtained. Examples include isolation of potential probiotics from kimchi and kefir (Lee et al., 2015; Wang et al., 2015), EPS-producing strains from fermented plant material (Siddiqui et al., 2013), olive de-bittering strains from cheeses and brines (Zago et al., 2013), and bacteriocin-producing strains from fermented vegetables (Hu et al., 2013).

Although bioprospecting approaches remain an important part of strain improvement programs, advances in molecular biology have dramatically changed how the industry obtains new and novel strains. Rapid and inexpensive genome sequencing, in particular, has made it possible to identify many of the specific genes responsible for the fermentation traits desired by starter cultures. In the dairy culture industry, for example, structural and regulatory genes for expression of exopolysaccharide production, phage resistance, and citrate fermentation are now established for many relevant strains.

Molecular approaches. The biochemical means for creating specific changes in the genome of these organisms are also well established. The most straightforward methods for generating such changes rely on recombinant DNA or gene modification (GM) technologies. There are hundreds of examples in the literature that describe novel strains that have phenotypes that would make them valuable in the fermented foods and beverage industry. However, because these strains are genetically modified (making them GM), almost without exception, none are used commercially.

The problem with GM? If GM starter culture organisms (GMO), possessing highly desirable properties, can be developed in the laboratory, then why aren't these cultures available in the marketplace? This simple question has many answers. First, GM products must satisfy the regulatory requirements of the country or countries in which the products will be marketed. Because regulations differ throughout the world (and even what constitutes a GMO varies from country to country), gaining approval from one jurisdiction in no way guarantees acceptance elsewhere. In the EU for example, the European Food Safety Authority's (EFSA) Panel on Genetically Modified Organisms requires a risk assessment for any GM product (Aguilera et al., 2013). Monitoring and detection plans must also be included.

The labeling issue has become one of the main issues – in fact, it may be a deal-breaker for a company considering GM strains. This is because many consumers are either opposed to GMOs or confused enough by the GMO declaration on the label that it influences their purchasing decisions. Labeling is required in the European Union (although no such requirement currently exists in the United States). Thus, it would appear that the U.S. market (and perhaps others) might be more receptive to GM cultures than the EU market. However, foods or food ingredients, including starter cultures, are marketed globally. Thus, it seems unlikely that a culture supplier would commit resources to development of GM microorganisms unless it was confident it could gain approval on a near worldwide basis.

Alternatives to GM. Despite the restrictions on using recombinant DNA technology, it is still possible to take advantage of molecular methods for developing new strains (Derkx et al., 2014; Kuipers, 2015). Even classical methods, such as transformation, transduction, and conjugation can be used. The latter has been especially useful, as lactococcal strains harboring phage resistance plasmids have been mated with cheese strains to generate phage-resistant transconjugants (Marcó et al., 2012). Likewise, transformation has been used to introduce and express DNA in naturally competent strains of *Streptococcus thermophilus* (Lecomte et al., 2014; Lecomte et al., 2016). The caveat, however, before such strains could be considered for use in food application is that plasmids and all other molecular tools must be "food-grade". That is, they must derived from GRAS organisms and not contain antibiotic-resistance genes as selection markers.

Two other general strategies, called pangenomics and evolutionary engineering, also rely on state-of-the-art genomics and bioinformatics. For example, for most starter culture organisms, there are now multiple sequenced genomes of the same species in publically available databases. When all of the genes for a given species are combined, the result is the pangenome (Garrigues et al., 2013). In contrast, those genes conserved or shared among the sequenced genomes represents the core genome. Analysis of the pangenomes and core genomes, along with knowledge of particular phenotypes, provides a basis for identifying potential traits consistent with that particular species, even if not all strains express that particular phenotype. Likewise, if the goal is to identify strains with unique phenotypes, then those genes outside the core would be useful targets.

Another tried and true approach to obtain new or modified strains having a phenotype of interest is referred to as evolutionary engineering (Bachmann et al., 2014). This method relies on continuous cultivation of a culture under selective conditions such that a particular phenotype is enriched. The phenotype could be acid production, gassing ability, or phage resistance. In reality, the starter culture industry has long used this approach, either as described above or combined with random mutagenesis.

Perhaps the best example of this approach is for isolating phage-resistant strains. Dairy cultures that have desirable performance characteristics in cheese or yogurt, but that have become sensitive to bacteriophage can be repeatedly exposed to those phage. After each round, mutants can be recovered that have become insensitive. Provided they still perform as well as the original strain, they can then be re-introduced as a production strain. These methods can be limited, however, by the appearance of mutations that negatively affect the performance of the strain. Now that genome sequencing has become routine, it is possible to identify and predict which of those mutations may be deleterious to culture performance.

References

Ainsworth, S., S. Stockdale, F. Bottacini, J. Mahony, and D. Sinderen. 2014. The *Lactococcus lactis* plasmidome: much learnt, yet still lots to discover. FEMS Microbiol. Rev. 38:1066–1088.

Bachmann, H., J.T. Pronk, M. Kleerebezem, and B. Teusink. 2014. Evolutionary engineering to enhance starter culture performance in food fermentations. Curr. Opin. Biotechnol., 32:1–7.

Cavanagh, D., G.F. Fitzgerald, and O. McAuliffe. 2015 From field to fermentation: The origins of *Lactococcus lactis* and its domestication to the dairy environment. Food Microbiol. 47:45–61.
Derkx, P.M., T. Janzen, K.I. Sørensen, J.E. Christensen, B. Stuer-Lauridsen, and E. Johansen. 2014. The art of strain improvement of industrial lactic acid bacteria without the use of recombinant DNA technology. Microb. Cell Fact. 13 (Suppl. 1):S5.
Garrigues, C., E. Johansen, and R. Crittenden. 2013. Pangenomics – an avenue to improved industrial starter cultures and probiotics. Curr. Opin. Biotechnol. 24:187–191.
Hu, M., H. Zhao, C. Zhang, J. Yu. and Z. Lin. 2013. Purification and characterization of plantaricin 163, a novel bacteriocin produced by *Lactobacillus plantarum* 163 isolated from traditional Chinese fermented vegetables. J. Agric. Food Chem. 61:11676–11682.
Johansen, E., G. Øregaard, K.I. Sørensen, and P.M.F. Derkx. 2015. Modern approaches for isolation, selection, and improvement of bacterial strains for fermentation applications. In: W. Holzapfel, ed. Advances in fermented foods and beverages. Woodhead Publishing, Cambridge, pp. 227–248.
Kuipers, O.P. 2015. Back to nature: a revival of natural strain improvement methodologies. Microb. Biotechnol. 8:17–18.
Lecomte, X., V. Gagnaire, V. Briard-Brion, J. Jardin, S. Lortal, A. Dary, and M. Genay. 2014. The naturally competent strain *Streptococcus thermophilus* LMD-9 as a new tool to anchor heterologous proteins on the cell surface. Microb. Cell Fact. 13:82.
Lecomte, X., V. Gagnaire, S. Lortal, A. Dary, and M. Genay. 2016. *Streptococcus thermophilus*, an emerging and promising tool for heterologous expression: advantages and future trends. Food Microbiol. 53:2–9.
Lee, N.K., K.J. Han, S.H. Son, S.J. Eom, S.K. Lee, and H.D. Paik. 2015. Multifunctional effect of probiotic *Lactococcus lactis* KC24 isolated from kimchi. LWT – Food Sci. Technol. 64:1036–1041.
Marcó, M.B., S. Moineau, and A. Quiberoni. 2012. Bacteriophages and dairy fermentations. Bacteriophage 2:149–158.
Pedersen, M.B., S.L. Iversen, K.I. Sørensen, and E. Johansen. 2005. The long and winding road from the research laboratory to industrial applications of lactic acid bacteria. FEMS Microbiol. Rev. 29:611–624.
Siddiqui, N.N., A. Aman, and S.A. Qader. 2013. Mutational analysis and characterization of dextran synthesizing enzyme from wild and mutant strain of *Leuconostoc mesenteroides*. Carbohydr. Polym. 91:209–216.
Wang, J., Z. Xing, W. Tang, Y. Zheng, and Y. Wang. 2015. Isolation, identification, and potential probiotic characterization of one *Lactococcus* from kefir grain. Food Sci. Biotechnol. 24:1775–1780.
Zago, M., B. Lanza, L. Rossetti, I. Muzzalupo, D. Carminati, and G. Giraffa. 2013. Selection of Lactobacillus plantarum strains to use as starters in fermented table olives: oleuropeinase activity and phage sensitivity. Food Microbiol. 34:81–87.

Phage resistance genes

Of course, if some strains are naturally resistant to phage infections, then there must be a genetic basis for the phage-resistant phenotypes described above. Indeed, genes responsible for these phage-resistant phenotypes have been identified and characterized. Importantly, these genes can be introduced into phage-sensitive strains, making them phage-resistant. In addition, it is also possible to obtain spontaneous phage-insensitive mutants by simply exposing sensitive, wild-type strains to lytic bacteriophage. The phage-resistant derivatives that are then selected must be evaluated for cheese making properties before being reintroduced into a starter culture, because pleiotropic mutations frequently occur that render them defective as cheese cultures. Although it would have been impossible and prohibitively expensive just a decade ago, it is now common practice to sequence the entire genome of such strains and identify the nature of the mutation.

Identifying the genetic basis for phage resistance in lactic acid bacteria continues to be a major goal among researchers. Genes in *Lactococcus lactis* subsp. *lactis* that conferred a phage-resistance phenotype were first identified in the early 1980s by the Klaenhammer lab at North Carolina State University. The genes were located on a plasmid (pTR2030) that was later shown to contain multiple phage-resistance determinants. Importantly, when the plasmid was transferred via a simple conjugal mating procedure into a phage-sensitive, industrial cheese making strain, transconjugants with resistance to a broad range of lytic industrial phages were obtained.

This was a seminal accomplishment, in part, because it represented the first application of biotechnology to improve dairy starter cultures, but also because the actual technique of gene transfer did not involve recombinant DNA technology. Thus, these modified strains could be used commercially. Indeed, this approach was successful in actual cheese manufacturing environments. However, with prolonged use, bacteriophage eventually appeared in cheese plants that were able to circumvent the resistance of these strains. Nonetheless, these early efforts marked the beginning of a new era in starter culture microbiology that led to the development of other molecular strategies aimed at controlling bacteriophage.

STARTER CULTURE TECHNOLOGY IN THE TWENTY-FIRST CENTURY

Phage, of course, is not the only concern of the dairy starter culture industry. How fast a given strain grows, what sugars it ferments, and what end-products are formed are also important issues that influence culture performance. Genetic tools now exist for manipulating metabolism and modifying phenotypic properties of lactic starter cultures for nearly every fermented food and beverage. Importantly, it's not only the academic labs that have been engaged in starter culture research. The starter culture companies have also maintained active research and development programs, often leading to major findings on the genetics and physiology of starter culture organisms.

Not surprisingly, these research efforts are focused on developing customized cultures for specific applications. This means that the phenotype and even the genotype of each strain in an industrial culture collection must be cataloged. Fermentation rates, product formation and sugar fermentation patterns, and osmotic- or halo-tolerance behavior, are among the standard properties that should be included in the strain bank data. However, this is only a partial list and depending on the application, other properties could be relevant.

In the case of lactic acid bacteria, for example, sensitivity and/or resistance to phage must be established for each strain. Likewise, expression and/or sensitivity to bacteriocins must also be determined, especially when multiple strains are used in culture blends. For some applications, lactic acid cultures may be routinely screened for their ability to form biogenic amines. Similarly, yeasts strains are also subjected to specific phenotypic assessments. Many yeasts are known to produce so-called killer toxins that inhibit sensitive strains. Thus, yeast cultures used for beer and wine may be assessed for their killer phenotype. The flocculation phenotype is also important for brewing yeasts.

Despite the opportunities that now exist to modify and improve starter culture organisms, strain improvement programs have been limited by regulatory and public perception considerations (see Box 4.5). While tools for introducing DNA into food grade starter microorganisms or for altering the existing genetic makeup of these organisms are now widely available, with perhaps just a few exceptions, commercialization of genetically

modified organisms (GMOs) has not occurred. Although plant-based GMOs are common in the United States, they are generally not marketed in Europe or the Far East. The debate over GMOs is not likely to abate anytime soon, even as researchers continue to demonstrate the potential benefits GMO technologies may offer.

Probiotics

Although most of the organisms produced by the culture industry are used for fermentations, there is one major exception. Specifically, probiotics have become a significant growth category for this industry. As currently defined, probiotics are "live microorganisms, that when administered in adequate amounts, confer a health benefit on the host." These organisms have long been added to yogurt and other cultured dairy products, but there is now a large and growing market for probiotic microorganisms in other fermented and non-fermented foods.

The main probiotic organisms include species of *Bifidobacterium* and *Lactobacillus*. In general, these organisms are produced industrially in the same manner as starter cultures. However, some strains require specialized nutrients or growth conditions to maximize cell density and viability. Also, because probiotic products are also sold as supplements, encapsulation and other technologies have been developed by the culture industry to promote stability and shelf-life.

Cultures adjuncts

Another culture application used by the cheese industry consists of lactic acid and related bacteria whose function is to accelerate and enhance cheese ripening and maturation. Specifically, these organisms, usually strains of *Lactobacillus helveticus* and *Lactobacillus casei*, produce peptidases and other protein-hydrolyzing enzymes that are necessary for proper flavor and texture development. Citrate-fermenting LAB that produce the flavor compound diacetyl as well as heterofermentative LAB that produce carbon dioxide may also be added as adjunct cultures for specific cheeses.

Finally, adjunct cultures and culture preparations are now available whose function is to enhance food safety and preservation. These are usually lactic acid bacteria that do not necessarily modify texture or flavor. Rather, they are used to inhibit pathogenic or spoilage organisms. The inhibitory activity of these organisms may be due to one of several substances they produce, including organic acids, hydrogen peroxide, and diacetyl. However, many of these strains also produce bacteriocins, heat-stable peptides that inhibits other closely related organisms.

Bacteriocins

Organisms representing nearly all of the food-related genera are capable of producing bacteriocins. Therefore, they can be used either as part of a lactic acid-producing starter culture or as an adjunct in dairy, meat, and other foods to inhibit pathogens and spoilage organisms and to enhance shelf-life. Furthermore, some of these organisms are capable of producing bacteriocin in the food, with minimal production of acids or other fermentation end-products. Thus, the sensory characteristics of the product are not affected by the producer organism, a property that would be especially important in non-fermented foods such as ready-to-eat meats.

In some food applications, adding live bacteriocin-producing organisms is not ideal. Therefore, another route by which bacteriocins can be introduced into a food without adding live organisms has been developed. In this process, the producer organism is grown in a dairy- or non-dairy-based medium, and then the spent fermentation medium is harvested, pasteurized, and concentrated. This material would contain the bacteriocin (as well as organic acids), that could then be added to foods as a natural preservative. Although these so-called bioprotective products are mainly effective against Gram-positive bacteria, some commercially-available products also inhibit yeasts, mold, and Gram-negative spoilage bacteria, including psychrotrophs that spoil refrigerated foods.

ANCILLARY PRODUCTS AND SERVICES

Although starter cultures are certainly the main product line, the starter culture industry, as noted previously, produces and markets a variety of other products. Dairy culture suppliers, in particular, offer culture media, coagulants, colors, and other ancillary products used in cheese manufacture. Starter culture companies also provide technical services and support, perhaps more so than any other ingredient suppliers. This is because the manufacture of cheese, sausage, wine, and all fermented products depends on the activity of an inherently unstable biological material, i.e., the starter culture. Although there may certainly be quality issues that arise during the manufacture of corn flakes, canned peas, granola bars, or other non-fermented foods, the failure of a biologically-dependent process is not one of them. A cheese plant manager who is responsible for converting 2 million kg of milk into 200,000 kg of cheese (worth at least $250,000) each day will not wait long before calling the culture supplier the minute he or she suspects a culture problem. Thus, a well-trained technical support staff is a necessary component of the modern culture industry.

BIBLIOGRAPHY

Bachmann, H., J.T. Pronk, M. Kleerebezem, and B. Teusink. 2015. Evolutionary engineering to enhance starter culture performance in food fermentations. Curr. Opin. Biotechnol. 32:1–7.

Bourdichon, F., S. Casaregola, C. Farrokh, J.C. Frisvad, M.L. Gerds, W.P. Hammes, J. Harnett, G. Huys, S. Laulund, A. Ouwehand, I.B. Powell, J.B. Prajapati, Y. Seto, E. Ter Schure, A. Van Boven, V. Vankerckhoven, A. Zgoda, S. Tuijtelaars, E.B. Hansen. 2012. Food fermentations: microorganisms with technological beneficial use. Int. J. Food Microbiol. 154:87–97.

Brandt, M.J. 2014. Starter cultures for cereal based foods. Food Microbiol. 37:41–43.

Douglas, G.L. and T.R. Klaenhammer. 2010. Genomic evolution of domesticated microorganisms. Ann. Rev. Food Sci. Technol. 1:397–414.

Frey, C.N. 1930. History and development of the modern yeast industry. Ind. Eng. Chem. 22:1154–1162.

Garneau. J.E. and S. Moineau. 2010. Bacteriophages of lactic acid bacteria and their impact on milk fermentations. Microb. Cell Fact. 10(Suppl. 1):S20.

Labrie, S.J., J.E. Samson, and S. Moineau. 2010. Bacteriophage resistance mechanisms. Nat. Rev. Microbiol. 8:317–327.

Leroy, F. and L. De Vuyst. 2004. Functional lactic acid bacteria starter cultures for the food fermentation industry. Trends Food Sci. Technol. 15:67–78.

Mahony, J., J. Murphy, and D. van Sinderen. 2012. Lactococcal 936-type phages and dairy fermentation problems: from detection to evolution and prevention. Front. Microbiol. 3:335.

Meng, X.C., C. Stanton, G.F. Fitzgerald, C. Daly, and R.P. Ross. 2008. Anhydrobiotics: the challenges of drying probiotic cultures. Food Chem. 106:1406–1406.

Mills, S., O. O'Sullivan, C. Hill, G. Fitzgerald, and R.P. Ross. 2010. The changing face of dairy starter culture research: from genomics to economics. Int. J. Dairy Technol. 63:149–170.

O'Shea, E.F., P.D. Cotter, R.P. Ross, and C. Hill. 2013. Strategies to improve the bacteriocin protection provided by lactic acid bacteria. Curr. Opin. Biotechnol. 24:130–134.

Pedersen, M.B., S.L. Iversen, K.I. Sørensen, and E. Johansen. 2005. The long and winding road from the research laboratory to industrial applications of lactic acid bacteria. FEMS Microbiol. Rev. 29:611–624.

Settanni, L. and G. Moschetti. 2010. Non-starter lactic acid bacteria used to improve cheese quality. Food Microbiol. 27:691–697.

5 Cultured dairy products

> In the spring of 1905, Parisians rushed in droves to a newly opened shop off a resplendent grand boulevard near Théâtre du Vaudeville. They weren't heading there to buy croissants or Camembert, but for pots of yogurt that they believed could prevent aging. At that time, a mania for yogurt was rapidly unfolding on both sides of the Atlantic, and its source was unexpected – a Russian-born biologist who would go on to receive a Nobel Prize in Physiology or Medicine.
>
> Luba Vikhanski, Metchnikoff biographer, as quoted in Smithsonian.com, April, 2016.

INTRODUCTION

It is very possible that fermented milks are among the oldest of all fermented foods. Consider that milk obtained from a domesticated cow or camel or goat, some several thousands of years ago, would have been fermented within hours by endogenous lactic acid bacteria, creating some sort of yogurt-like product. In fact, the ability to maintain milk in a fresh state before souring and curdling had occurred would have been quite some trick, especially in warm environments. Of course, fermentation and acid formation would have probably been a very good thing since, in the absence of lactic acid bacteria, the milk would have supported growth of undesirable bacteria, including possible pathogens.

Almost all bacteria like milk. It is particularly suitable as a fermentation substrate owing to its carbohydrate-rich, nutrient-dense composition. Fresh bovine milk contains 5% lactose and 3.3% protein, and has a water activity near 1.0 and a pH of 6.6 to 6.7, perfect conditions for most microorganisms. Lactic acid bacteria are saccharolytic and fermentative, and, therefore, are ideally suited for growth in milk. In general, they will out-compete other microorganisms for lactose, and by virtue of acidification, will produce an inhospitable environment for would-be competitors. Therefore, when properly made, cultured dairy products have long shelf-lives and, although growth of acid-tolerant yeast and molds is possible, growth of pathogens rarely occurs.

Archaeological evidence suggests that cows were domesticated and used as a source of milk around 10,000 years ago. Given the early recognition of the importance of milk in human nutrition, consumption of milk and milk products spread around the world. Thus, it is not surprising that cultured dairy products, in particular, have evolved on every continent. Their manufacture was already well established thousands of years ago, again based on data

Microbiology and Technology of Fermented Foods, Second Edition. Robert W. Hutkins.

obtained by archaeo-biologists. In addition, cultured dairy products are mentioned in the early written record, including the Old Testament as well as other ancient religious texts. Yogurt is also mentioned in Hindu sacred texts and mythology. And although the manufacturing procedures, the sources of milk, and the names of these products may vary considerably, they share many common characteristics. Thus, dahi (India), laban (Egypt, Lebanon), and jugart (Turkey) are all yogurt-like products whose manufacture involves similar milk handling procedures and depends on the same types of thermophilic lactic acid bacteria. Other products, in particular, kefir and koumiss, evolved from Asia, and are made using various lactose-fermenting yeasts in addition to lactic acid bacteria.

CONSUMPTION OF CULTURED DAIRY PRODUCTS

Yogurt, sour cream (and sour cream-based dips), and cultured buttermilk are the most popular cultured dairy products in the United States. Yogurt, however, accounts for well over half of all cultured dairy products consumed in the United States (Figure 5.1), with nonfat and low-fat versions being the most popular (about 90% of total yogurt sales). Over the past three decades, per capita consumption of buttermilk in the United States has decreased more than 50%, whereas consumption of sour cream (including sour cream-based dips) has doubled. It is the yogurt category that has seen the most growth – since its commercial introduction in the mid-1960s, consumption has increased nearly 5000%!

Despite the increased demand for yogurt in the US, total per capita consumption for all cultured dairy products in 2015 in the United States was only about 9 kg (about 20 pounds). This is in contrast to Europe, where in some countries yogurt consumption alone is more than 20 kg per person per year – the equivalent of ninety 8-ounce cups (Figure 5.1). Furthermore, in the Netherlands, France, and other European countries, the dairy sections of food markets and grocery stores contain numerous other traditional as well as new forms of cultured milk and cream products. Many of these new product trends have begun to catch on in the United States, and now kefir, fluid yogurts, crème fraîche (a 50% fat sour cream), and other "new" cultured dairy products are readily available in the marketplace. It is important to note that the largest market for yogurt, in terms of actual dollar value, is China. In fact, despite per capita consumption of less than 5 kg, China (and the Far East region) represents the greatest opportunity for growth. In contrast, in Europe where per capita yogurt consumption has historically been high, little, if any, growth has occurred in recent years.

Cultured dairy products and probiotic bacteria

Several factors have undoubtedly contributed to the increased worldwide consumption of yogurt and related products. They can be low in calories, delivered in convenient packages, and they come in many flavors and textures. For example, over the past decade, the so-called Greek-style yogurts captured a large share of US yogurt market. More recently, fluid yogurt drinks and smoothies have become popular. However, perhaps the main reason for their popularity, on a worldwide basis, is due to positive nutritional benefits these products are perceived to provide. Consumption of yogurt, and specifically, the live cultures present in yogurt, has been promoted on the basis of the health benefits conferred by those bacteria. Although the actual species that comprise the yogurt starter culture – *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, may have a role in

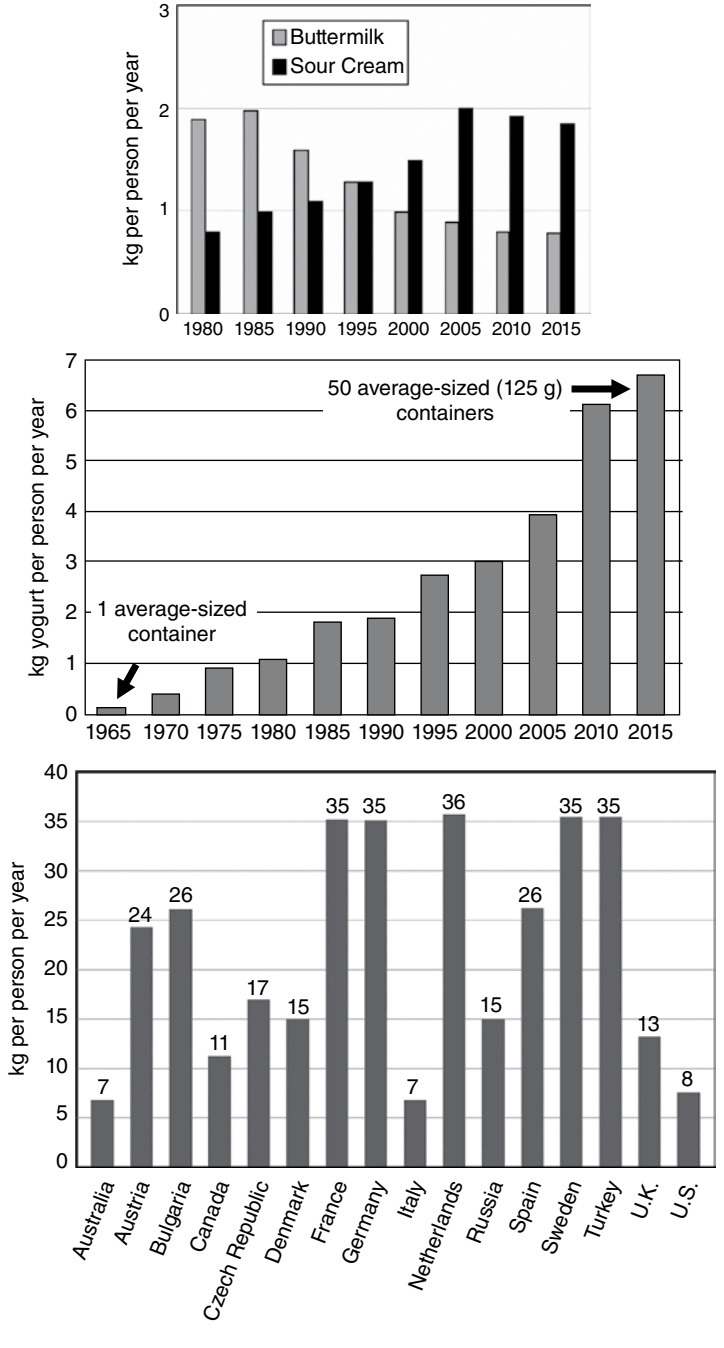


Figure 5.1 Consumption of cultured dairy products. Top panel shows per capita consumption of sour cream (including dips) and cultured buttermilk in the US Yogurt consumption in the US and globally is shown in the middle and lower panels, respectively. Sources: USDA, International Dairy Foods Association, Euromonitor, Mintel, and other regional sources.

gastrointestinal health, it's the probiotic bacteria added to yogurt that have made it one of the most recognizable "health foods" (Box 5.1). Indeed, the term, "probiotic yogurt" has become nearly synonymous with the yogurt category.

Box 5.1 Probiotics and prebiotics in cultured dairy products

The early days

Humans began consuming cultured dairy products thousands of years ago. These early products probably resembled yogurt and the many yogurt-like products that are now common in the grocery store. Even in the absence of modern technology, they were easy to produce, had a reasonably good shelf-life, were generally free of harmful substances, and had a pleasant sensory appeal. Eventually, cultured dairy products were also regarded as having therapeutic value, even though there was hardly a scientific basis for this notion (Shortt, 1999). After all, the existence of bacteria and their role in fermentation were only established by Pasteur in the 1860s.

It wasn't until the beginning of the twentieth century when the Russian scientist Elie Metchnikoff suggested that the health benefits of fermented milk were due to the bacteria involved in the fermentation (Mackowiak, 2013). Metchnikoff (who was working at the Pasteur Institute in Paris) had observed that Bulgarian peasants regularly consumed cultured dairy products and that there were many centenarians (100 years of age) among this population. He suggested their longevity was due to the bacteria responsible for fermentation, presumably the not-yet-named yogurt bacteria, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* or perhaps some other lactobacilli. He even proposed a mechanism – that these bacteria inhibited so-called putrefactive bacteria in the intestinal tract, thereby influencing the intestinal microflora such that overall health and longevity could be enhanced.

The reader is reminded that the germ theory of disease had been conceived only one generation earlier. The discovery of antibiotics was still more than a generation away. Thus, in the early part of the twentieth century, physicians and health care practitioners had few resources available for treating or preventing infectious diseases. That's why the medical community was very receptive to Metchnikoff's ideas. Indeed, as a Nobel Laureate, he was one of the most famous scientists of the era (with rock star status), both in Europe as well as the United States (Vikhanski, 2016). His lectures in London, Paris, and New York attracted tremendous attention among the public and were widely reported in the media (alas, only print media, no social media in the early 1900s).

At around the same time and shortly thereafter, other scientists (as recounted by Shortt, 1999) reported that consumption of other lactobacilli (including *Lactobacillus acidophilus*) and bifidobacteria also had positive health effects, including reducing the rate of infant diarrhea. Interestingly, the observation a full century ago that bifidobacteria were present in the fecal contents of breast-fed infants suggested that these bacteria were associated with intestinal health and foreshadowed the concept of prebiotics (see below).

Collectively, these early reports, by highly regarded scientists at prestigious research institutes, attracted even greater attention among the medical community. By the 1920s, studies using bacteria therapy (with milk as the carrier vehicle) had begun.

Unfortunately, many of these early studies suffered from the absence of established measurement criteria, the use of mis-identified strains, and other design flaws. As described elsewhere in this chapter, in more recent years, these experimental limitations have been recognized and addressed, and now rigorous and appropriate methodologies are being used (Sanders et al., 2014).

Probiotics and prebiotics defined

The term "probiotics," was first used in 1965, and referred to "growth-promoting factors" produced by one microorganism that stimulated growth of another (i.e., the opposite of antibiotics). This definition went through several permutations and by 1989, probiotics were defined as a "live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance" (Fuller, 1989). The current definition, adopted by the World Health Organization (part of the United Nations' Food and Agriculture Organization) in 2001, defines probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host." This latest derivation is important because it recognizes both the relevance of a live and sufficient dose as well as the many reports that indicate probiotics may have health benefits that extend beyond the gastrointestinal tract.

Recently, a consensus panel of probiotic experts met to review this definition. They ultimately decided to leave it nearly the same (Hill et al., 2014). However, in the report that followed, several key issues were raised, mostly having to do with health benefits. In particular, they considered whether potential health benefits are conferred only by specific strains or if there are "core benefits" common to species or even genera. They concluded that while some benefits are widespread, occurring among a range of organisms, others are species or strain specific (Table 5.1.1). More recently, the concept of shared probiotic mechanisms among related species and even genera was further described (Sanders et al., 2017). Among the proposed shared benefits were production of short chain fatty acids and similar cell surface architecture that affect host response. Ultimately, however, one of the most important issues for scientists, health professionals, regulators, and consumers, concerns the level of evidence or clinical proof that a given probiotic actually confers a health benefit in the first place (addressed elsewhere in this chapter).

The notion that foods or food constituents could influence gut bacteria and also promote specific groups also dates back nearly 100 years. However, it was not until 1995

Table 5.1.1 Suggested health benefits of probiotic bacteria[1].

Widespread (general-level effects)	Frequent (species-level effect)	Rare (strain-specific effects)
Colonization resistance	Synthesize vitamins	Modulate immune system
Inhibition of pathogens	Gut barrier protection	Neurological effects
Regularity	Detoxification	Endocrinological effects
Short chain fatty acid production	Bile salt metabolism	Produce bioactive agents
Normalize microbiota	Enzymatic activity	

[1] Adapted from Hill et al., 2014, with permission.

when Gibson and Roberfroid devised the term “prebiotics” to refer to "non-digestible food ingredient(s) that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" (Gibson and Roberfroid, 1995). Although this definition has also been revised multiple times, they generally refer to substrates that are selectively metabolized by members of the commensal microbiota, conferring a health benefit on the host (Gibson et al., 2017). Prebiotics, therefore, enrich for desirable commensal organisms, including, but not limited to lactobacilli and bifidobacteria.

Most of the prebiotics that are used commercially and that have received the most research attention are either polysaccharides or oligosaccharides. They are either derived from plant materials or are synthesized from natural disaccharide precursors (Figure 5.1.1). For example, inulin is a naturally-occurring plant polysaccharide (consisting of fructose units, linked β-1,2 with a terminal glucose residue) that can be used in its intact form or as a mixture of partially hydrolyzed fructooligosaccharide (FOS)

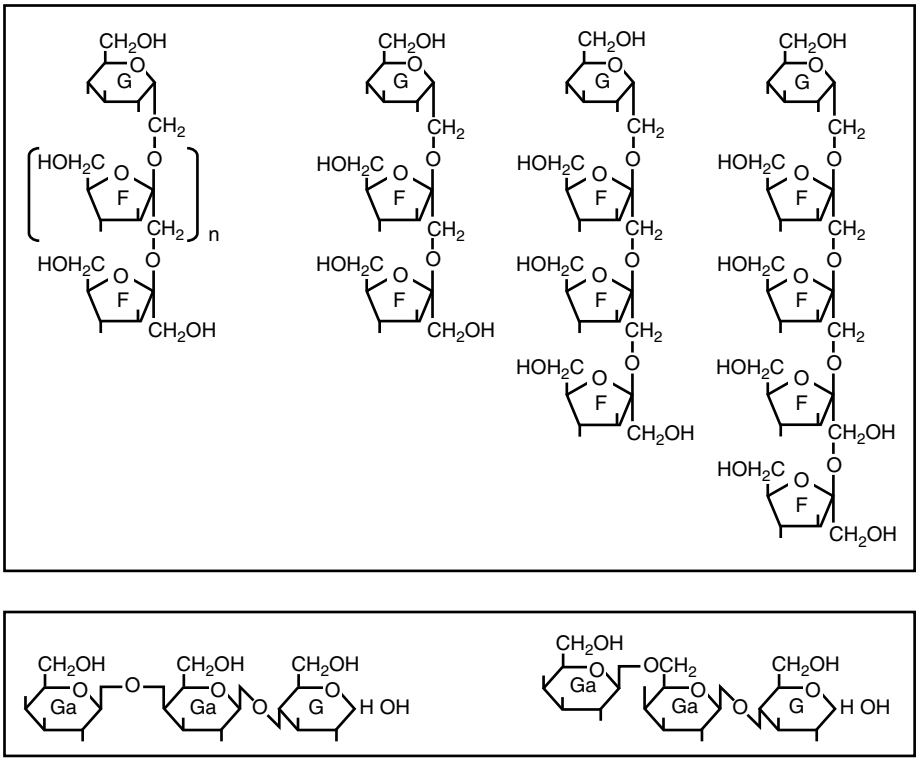


Figure 5.1.1 Structures of common prebiotics added to cultured dairy products and other foods. Shown on the upper panel, far left is inulin, a polysaccharide consisting of 20 or more fructose units, linked β-1,2 attached to terminal glucose molecule. Also shown are short chain fructooligosaccharides (FOS) comprised of 2, 3, or 4 fructose units, also attached to a terminal glucose. The latter can be enzymatically produced from sucrose or hydrolyzed from inulin (which may result in FOS sans glucose). Galactooligosaccharides (GOS) are shown in the lower panel. They are synthesized from lactose, with 1–6 additional galactose units attached via β-1,4 (left) or β-1,6 linkages (right).

molecules. The latter can also be synthesized from sucrose via a transfructosylating enzyme that adds one, two, or three fructose units to the sucrose backbone. Another type of prebiotic oligosaccharide that has attracted considerable attention are galactooligosaccharides (GOS). These oligosaccharides are built from lactose via addition of galactose residues by β-galactosidases with high galactosyltransferase activity. Because GOS molecules somewhat resemble the oligosaccharides found in human milk (albeit with considerably less structural complexity), they are attractive as supplements in infant formula.

It is now established that human milk oligosaccharides are responsible for the bifidogenic properties associated with human milk (Garrido et al., 2012; Zivkovic et al., 2011). In fact, it had long been suggested that there was something in human milk that promoted growth of bifidobacteria (the so-called "bifidus" factor) and that this factor accounted for the dominance of these bacteria in the colon of nursed infants. That infants fed mother's milk suffered fewer intestinal infections and were generally healthier than formula-fed infants provided circumstantial evidence that having a greater proportion of bifidobacteria (and perhaps lactobacilli) in the colon would be desirable, not just for infants, but for the general population as well.

Applications in cultured dairy products

In the US, Canada, Europe, Japan, and Australia, probiotics are now so commonly associated with yogurt that there are few brands that do not contain added "probiotic" species of *Lactobacillus* or *Bifidobacterium*. Although many fermented non-dairy foods also contain probiotics, the historical applications noted above likely account for why dairy is the most popular food vehicle. Although much newer to the marketplace, prebiotics are also becoming more common in cultured dairy foods. Many prebiotics have the added marketing benefit of being considered (in 2017, at least) as fiber. This makes them amenable in a range of other foods such as bread and baked foods. Collectively, the market for probiotics and prebiotics in cultured dairy products is in the multi-billions, with no indication that the demand for such products is slowing down anytime soon.

References

Fuller, R. 1989. Probiotics in man and animals. J. Appl. Bacteriol. 66:365–378.

Gibson, G.R., R. Hutkins, M.E. Sanders, S.L. Prescott, R.A. Reimer, S.J. Salminen, K. Scott, C. Stanton, K.S. Swanson, P.D. Cani, K. Verbeke, and G. Reid. 2017. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. Nat. Rev. Gastroenterol. Hepatol. 14:491–502.

Gibson, G.R., and M.B. Roberfroid. 1995. Dietary modulation of the colonic microbiota: introducing the concept of prebiotics. J. Nutr. 125:1401–1412.

Garrido, D. D. Barile, and D.A. Mills. 2012. A molecular basis for bifidobacterial enrichment in the infant gastrointestinal tract. Adv. Nutr 3:415S–421S.

Hill, C., F. Guarner, G. Reid, G.R. Gibson, D.J. Merenstein, B. Pot, L. Morelli, R.B. Canani, H.J. Flint, S. Salminen, P.C. Calder and M.E. Sanders. 2014. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat. Rev. Gastroenterol. Hepatol. 11:506–514.

Mackowiak, P.A. 2013. Recycling Metchnikoff: probiotics, the intestinal microbiome and the quest for long life. Front. Public Health. 1:52.

Sanders, M.E., A. Benson, S. Lebeer, D.J. Merenstein, and T.R. Klaenhammer. 2018. Shared mechanisms among probiotic taxa: implications for general probiotic claims. Curr. Opin. Biotechnol. 49:207–216.
Sanders, M. E., I. Lenoir-Wijnkoop, S. Salminen, D.J. Merenstein, G.R. Gibson, B.W. Petschow, M. Nieuwdorp, D.J. Tancredi, C.J. Cifelli, P. Jacques, and B. Pot. 2014. Probiotics and prebiotics: prospects for public health and nutritional recommendations. Ann. N.Y. Acad. Sci. 1309:19–29.
Shortt, C. 1999. The probiotic century: historical and current perspectives. Trends Food Sci. Technol. 10:411–417.
Vikhanski, L. 2016. Immunity: How Elie Metchnikoff Changed the Course of Modern Medicine. Chicago Review Press.
Zivkovic, A.M., J.B. German, C.B. Lebrilla, and D.A. Mills. 2011. Human milk glycobiome and its impact on the infant gastrointestinal microbiota. Proc. Natl. Acad. Sci. USA 108:Suppl 1:4653–4658.

The most common bacteria added as probiotics to yogurt are *Lactobacillus acidophilus* and various species of *Bifidobacterium*. Again, it is important to emphasize that these organisms are not part of the yogurt starter culture, nor are they involved in any way in the actual yogurt fermentation. Their only contribution is as a culture adjunct intended to promote good health. Still, according to marketing data, about 80% of the yogurt products produced in the United States contain *L. acidophilus* or species of *Bifidobacterium*. The mechanisms by which these suggested health benefits actually occur and the evidence to support these claims will be discussed below.

In addition to probiotic bacteria, a group of compounds called prebiotics (Box 5.1) are also now being added to cultured milks – mainly yogurt and kefir in the United States, but many more products in Europe, Japan, and Korea. Prebiotics are oligosaccharide-containing materials that are neither degraded nor absorbed during transit through the stomach or small intestine. They end up reaching the colon, where they are preferentially fermented by intestinal strains of lactobacilli and bifidobacteria. Thus, they enrich the population of those bacteria thought to contribute to gastrointestinal health.

Probiotics (and prebiotics) can now be found not only in yogurt, but also in a variety of other cultured and non-cultured dairy products. In most of these products, the probiotic bacteria are not involved in the fermentation. However, other products have been developed to take advantage of their fermentative ability as well as their probiotic activity. Examples include Yakult, a product developed in Japan and made using a strain of *Lactobacillus casei* (strain Shirota) and Cultura, a European "bioyogurt" also made with *L. casei* (strain F19).

There are also various dairy products containing probiotic lactic acid bacteria that are not fermented; thus, these products serve strictly as carriers. The product known as sweet acidophilus milk, for example, is simply fluid milk supplemented with *L. acidophilus* (added after pasteurization). Similar unfermented products may also contain other probiotic bacteria. Maintaining the product at low refrigeration temperatures (the same as for normal fluid milk products) prevents the bacteria from fermenting and souring the milk.

FERMENTATION AND MANUFACTURING PRINCIPLES

The basic principles involved in the manufacture of cultured dairy products, which lactic acid bacteria are used for these products, and the attributes and properties desired by these

bacteria are the focus of this chapter. The emphasis will be on yogurt, sour cream, and other popular products, but cultured dairy foods from around the world, including India, Africa, Iceland, and other countries, will also be described.

In theory, and also in practice, some of these products can be made in the absence of a starter culture simply by adding food-grade acidulants to the milk mixture. Indeed, there are manufacturing advantages for following this practice. However, these acid-set products are not fermented – in fact, they must be labeled as "directly set" to denote this manner of acidification. They also often lack the flavor and texture properties of the fermented versions, and they certainly lack the nutritional benefits that are contributed by live active cultures.

The function of lactic acid bacteria in the manufacture of cultured dairy products is quite simple – they should ferment lactose to lactic acid such that the pH decreases and the isoelectric point of casein, the major milk protein, is reached. By definition, the isoelectric point of any protein is the pH at which the net electrical charge is zero and the protein is at its minimum solubility. In other words, as the pH is reduced, acidic amino acids (e.g., aspartic acid and glutamic acid), basic amino acids (e.g., lysine and arginine), and partial charges on other amino acids become protonated and more positive such that at some point (i.e., the isoelectric point), the total number of positive and negative charges on the amino acids are in equilibrium.

For casein, which ordinarily has a negative charge, the isoelectric point is 4.6. At the pH of milk (about 6.5), repulsive forces keep the negatively charged casein micelles apart. However, when sufficient acid has been produced to protonate the negatively charged amino acids and overcome the natural buffering capacity of milk, casein precipitates and a coagulum is formed. Along the way and depending on the culture, other small organic molecules, including acetaldehyde, diacetyl, acetic acid, and ethanol may be produced. Although these latter compounds are usually produced in relatively low concentrations, they may still make important contributions to the overall flavor profile of the finished product. The culture may also produce other compounds that contribute to the viscosity, body, and mouth feel of the product (see below). The choice of culture, therefore, is dictated by the product being produced, since different cultures generate flavor and aroma compounds specific to that product (Table 5.1).

Table 5.1 Organisms used as starter cultures in the manufacture of fermented dairy products.

Product	**Organisms**
Yogurt	*Streptococcus thermophilus*
	Lactobacillus delbrueckii subsp. *bulgaricus*
Buttermilk	*Lactococcus lactis* subsp. *lactis*
	Lactococcus lactis subsp. *cremoris*
	Lactococcus lactis subsp. *lactis* biovar. *diacetylactis*
	Leuconostoc lactis
	Leuconostoc mesenteroides subsp. *cremoris*
Sour cream	*Lactococcus lactis* subsp. *lactis*
	Lactococcus lactis subsp. *cremoris*
	Lactococcus lactis subsp. *lactis* biovar. *diacetylactis*
	Leuconostoc lactis
	Leuconostoc mesenteroides subsp. *cremoris*

The actual manufacture of cultured dairy products requires only milk (skim, low-fat, or whole, or cream, depending on the product) and a suitable lactic starter culture. However, the process is not quite so simple, because defects associated with flavor, texture, and appearance are not uncommon. One of the most frequent and serious problems in the manufacture of many of these products, especially yogurt, is syneresis.

Syneresis is defined as the separation (or squeezing out) of water from the coagulated milk. For many consumers, the appearance of these pools of slightly yellow-green water (which is actually just whey) from the top of the product is considered unnatural and objectionable. Thus, to minimize syneresis problems, and to improve the body and texture of the finished product, manufacturers perform several steps whose purpose is to enhance the water binding capacity of the milk mixture. First, the milk solids are increased, either by adding dry milk powder or by concentrating milk. Second, the milk mixture is heated well above ordinary pasteurization conditions to denature the whey proteins, exposing more amino acid residues to the aqueous environment. Finally, most manufacturers have incorporated stabilizers, thickening agents, and other ingredients into the formulation to further reduce syneresis. However, a few countries, France, in particular, prohibit many of these additional ingredients and instead rely on other means to reduce syneresis (discussed below).

YOGURT PRODUCTION

Yogurt manufacture involves several distinct steps, including formulation and processing of the mix, culture addition and fermentation, and packaging and post-process handling. Each of these steps are described below. The type of yogurt will dictate specific steps.

1. Mix formulation

Yogurt can be made from skim (fat-free), reduced fat, or whole milk. In the US, reduced fat yogurts (1–2%) are the most popular, whereas in many other parts of the world, whole fat yogurts are more common. As is true for all dairy products, but especially for yogurt and other fermented milks, it is important to use good quality milk, free of antibiotics and other inhibitory substances. The first step (Figure 5.2) involves adding nonfat dry milk to the milk to increase the total solids to 12% to 13%, sometimes to as high as 15%. Another common alternative is to increase the total milk solids by concentrating the milk via evaporation. Other permitted ingredients (see below) may be added, and, if the mix contains fat, it is then homogenized. There are, however, some manufacturers that produce unhomogenized, "cream-on-the-top" whole milk versions.

2. Heat treatment

Yogurt is considered a fluid milk product by the US Food and Drug Administration (FDA) and must be made using pasteurized milk. However, most yogurt mixes receive a heat treatment well above that required for pasteurization. Thus, instead of pasteurizing milk for 71.7°C for 15 seconds (the minimum required), mixes are heated to between 85°C and 88°C for up to 30 minutes. Other time-temperature conditions can also be used, but kinetically they are usually equivalent. Heating can be done in batch mode (i.e., in vats), but continuous heating in plate or tube type heat exchangers is far more common.

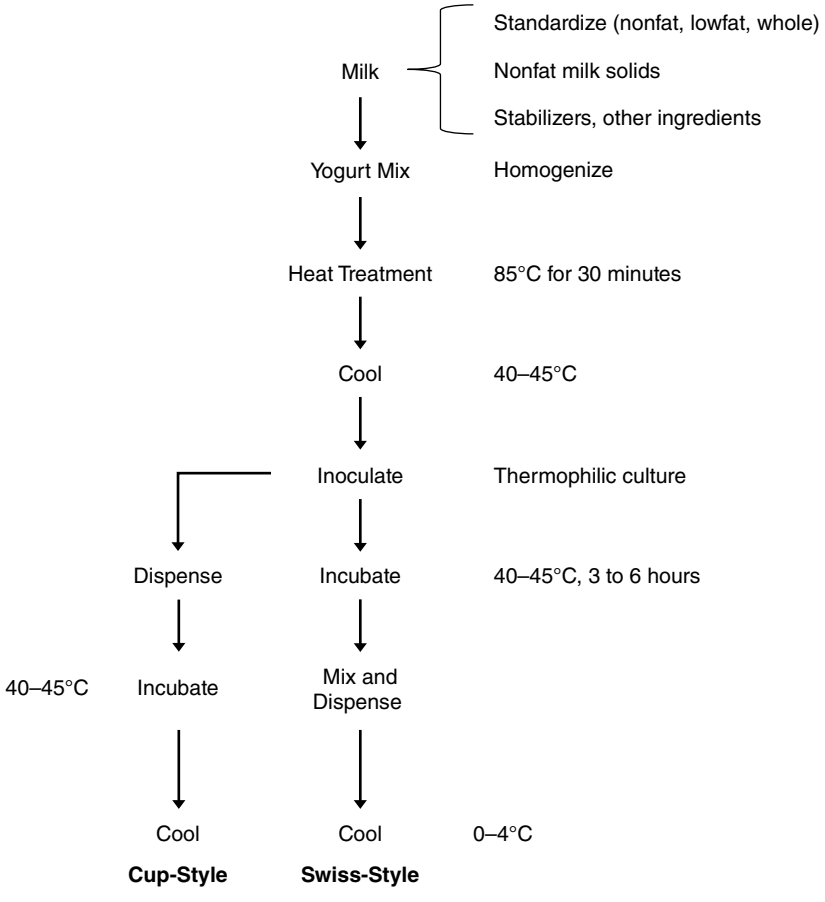


Figure 5.2 Manufacture of yogurt.

The high temperature treatment not only satisfies all of the normal reasons for pasteurization (i.e., killing pathogens and spoilage organisms and inactivating enzymes), but these severe heating conditions also perform two additional functions. First, even heat-resistant bacteria and their spores are killed, making the mixture essentially free of competing microorganisms. Second, the major whey proteins, alpha-lactalbumin and beta-lactoglobulin, are nearly 100% denatured at the high pasteurization temperatures. These proteins exist in globular form in their native state, but once denatured, amino acid residues are exposed and their ability to bind water, via hydrogen bonding, is significantly enhanced. Denatured whey proteins also reduce the Eh and stabilize the milk gel.

3. Yogurt styles

The pasteurized milk is then cooled via a heat exchanger to the desired incubation temperature, usually between 40°C and 45°C. Alternatively, the milk can be cooled as for conventional processing to 2°C to 4°C, and then warmed to the higher temperature later. The incubation temperature is critical, since it will influence the activity of the culture and ultimately the properties of the finished yogurt (see below).

The route the mix takes next depends on the type or style of yogurt being made. There are two general styles of yogurt. Yogurt that reaches the consumers in a form pre-mixed with flavors, fruit, or other bulky ingredients is called stirred or Swiss-style yogurt. For this type, the milk mixture is pumped into vats and the culture is added. The yogurt is then incubated such that the entire fermentation occurs in the vat. At the end of the fermentation, the mixture is gently agitated and cooled, and the flavor and other ingredients are introduced. The mixture is then pumped into containers.

In contrast, the milk mixture can be inoculated with culture, and pumped immediately into the container. The yogurt is incubated and fermented directly in the container. This so-called fermented-in-the-cup style yogurt was the most popular form for many years and still is in much of the world. If fruit or other bulky flavorings are desired, the fruit or flavoring material is first dispensed into the cup and the yogurt mix is layered on top, followed by incubation and fermentation. For these fruit-on-the-bottom-style yogurts, the consumer must do the stirring and mixing to incorporate the flavoring throughout the product.

4. Yogurt cultures

For either style, the lactic culture is the key ingredient and requires careful selection. Yogurt cultures, in general, and the actual strains, in particular, have a profound influence on the flavor, texture, appearance, and overall quality attributes of the finished product. As noted above, starter cultures for yogurt consist of two organisms, *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*. Many commercial products contain more than one strain of each organism, but the traditional ratio of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* (or rod to coccus) has usually been about 1:1. Although this ratio can be adjusted, depending on the desired properties of the final product, it is important that both organisms be present.

Yogurt made with only one of these two organisms usually will not turn out well, for several reasons. It has long been known that these bacteria grow faster and perform better when grown as a pair compared to when they are grown individually. The basis for this synergistic growth (or "proto-cooperation") has been the subject of considerable study since the 1960s. That *S. thermophilus* always appeared to grow first in co-culture experiments suggested that the initial milk environment was somehow less conducive for *L. delbrueckii* subsp. *bulgaricus*. It is now thought that *S. thermophilus* lowers the pH and Eh to levels preferred by *L. delbrueckii* subsp. *bulgaricus.* In contrast, the ability of *S. thermophilus* to produce the enzyme urease (and form NH_3) may also serve to maintain pH homeostasis when the pH is too low.

Growth of *S. thermophilus* in milk is limited by nitrogen sources. Most strains are weakly proteolytic and lack the ability to hydrolyze casein. They can still make do, at least initially, by relying on the small pool of free amino acids present in the milk. Later, when those amino acids are depleted, *S. thermophilus* benefits by its association with *L. delbrueckii* subsp. *bulgaricus*, since the latter produces a proteinase that makes peptides and amino acids available for all organisms that happen to be present.

Eventually, however, *L. delbrueckii* subsp. *bulgaricus* may produce more acid than can be tolerated by *S. thermophilus*. In yogurt, therefore, the *S. thermophilus* population may begin to decrease. If these organisms were continually propagated as a single mixed culture, the *S. thermophilus* would likely be displaced after several transfers. Thus, while it is possible to make yogurt on a small scale (e.g., in the home) using the backslopping technique, these cultures will eventually become less reliable. In contrast, commercial cultures are optimized to provide the strain balance preferred by the yogurt manufacturer.

Depending on the form of the culture and the manufacturer's instructions, the cultures are added to the yogurt mix to give an initial cell concentration of about 10^7 cells per gram. The inoculated mixes are then incubated (either in the cup or vat) at 40°C to 45°C for four to six hours or until a titratable acidity (as lactic acid) of 0.8% to 0.9% is reached and the pH is about 4.4 to 4.6. Depending on the culture activity and desired final pH, the fermentation may be even shorter (i.e., three hours or less). The recent trend in the United States toward low acid yogurts means that the fermentation may be judged, by some manufacturers, as complete when the pH reaches 4.8 to 4.9, or just as the mixture begins to coagulate. In contrast, many traditional-style yogurts are fermented until the pH is near 4.0.

As noted above, the incubation temperature can have a profound effect on the fermentation and the overall product characteristics. In general, most strains of *L. delbrueckii* subsp. *bulgaricus* have higher temperature optima than *S. thermophilus*. Thus, growth of *S. thermophilus* would be favored at temperatures below 42°C, whereas *L. delbrueckii* subsp. *bulgaricus* is favored above 42°C. By shifting the incubation temperature by just a few degrees, it is possible to influence the growth rates of the two organisms, as well as the metabolic products they produce. Since, for example, *L. delbrueckii* subsp. *bulgaricus* is capable of producing greater amounts of lactic acid and acetaldehyde compared to *S. thermophilus*, high incubation temperatures may result in a more acidic and flavorful yogurt.

5. Culture metabolism

Both *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* are homofermentative, meaning lactic acid is their primary end-product from sugar metabolism. Both also ferment lactose in a similar manner. Moreover, the specific means by which lactose metabolism occurs in these bacteria not only dictate product formation, but also have an important impact on the health-promoting activity these bacteria provide.

The first step involves transport of lactose across the cell membrane. As reviewed in Chapter 3, there are two general routes by which this step can occur in lactic acid bacteria. Most mesophilic lactococci (i.e., *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*) use the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS), in which lactose is phosphorylated during its transport across the cytoplasmic membrane. The product that accumulates in the cytoplasm, therefore, is lactose-phosphate. In contrast, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus,* the thermophilic culture bacteria, use a secondary transport system (called LacS) for lactose uptake. Transport of lactose occurs via a symport mechanism. Lactose is not modified during transport and instead accumulates inside the cell as free lactose.

For both the lactose PTS and LacS systems, the next step is hydrolysis, but since the substrates are different, the hydrolyzing enzymes must be different, too. In lactococci, lactose-phosphate is hydrolyzed by phospho-β-galactosidase, forming glucose and galactose-6-phosphate. Glucose is subsequently phosphorylated to form glucose-6-phosphate, which then feeds directly into the Embden-Meyerhof-Parnas glycolytic pathway, and ultimately leading to formation of lactic acid. The other product of the phospho-β-galactosidase reaction, galactose-6-phosphate, is metabolized simultaneously by a parallel pathway called the tagatose pathway, which also results in lactic acid production.

In *S. thermophilus,* the intracellular lactose that accumulates is hydrolyzed by cytoplasmic β-galactosidase, releasing glucose and galactose. The glucose is metabolized to lactic acid, the same as in the lactococci. However, most strains of *S. thermophilus* lack the

ability to metabolize galactose and instead secrete galactose back into the milk. This is despite the observation that a pathway for galactose metabolism indeed exists in these bacteria.

The reason why this pathway does not function in *S. thermophilus*, and why this apparent metabolic defect occurs, has been the subject of considerable study. It is now evident that the genes coding for the enzymes necessary to metabolize galactose, the so-called Leloir pathway, are not transcribed or expressed. Rather, the genes are repressed, and the unfermented galactose is instead secreted into the extracellular medium (i.e., the milk).

Although it would appear that *S. thermophilus* is being wasteful by not making efficient use of both monosaccharide constituents of lactose, this is not the case. When *S. thermophilus* grows in milk, where the lactose concentration is 5% (more than 140 mM), or in yogurt mix, which has an even higher lactose concentration due to added milk solids, sugar limitation is not an issue. Rather, growth cessation in yogurt occurs due to low pH or low temperature, not sugar availability. Interestingly, when *S. thermophilus* excretes galactose it does so via the same LacS permease used for lactose uptake. Thus, LacS acts as an exchange system, trading one out-bound galactose for one in-coming lactose (see Chapter 3). This product-substrate exchange reaction spares the cell of energy that it would normally be required to spend for lactose transport. In fact, *S. thermophilus* may be so well-adapted to growth in milk that the galactose efflux reaction is kinetically favored, even in variant strains where the galactose genes are de-repressed and galactose pathway enzymes are expressed. In yogurt, galactose accumulation, even at concentrations as high as 0.7% to 1.0%, is ordinarily of no major consequence (the exception is for individuals who have a defect in galactose metabolism). However, in cheese manufacture, galactose can be the cause of serious technological problems, and galactose-fermenting strains of *S. thermophilus* may be of considerable value (Chapter 6).

Regardless of the metabolic details that occur during the yogurt fermentation, it is worth noting that most of the lactose is actually left unfermented. Yogurts ordinarily contain about 1% lactic acid, all of which come from lactose fermentation. Thus, if the milk contained 5% lactose, 4% would remain. However, since yogurt mixes typically contain added milk solids or are made from concentrated milk, the lactose concentration is often much higher than plain milk. Thus, the finished yogurt could still contain 5% or more lactose.

6. Post-fermentation

The fermentation is considered complete when the target acidity is reached and the yogurt is cooled quickly to below 4°C. For all practical purposes, cooling is really the only way to arrest the fermentation and stop further acid production. Cup-set yogurt must be very carefully moved to coolers (0°C to 4°C) to avoid agitation which may disturb the gel and promote syneresis. For Swiss style yogurt, where the fermentation occurs in a vat, the yogurt is typically stirred and cooled in the vat, then mixed with fruit or other flavoring, and pumped into cups or containers.

It is important to recognize that during the cooling period the pH may continue to drop by an additional 0.2 to 0.3 pH units, so initiating the cooling step even when the pH is 4.8 to 4.9 may be warranted. In addition, some culture strains may continue to produce acid during refrigerated storage, albeit slowly. Over-acidification and post-fermentation acidification are major problems, since US consumers generally prefer less acidic yogurts (see below).

7. Yogurt flavor and texture

The most pronounced or dominant flavor of yogurt is sourness, due to lactic acid produced by the starter culture. Most yogurts contain between 0.8% and 1.0% lactic acid and have a pH below 4.6. In the absence of sweetener or added flavors, most consumers can detect sourness when the pH is below 5.0. Other organic acids, including formic and acetic, may also be produced by the culture, but at much lower concentrations, and they generally make only modest contributions to yogurt flavor.

Other metabolic products that contribute significantly to flavor development are also produced by the culture. The most important of these is acetaldehyde, a two carbon aldehyde. Although normally present at less than 25 ppm concentration, this is still sufficient to give yogurt its characteristic tart or green apple flavor. Both *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* can produce acetaldehyde; however, the rate and amount produced depends on the strain and on the growth conditions.

There appear to be at least two metabolic pathways by which acetaldehyde can be produced (Figure 5.3). In one pathway, the enzyme, serine hydroxymethyl transferase hydrolyzes threonine, and acetaldehyde and glycine are formed directly. Although this appears to be the primary pathway (a mutation in the *glyA* gene eliminates acetaldehyde formation), it is theoretically possible to form acetaldehyde from pyruvate. The latter can either be decarboxylated, forming acetaldehyde directly, or converted first to acetyl CoA, and then oxidized

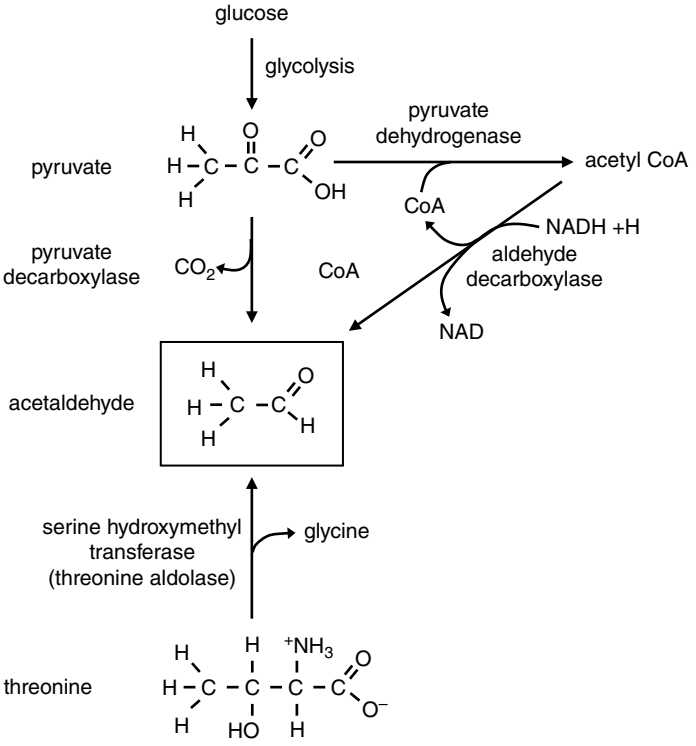


Figure 5.3 Formation of acetaldehyde by yogurt bacteria from pyruvate and threonine. Adapted from Chaves et al., 2002.

to acetaldehyde. Finally, diacetyl and acetoin may also be produced by the yogurt culture; however, they are usually present at concentrations below a typical taste threshold.

Whereas unflavored or "plain" yogurt is popular in Europe and the Middle East, most of the yogurt consumed in the United States is flavored with fruit and other flavorings. Fruits are usually added in the form of a thick puree, with or without real pieces of fruit. These ingredients obviously dilute or mask the lactic acid and acetaldehyde flavors. In fact, the trend in the United States is to produce mild-flavored yogurts with less characteristic yogurt flavor. Thus, strains that produce little acetaldehyde are often used in yogurt cultures.

The texture and rheological properties of yogurt are, perhaps, just as important to consumers as flavor. Coagulated milk is essentially a protein gel that imparts viscosity, mouth feel, and body to the finished product. Formation and maintenance of the gel structure, therefore, is important for yogurt quality. The gel properties are affected by the ingredients in the yogurt mix, how the yogurt mix is processed and produced, culture activity, and post-fermentation factors. For example, proper heat treatment of the milk has a profound effect on gel strength and, specifically, the water holding capacity. If the gel is poorly formed or disrupted syneresis will occur, leading to the defect known as "wheying off" (see below). To control syneresis and maintain a suitable gel structure, yogurt manufacturers in the United States commonly add stabilizers to the mix. Typically, stabilizers are hydrophilic polysaccharides whose purpose is to bind water. The most popular stabilizers are naturally derived gums and starches, including carrageenan, locust bean, and guar gums; corn starch; tapioca; and pectin. The protein, gelatin, is also frequently added as a stabilizer.

In some countries (e.g., France and the Netherlands), stabilizers are not permitted in plain yogurt. Therefore, the ability of some strains of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* to produce and secrete natural polysaccharide material directly in the yogurt during the fermentation is an especially important trait. This is because many of these extracellular polysaccharides (exopolysaccharides or EPS) have excellent stabilizing and rheological properties. The producer strains are now widely used and included in yogurt starter cultures to provide the desired body characteristics. A variety of EPS produced by these bacteria have been identified (Box 5.2). Some EPS contain only a single sugar (homopolysaccharides). In contrast, other EPS consist of a heterogenous mixture of different sugar monomers (heteropolysaccharides) in varying ratios. The latter contain isomers of galactose and glucose, along with rhamnose and other sugars.

Box 5.2 Exopolysaccharide production by lactic acid bacteria

Introduction

Many microorganisms are able to synthesize exopolysaccharides (EPS). These biopolymers serve several important ecological and physiological functions. For example, bacterial EPS can protect the producer cells against toxic chemicals, predatory protozoa, bacteriophage, phagocytes, and environmental antagonists (Nwodo et al., 2012). They are also involved in aggregation, biofilm formation, and quorum sensing (Badel et al., 2011).

In addition to their ecological role, EPS are also important industrially, serving as alternatives to plant-derived gums (Freitas et al., 2011). Several have been approved for food applications. Examples include xanthan gum, produced by *Xanthomonas*

campestris and gellan gum, produced by species of *Sphingomonas*. These EPS are produced in industrial fermenters and are subsequently separated and marketed as stabilizers or gums.

Practical considerations

For lactic acid bacteria (LAB), EPS are physiologically relevant for many of the reasons noted above, i.e., for ecological or survival purposes. However, their ability to produce EPS *in situ* is of particular practical importance in fermented foods. This is because LAB-produced EPS provide several functional benefits. EPS are especially applicable in yogurt and other cultured dairy products since they can reduce syneresis, increase viscosity, and enhance gel strength (Torino et al., 2015). They also improve body and emulsification and can act as fat replacers (Ryan et al., 2015). Incorporating EPS-producing strains in dairy starter cultures could therefore eliminate the necessity for adding gums and other stabilizing agents. Interest in naturally-produced gelling agents has also been fueled by consumer demand for clean labels. Likewise, in some European countries, the addition of stabilizers in cultured dairy products is restricted.

In addition to cultured dairy products, EPS have also been reported to confer functional benefits in other fermented foods. For example, in sourdough bread, EPS produced *in situ* by sourdough lactobacilli may enhance flavor, texture, and shelf-life (Galle and Arendt, 2014; Di Monaco et al., 2015; Wolter et al., 2014), as well as improve properties of gluten-free bread (Galle et al., 2012). EPS-producing lactic acid bacteria were also reported to improve texture of fermented and pureed carrots (Juvonen et al., 2015). EPS production by *Oenococcus oeni* may promote colonization on barrels and wine-making equipment and subsequent inoculation into wine (Dimopoulou et al., 2014). Finally, EPS may also have nutritional or health benefits (Ryan et al., 2015). For example, like other polysaccharides, some EPS may have prebiotic activity, promoting growth of beneficial members of the gastrointestinal tract (Salazar et al., 2016, Welman, 2014). They may also have immunomodulatory, cholesterol-binding, and pathogen inhibition activities (Welman, 2014, Zannini et al., 2016).

General types of EPS

Lactic acid bacteria that produce EPS do so in one of two general ways. Some strains synthesize EPS as released or dis-attached material (referred to as "ropy" strains), whereas others produce capsular polysaccharide that remains attached to the cell surface (Broadbent et al., 2003). However, it appears that the EPS from both ropy and capsular strains have useful functional properties in cultured milk products, as noted above, regardless of how synthesis and attachment occurs. Lactic acid bacterial EPS may also serve as a defense mechanism against bacteriophages by preventing their adsorption (Forde and Fitzgerald, 1999; Lamothe et al., 2002). In addition, some LAB may produce EPS to adhere or stick to various surfaces, thereby promoting colonization of desirable habitats (Lamothe et al., 2002). The latter could include the gastrointestinal tracts of humans and other animals.

Structure of exopolysaccharides

The molecular masses of EPS from LAB range from 10 kDa to greater than 1000 kDa (Welman and Maddox, 2003). The number of monomers present within these EPS range from about 50 to more than 5000. The most frequently found monosaccharides are glucose and galactose, but rhamnose, mannose, fructose, arabinose and xylose, and the sugar derivatives, *N*-acetylgalactosamine and *N*-acetylglucosamine, are also common.

In general, the EPS can be subdivided, based on their chemical composition, into two major groups: homopolysaccharides and heteropolysaccharides (Ryan et al., 2015). Homopolysaccharides are composed of only one type of monosaccharide, whereas heteropolysaccharides are composed of a repeating unit that contains two or more different monosaccharides. Examples of the homopolysaccharides include: (1) dextrans and mutans, α-1,6 and α-1,3-linked glucose polymers produced by strains of *Leuconostoc mesenteroides* and *Streptococcus mutans*, respectively; (2) β-glucans, β-1,3-glucose polymers, produced by strains of *Pediococcus* and *Lactobacillus*; (3) fructans, β-2,6-linked D-fructose polymers produced by strains of *Streptococcus salivarius*, *Lactobacillus sanfranciscensis*, and *Lactobacillus reuteri*; and (4) polygalactans, produced by strains of *Lactococcus lactis*.

Most of the lactic acid bacteria-produced EPS, however, are heteropolysaccharides (De Vuyst and Degeest, 1999). These EPS are structurally more diverse than the homopolysaccharides, and their overall composition and physicochemical properties differ considerably between species and strains. Heteropolysaccharides most often contain a combination of glucose, galactose, and rhamnose, as well as lesser amounts of fructose, acetylated amino sugars, ribose, glucuronic acid, and noncarbohydrate constituents such as glycerol, phosphate, pyruvyl, and acetyl groups. These EPS are the ones most commonly associated with yogurt, due to their production by *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. The majority of the EPS produced by *S. thermophilus* contain repeating tetrasaccharide units; however, several strains produce EPS consisting of hexasaccharides or heptasaccharides. Strains of *L. delbrueckii* subsp. *bulgaricus* typically produce heptasaccharides composed of glucose, rhamnose, and a high ratio of galactose, whereas *L. helveticus* produces heteropolysaccharides made up almost exclusively of glucose and galactose. Some mesophilic LAB, including *L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris, L. sakei,* and *L. rhamnosus,* are also heteropolysaccharide EPS producers.

Production of exopolysaccharides

The enzymatic machinery for synthesizing homopolysaccharides are located at the exterior surface of the cell. Thus, the main enzymes for elongation, i.e., glycosyltransferases, are attached to the cell surface, such that the EPS is built entirely outside the cell. In contrast, heteropolysaccharides are synthesized in the cytoplasm and are translocated to the exterior via an export mechanism presumably associated with "flippase" enzymes similar to those used for cell wall synthesis (Zivkovic et al., 2015).

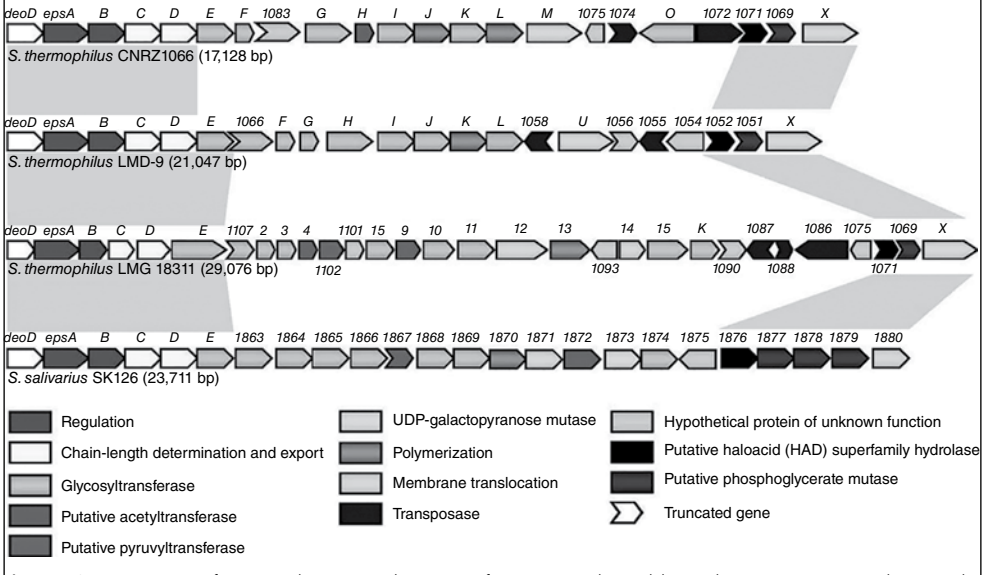


Figure 5.2.1 Comparison of *eps* gene cluster among three strains of *Streptococcus thermophilus*. Similarity to *Streptococcus salivarius* is also shown. From Goh et al., 2011, with permission.

Genetic organization

Genes encoding for EPS production exist as clusters, with specific structural, regulatory, and transport functions. As noted above, these gene clusters are often located on plasmids in mesophilic LAB and on the chromosome in thermophilic LAB (Goh et al., 2011; Wu et al., 2014). In general, these clusters reveal a common operon structure, with the genes positioned in a particular order. An example of such an arrangement for the EPS produced by several strains of *S. thermophilus* is shown in Figure 5.2.1.

At the beginning of the cluster encoded in strain CNRZ 1066 are *epsA* and *epsB,* genes involved in EPS regulation. The central region of the gene cluster, consisting of *epsE, epsF, epsG, epsH,* and *epsI,* encodes for enzymes involved in biosynthesis (glycosyltransferase and acetylransferase) of the EPS repeating unit. Other products of *epsC, epsD, epsJ, epsL* and *epsM* are responsible for polymerization and export of the EPS. Interestingly, some *eps* clusters contain mobile elements (i.e., transposase genes) that may promote gene transfer as well as account for the apparent instability of EPS production (Zivkovic, et al., 2015).

References

Badel, S., T. Bernardi, and P. Michaud. 2011. New perspectives for Lactobacilli exopolysaccharides. Biotechnol. Adv. 29:54–66.

Broadbent, J.R., D.J. McMahon, D.L. Welker, C.J. Oberg, and S. Moineau. 2003. Biochemistry, genetics, and applications of exopolysaccharide production in *Streptococcus thermophilus*: a review. J. Dairy Sci. 86:407–423.

De Vuyst, L. and B. Degeest. 1999. Heteropolysaccharides from lactic acid bacteria. FEMS Microbiol. Rev. 23:153–177.

Di Monaco, R., E. Torrieri, O. Pepe, P. Masi, and S. Cavella. 2015. Effect of sourdough with exopolysaccharide (EPS)-producing lactic acid bacteria (LAB) on sensory quality of bread during shelf life. Food Bioprocess Technol. 8:691–701.

Dimopoulou, M., M. Vuillemin, H. Campbell-Sills, P.M. Lucas, P. Ballestra, C. Miot-Sertier, M. Favier, J. Coulon, V. Moine, T. Doco, and M. Roques. 2014. Exopolysaccharide (EPS) synthesis by *Oenococcus oeni*: from genes to phenotypes. PLoS ONE 9:e98898.

Freitas, F., V.D. Alves, and M.A.M. Reis. 2011. Advances in bacterial exopolysaccharides: from production to biotechnological applications. Trends Biotechnol. 29:388–398.

Forde, A., and G.F. Fitzgerald. 1999. Analysis of exopolysaccharide (EPS) production mediated by the bacteriophage adsorption blocking plasmid, pCI658, isolated from *Lactococcus lactis* subsp. *cremoris* HO2. Int. Dairy J. 9:465–472.

Galle S. and E.K. Arendt. 2014. Exopolysaccharides from sourdough lactic acid bacteria. Crit. Rev. Food Sci. Nutr. 54:891–901.

Galle, S., C. Schwab, F. Dal Bello, A. Coffey, M.G. Ganzle, and E.K. Arendt. 2012. Influence of *in-situ* synthesized exopolysaccharides on the quality of gluten-free sorghum sourdough bread. Int. J. Food Microbiol. 155:105–112.

Goh, Y.J., C. Goin, S. O'Flaherty, E. Altermann, and R. Hutkins. 2011. Specialized adaptation of a lactic acid bacterium to the milk environment: the comparative genomics of *Streptococcus thermophilus* LMD-9. Microb. Cell Fact. 10(Suppl 1):S22.

Juvonen, R., K. Honkapää, N.H. Maina, Q. Shi, K. Viljanen, H. Maaheimo, L. Virkki, M. Tenkanen, and R. Lantto. 2015. The impact of fermentation with exopolysaccharide producing lactic acid bacteria on rheological, chemical and sensory properties of pureed carrots (*Daucus carota* L.). Int. J. Food Microbiol. 207:109–118.

Lamothe, G., L. Jolly, B. Mollet, and F. Stingele. 2002. Genetic and biochemical characterization of exopolysaccharide biosynthesis by *Lactobacillus delbrueckii* subsp. *bulgaricus*. Arch. Microbiol. 178:218–228.

Nwodo, U.U., E. Green, and A.I. Okoh. 2012. Bacterial exopolysaccharides: functionality and prospects. Int. J. Mol. Sci. 13:14002–14015.

Ryan, P.M., R.P. Ross, G.F. Fitzgerald, N.M. Caplice, and C. Stanton. 2015. Sugar-coated: exopolysaccharide producing lactic acid bacteria for food and human health applications. Food Funct. 6:679–693.

Salazar, N., M. Gueimonde, C.G. de Los Reyes-Gavilán, and P. Ruas-Madiedo. 2016. Exopolysaccharides produced by lactic acid bacteria and bifidobacteria as fermentable substrates by the intestinal microbiota. Crit. Rev. Food. Sci. Nutr. 56:1440–1453.

Torino, M.I., G. Font de Valdez, and F. Mozzi. 2015. Biopolymers from lactic acid bacteria. Novel applications in foods and beverages. Front. Microbiol. 6:834.

Welman, A.D., and I.S. Maddox. 2003. Exopolysaccharides from lactic acid bacteria: perspectives and challenges. Trends Biotechnol. 21:269–274.

Welman, A.D. 2014. Exopolysaccharides from fermented dairy products and health promotion. In: Holzapfel, W., ed. Advances in Fermented Foods and Beverages: Improving Quality, Technologies and Health Benefits, Woodhead Publishing, Cambridge, pp. 23–38.

Wolter, A., A.S. Hager, E. Zannini, M. Czerny, and E.K. Arendt. 2014. Influence of dextran-producing *Weissella cibaria* on baking properties and sensory profile of gluten-free and wheat breads. Int. J. Food Microbiol. 172:83–91.

Wu, Q., H.M. Tun, F.C.-C. Leung, and N.P. Shah. 2014. Genomic insights into high exopolysaccharide-producing dairy starter bacterium *Streptococcus thermophilus* ASCC 1275. Sci. Rep. 4:4974.

Zannini, E., D.M. Waters, A. Coffey, and E.K. Arendt. 2016. Production, properties, and industrial food application of lactic acid bacteria-derived exopolysaccharides. Appl. Microbiol. Biotechnol.100:1121–1135.

Zivkovic, M., M. Miljkovic, P. Ruas-Madiedo, I. Strahinic, M. Tolinacki, N. Golic, and M. Kojic. 2015. Exopolysaccharide production and ropy phenotype are determined by two gene clusters in putative probiotic strain *Lactobacillus paraplantarum* BGCG11. Appl. Environ. Microbiol. 81:1387–1396.

DEFECTS

Commercial yogurt, produced and packaged in modern facilities, can easily have a shelf-life as long as 4–6 weeks. Nonetheless, yogurt can suffer from flavor, texture, and appearance defects. In some cases, these defects are caused by microorganisms (including the starter culture), but often they are caused by manufacturing issues. This is especially true for the wheying off or syneresis defect that is probably the single most serious issue for yogurt consumers.

Although relatively rare, some chemically-derived flavor defects in yogurt can be caused by using poor-quality milk. For example, rancid or oxidized flavors are almost always due to mishandling of the milk (or nonfat dry milk). The most common flavor defects, however, are caused by microorganisms. In some cases, microbial spoilage is due to the presence of spore-forming bacteria, including various species of *Bacillus* that can survive pasteurization and grow in the milk, producing bitter or rancid flavors. Yeasts and molds that are present in the fruit flavoring material can also produce off-flavors, although it is usually just the appearance of yeast or mold colonies that cause consumers to reject such products. Of course, bacteria, such as psychrotrophs that grew in the raw milk prior to pasteurization, can produce bitter and rancid flavors.

Despite the acidity and acetaldehyde flavor normally expected in yogurt, these attributes can also be considered as defects if present at high enough concentrations or if they exceed

consumer tastes. Yogurts within a range of pH 4.2 to 4.5 (0.8% to 1% lactic acid) are generally acceptable, but the pH can drop to as low as 4.0 (and about 2% lactic acid) under certain circumstances. Excessive acid production, for example, can occur if the yogurt is incubated at too high a temperature or if cooling rates are too slow (both promote growth of *L. delbrueckii* subsp. *bulgaricus*). Even properly produced yogurt can eventually become too acidic during low temperature storage due to the ability of the culture to produce, albeit slowly, lactic acid. This post-fermentation acidification is one of the main reasons why consumers may consider an otherwise perfectly fine yogurt to be spoiled.

One way to prevent over-acidification is to control culture activity. Strains have been identified (either naturally or via molecular interventions) that are sensitive to low pH or low temperatures, such that growth and acid formation are halted when the pH reaches a critical value or when the temperature is sufficiently low. Of course, another way to control acidity and to increase shelf-life would be to heat pasteurize the yogurt after the fermentation. This approach has been largely rejected by the industry because it would negate most of the health benefits conferred by the culture and would harm the image yogurt enjoys as a healthy food product. Moreover, the National Yogurt Association, a trade organization representing yogurt manufacturers, has suggested that refrigerated yogurt contain at least 10^8 viable organisms per gram at the time of manufacture.

Texture, body, and appearance defects are not uncommon in yogurt. Two of the more serious defects, weak bodied yogurt and free whey formation (i.e., wheying off), have several causes. They may occur when the solids content is too low or when the mix was pasteurized at too low of a temperature, both of which ultimately influence how much denatured whey proteins are present. The gel structure will shrink and syneresis will occur if the culture produces too much acid too fast during the fermentation, causing the pH to become too low. The same result will also occur if the temperature was lowered too rapidly during cooling. When yogurt is mixed with fruit or other flavorings, in the case of Swiss or stirred style yogurts, or when stirred by consumers prior to consumption, the gel is further disrupted and thinning and syneresis occurs.

NUTRITIONAL BENEFITS OF YOGURT

One of great all-time television commercials aired in the late 1970s and featured an interview with a Russian octogenarian who claimed that his longevity was due to his daily consumption of yogurt. When asked who got him started on his yogurt regimen, he proudly stated it was his mother, whose smiling face then moves into the television frame. Indeed, the popularity of yogurt, as implied by this advertisement and as mentioned previously in this chapter (Figure 5.4), has long been due, in large part, to the purported health benefits ascribed to yogurt consumption. In fact, this notion of yogurt as an elixir that fends off aging and promotes human health originated at least a century ago, when the Russian immunologist and Nobel laureate Elie (Elia or Ilya) Metchnikoff published *The Prolongation of Life* in 1906. As Metchnikoff and many other microbiologists have since reported, it is the specific bacteria that either conduct the fermentation (i.e., *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*) or that are added to yogurt in the form of culture adjuncts (e.g., lactobacilli and bifidobacteria) that are responsible for the desirable health benefits of yogurt.

Of course, yogurt has nutritional properties other than those derived from the culture organisms. A single 170 g (6 ounce) serving of plain, nonfat yogurt contains about 170 calories and supplies 18% of the Daily Value requirements for protein, 30% for calcium, and 20% for vitamin B_{12}. Still, these are the same nutrient levels one would get from milk

Figure 5.4 A 1977 advertisement for yogurt, which appeared in television and print media.

(provided one had accounted for the milk solids normally added to the yogurt mix). Although there are reports that yogurt contains more vitamins than the milk from which it was made (due to microbial biosynthesis), these increases do not appear to be significant. Thus, if indeed yogurt has enhanced nutritional quality compared to milk, those differences must be due to the microorganisms found in yogurt.

As noted earlier, there are many health claims ascribed to yogurt and to the probiotic bacteria added as nutritional adjuncts. Among the claims are that these bacteria enhance mineral absorption, promote gastrointestinal and systemic health, and reduce the incidence of enteric infections. Despite substantial epidemiological and clinical research in support of these claims, however, regulators in North American and Europe remain mostly unconvinced (Box 5.3).

Box 5.3 Making claims for probiotic yogurt

The remarkable increase in yogurt consumption in the US and other countries over the past twenty years has been driven by several factors. Certainly, the Greek yogurt category continues to be a major driver (as much as 40% of total sales). However, another main reason for the popularity of yogurt has been because consumers associate yogurt with health, and with probiotic bacteria, in particular. Several major brands propelled sales of probiotic yogurts to billion dollar levels. Their advertisement campaigns also helped to inform consumers about the potential health benefits of probiotics. Indeed, thanks to major advertising campaigns, the rather unappetizing subject of gut health has become a common topic of polite conversation.

Science of probiotics in 2017

It's not just consumers, of course, who have become interested in probiotics. In the food and nutritional sciences, few subjects have received as much attention as has the

subject of probiotics. Since the late 1980s, probiotics and their role in gut health have arguably been the most researched topic in all of food science (Figure 5.3.1). Indeed, interest in probiotics now extends throughout the biomedical and basic science fields. Almost every week, major scientific journals publish papers on the role of the microbiota on health and disease and how probiotic bacteria can influence health status. Many of the studies are conducted as clinical trials using the same methodology (i.e., randomized, double-blinded, placebo-controlled) used for other biomedical studies.

The challenge for probiotic researchers, perhaps more so than for other health studies, has been to establish mechanisms and causality. These efforts are complicated by many factors, including: (1) the complexity and individuality of microbial communities in the gastrointestinal tract; (2) the variety and diversity of potential probiotic organisms to evaluate; (3) the range of clinical conditions that might be influenced by probiotics; and (4) the need for validated biomarkers (Sanders et al., 2013). The latter are especially critical because they represent the biological characteristics that predict underlying health or disease and that can be objectively measured. In addition, many consumers of probiotic products are already healthy, and any health-related benefits would be difficult to detect or measure.

Health claims and probiotic yogurt

As noted above, determining which health claims are supported by scientific evidence and identifying the appropriate criteria to evaluate this evidence have become critical goals (Martinez et al., 2015). Yogurt and other food manufacturers are especially

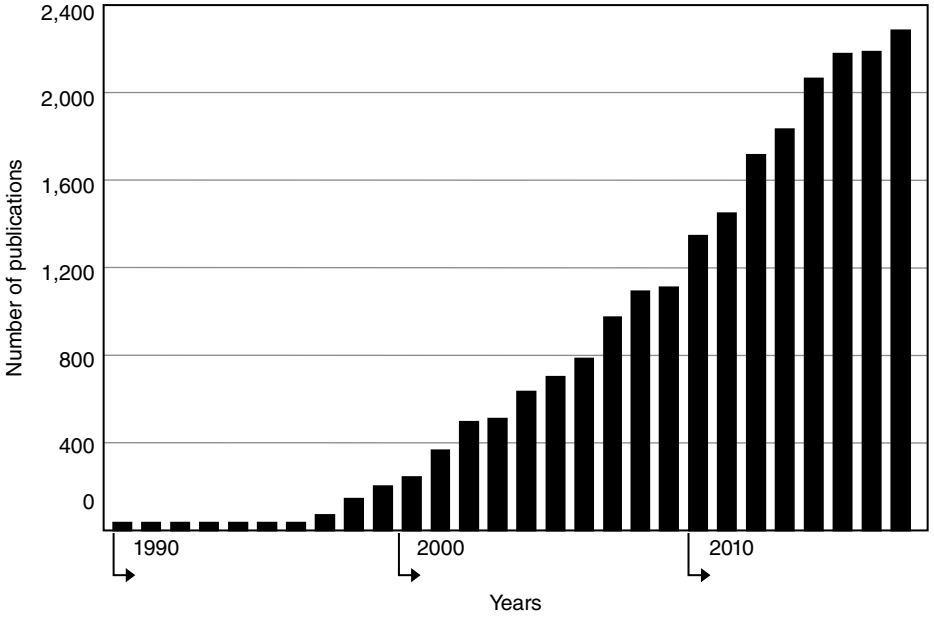


Figure 5.3.1 Publications on probiotics in the PubMed database. Citations were obtained using "probiotic" or "probiotics" as key words. Data for 2017 are through October.

motivated to promote health benefits on the package. However, health claims require approval by government agencies, and relatively few are allowed. In the US, European Union, and Canada, there are no health claims allowed for probiotic yogurts. The ruling bodies, including the US Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA) have determined that the evidence is insufficient or lacks physiological, biochemical, or cause-effect mechanisms.

In the US, so-called structure/function claims are permitted, and these are common on yogurt and other cultured dairy foods. According to the FDA, these claims "may describe the role of a nutrient or dietary ingredient intended to affect the normal structure or function of the human body", and they must be "truthful and not misleading". Examples for a probiotic-containing yogurt would be "probiotics promote digestive health" or "supports healthy immune function".

Indeed, although "contains probiotics" is commonly found on labels for yogurts and other products in the US, the use of the word "probiotics" is not allowed on food packages in Europe, since, by definition, it would imply a health benefit. This position has been challenged, in part, on the basis that the probiotics are held to a higher standard of evidence that are other nutritional products such as vitamins C and D (Hill et al., 2014). Although a claim for yogurt for lactose malabsorption was approved by EFSA, it was not for probiotics per se, but rather for the yogurts containing live yogurt culture. There are also individual countries (Switzerland, Italy, Canada, and Japan) that have approved claims for probiotics (Szajewska et al., 2016).

Another hurdle faced by manufacturers of probiotic-containing yogurts and other cultured milk products is that health benefits have generally been considered to be strain-specific. As noted previously, this view has evolved in recent years, and now the concept of shared probiotic mechanisms among species and genera has been suggested (Sanders et al., 2017).

Some researchers have expressed the view that regulators have attached an excessively high burden of proof for probiotic yogurts that is detrimental to researchers as well as the public (Reid, 2016; Hill et al., 2047). The relevant regulatory agencies would be advised, according to the latter researchers, to consider changes in the "labeling laws that allow for clinically proven health claims to be present on fermented dairy products". Despite these challenges, however, the opportunity exists for researchers, industry, and regulators to develop cultured dairy product that provide nutritional benefits to consumers (German, 2014).

References

German, J.B. 2014. The future of yogurt: scientific and regulatory needs. Am. J. Clin. Nutr. 99 (Suppl):1271S–1278S.

Hill, C, F. Guarner, G. Reid, G.R. Gibson, D.J. Merenstein, B. Pot, L. Morelli, R.B. Canani, H.J. Flint, S. Salminen, P.C. Calder, and M.E. Sanders. 2014. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat. Rev. Gastroenterol. Hepatol. 11:506–514.

Martinez, R.C.R., R. Bedani, and S.M.I. Saad. 2015. Scientific evidence for health effects attributed to the consumption of probiotics and prebiotics: an update for current perspectives and future challenges. Brit. J. Nutr. 114:1993–2015.

Reid, G. 2016. Probiotics: definition, scope and mechanisms of action Best Pract. Res. Clin. Gastroenterol. 30:17–25.
Sanders, M.E., A. Benson, S. Lebeer, D.J. Merenstein, and T.R. Klaenhammer. 2018. Shared mechanisms among probiotic taxa: implications for general probiotic claims. Curr. Opin. Biotechnol. 49:207–216.
Sanders, M.E., F. Guarner, R. Guerrant, P.R. Holt, E.M. Quigley, R.B. Sartor, P.M. Sherman, and E.A. Mayer. 2013. An update on the use and investigation of probiotics in health and disease. Gut 62:787–796.
Szajewska, H., Z. Konarska, and M. Kołodziej. 2016. Probiotic bacterial and fungal strains: claims with evidence. Dig. Dis. 34:251–259.

Perhaps the one health benefit that is most generally accepted is the claim that yogurt organisms can reduce the symptoms associated with lactose maldigestion, a syndrome whose symptoms are referred to as lactose intolerance. Lactose intolerance is a condition that is characterized by the inability of certain individuals to digest lactose. Its specific cause is due to the absence of the enzyme β-galactosidase, which is ordinarily produced and secreted by the cells that line the small intestine (Figure 5.5). In individuals expressing β-galactosidase,

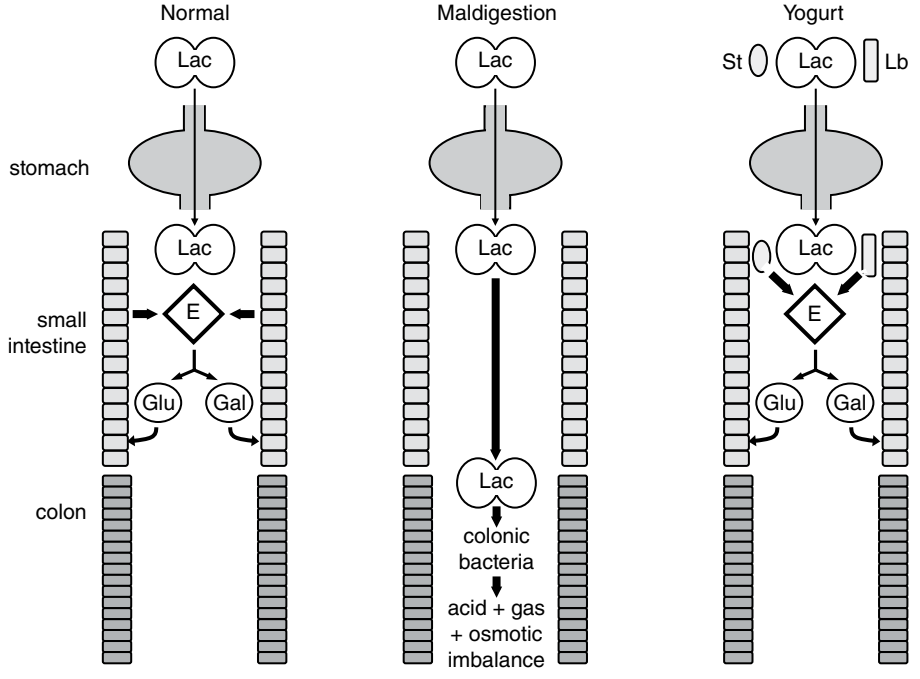


Figure 5.5 In lactose-tolerant individuals (left panel), ingested lactose (Lac) passes through the stomach. Hydrolysis occurs in the small intestine via β-galactosidase (E) produced by epithelial cells that line the surface of the intestinal wall. The hydrolysis products, glucose (Glu) and galactose (Gal) are subsequently adsorbed. In lactose-digesters (center panel), those intestinal cells do not secrete β-galactosidase, and the lactose escapes hydrolysis and instead reaches the colon where it causes colonic distress. When yogurt is consumed (right panel), *Streptococcus thermophilus* (St) and *Lactobacillus bulgaricus* (Lb) serve as a source of exogenous β-galactosidase (after permeabilization in the small intestine) and restore normal lactose hydrolyzing ability to the host.

lactose is hydrolyzed and the glucose and galactose are absorbed across the epithelial cells and eventually enter into the blood stream (in the case of galactose, only after conversion to glucose in the liver). If β-galactosidase is not produced in sufficient levels, however, the lactose remains undigested and is not absorbed. Instead, it passes to the large intestine, where it either causes an increase in water adsorption into the colon (via osmotic forces) or is fermented by colonic organisms. The resulting symptoms can include diarrhea, gas, and bloating, leading many lactose intolerant individuals to omit milk and dairy products from their diet. Lactose maldigestion has a genetic basis, affecting African, Asian, American Indian, and other non-Caucasian populations far more frequently than Caucasian groups. These individuals could typically tolerate lactose (i.e., milk) when young, but lose this ability during adulthood. As many as 50 million people in the United States may be lactose intolerant.

It has often been noted that lactose intolerant individuals could consume yogurt without ill effect. It is now known that the bacteria in yogurt are largely responsible for this effect, since heat-treated yogurt is not as effective at alleviating lactose intolerance symptoms. It is interesting, however, that while yogurt can be tolerated, other cultured milk products such as sour cream and buttermilk cannot. The explanation for this observation is related directly to the different routes by which lactose is metabolized by the cultures used in the manufacture of these products.

The fermentation that occurs in sour cream and cultured buttermilk production is performed mainly by lactococci (described below), whereas *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* are responsible for the yogurt fermentation. Recall that the latter organisms hydrolyze lactose via β-galactosidase. When yogurt is consumed and these bacteria reach the small intestine, cell lysis occurs (due to bile salts), intracellular β-galactosidase is released, and lactose is hydrolyzed. Thus, the yogurt culture serves as an exogenous source of this enzyme, substituting for the β-galactosidase the host no longer produces. In contrast, lactococci produce phospho-β-galactosidase. The substrate of this enzyme is lactose-6-phosphate (the product of the lactose PTS), which can only be formed by intact cells. This enzyme does not hydrolyze free lactose. Thus, when lactococci are ingested and lysed in the small intestine, there will be little, if any, impact on lactose digestion, and lactose intolerant individuals will, unfortunately, get no relief.

FROZEN YOGURT AND OTHER YOGURT PRODUCTS

Frozen yogurt is a product for which no U.S. federal standard of identity exists. This has led to confusion and even mis-representations about what this product actually is. Several states now have their own definitions of frozen yogurt (mainly dealing with fat levels), and the FDA has been petitioned to implement standards of identity. The main reason why such measures are thought necessary is to protect the image of yogurt as a healthy, nutritious food – one low in fat and calories and containing health-promoting bacteria. Obviously, there are marketing reasons for including "yogurt" on the label of a product, whether or not that product actually contains yogurt, or what consumers think is yogurt. For example, yogurt-covered pretzels, yogurt-covered raisins, and yogurt-containing salad dressings are made using material that may have once been yogurt, but after processing and dilution with other ingredients, little of the original yogurt character may actually be present. Similarly, ice cream mix containing only a trace of yogurt could, in theory, be called frozen yogurt and gain the market advantages associated with yogurt. Certainly, and perhaps most importantly, the presence of live yogurt organisms should not be assumed for these products.

As noted, some states have written definitions for frozen yogurt. However, while there may be requirements for the presence of particular organisms, there are generally no such requirements for minimum numbers. In Minnesota, for example, frozen yogurts refer to a "Frozen dairy food made from a mix containing safe and suitable ingredients including, but not limited to, milk products. All or a part of the milk products must be cultured with a characterizing live bacterial culture that contains the lactic acid producing bacteria *Lactobacillus bulgaricus* and *Streptococcus thermophilus* and may contain other lactic acid producing bacteria." Some states require that the frozen yogurt products have a minimum acidity, ranging from 0.3% to 0.5%.

STRAINED (GREEK-STYLE) YOGURT

The Greek yogurt phenomenon is one of the most remarkable marketing achievements in the dairy industry. Although this style of yogurt had long been known throughout Europe, the Middle East, and India, its entry into the US market in the late 2000s led to dramatic increases in yogurt consumption. Currently, in the US, Greek-style yogurts have captured about 40% of the total yogurt market.

The main distinguishing features of Greek yogurts are their high protein content and thicker body. Both are achieved by removing water (or whey) after the fermentation. Water removal is most often performed by straining or centrifugal separation, but other physical means can also be used. Whereas conventional yogurt typically contains about 4–5% protein, the protein content of Greek yogurts can be more than double; some contain as much as 12% protein. Some manufacturers simply add more protein (in the form of milk protein concentrate) or thickening agents to yogurt to mimic the Greek yogurt style.

CULTURED BUTTERMILK

Buttermilk is the fluid remaining after cream is churned into butter. It is a thin, watery liquid that is rarely consumed as a fluid drink. Buttermilk, however, is rich in phospholipids derived from the rupture of milk fat globules during churning. Thus, it has excellent functional properties and is an especially good source of natural emulsifiers. It is typically spray dried and used as an ingredient in processed food products.

Cultured buttermilk, in contrast, is an entirely different product. It is made from skim or low-fat milk that is fermented by suitable lactic acid bacteria. The only relation this product has to buttermilk is that butter granules or flakes are occasionally added to provide a buttery flavor and mouth feel. How, then, did this product come to be called buttermilk? In the traditional manufacture of butter, it was common practice to let the cream ferment naturally or to add a mixed, undefined lactic culture to cream prior to churning. The cream, and cream-ripened butter, would be better preserved and the lactic acid would provide a pleasant tart flavor. The resulting by-product, the buttermilk, would also be fermented.

Cultured buttermilk manufacture

Cultured buttermilk is usually made from low-fat milk, although nonfat-free and whole milk versions also exist. Similar to yogurt manufacture, nonfat dry milk is frequently added to give about 10% to 12% nonfat solids (Figure 5.6). Next, the milk is heated to 85°C to 88°C

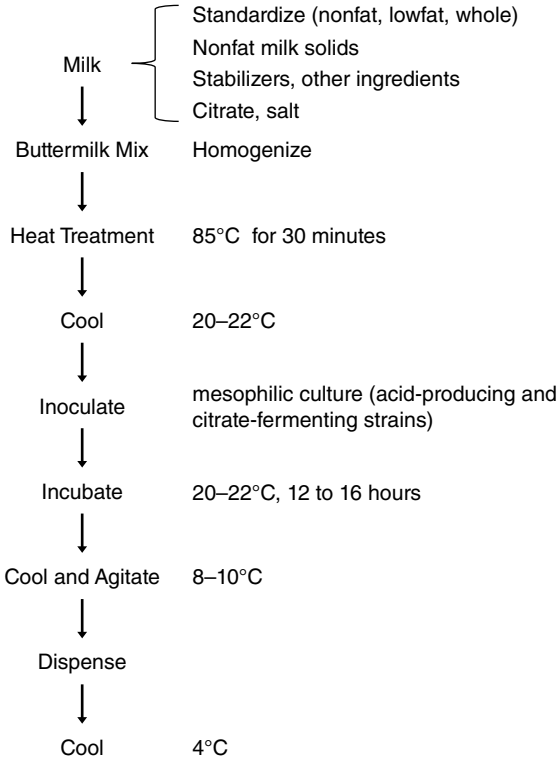


Figure 5.6 Manufacture of cultured buttermilk.

for 30 minutes. This not only pasteurizes the milk, but also satisfies other functional requirements, as described above for yogurt. The mix is then cooled to 21°C to 22°C and inoculated with a mesophilic starter culture, specific for cultured buttermilk.

The starter culture for buttermilk usually contains a combination of acid-producing bacteria and flavor-producing bacteria, in a ratio of about 5:1. Many culture suppliers now also offer body-forming (i.e., EPS-producing) strains. The acid producers include strains of *L. lactis* subsp. *lactis* and/or *L. lactis* subsp. *cremoris*. These bacteria are homolactic, and their function is simply to produce lactic acid and lower the pH. For manufacturers who prefer a decidedly tart, acidic product, the acid-producing strains are sufficient.

It is more common, however, to include flavor-producers in the starter culture. Among the flavor-producing bacteria used in buttermilk cultures are *L. lactis* subsp. *lactis* biovar. *diacetylactis*, *Leuconostoc mesenteroides* subsp. *cremoris*, and *Leuconostoc lactis*. The latter organisms are heterofermentative, producing lactic acid, as well as small amounts of acetic acid, ethanol, and carbon dioxide. These metabolic end-products contribute to the flavor and, in the case of the carbon dioxide, to the mouth feel of the product. Importantly, these bacteria also have the metabolic capacity to ferment citrate and to produce diacetyl, a compound that provides the characteristic flavor of cultured buttermilk. In particular, diacetyl imparts a "delicate" buttery aroma and flavor. The ability of the starter culture to perform the citrate fermentation, therefore, is a critical trait.

Several requirements must be satisfied for the citrate-to-diacetyl pathway (described in Chapter 3) to function in cultured dairy products. First, the activity of the citrate transport

permease has an optimum activity between pH 5.0 and 6.0. Thus, acid production is required. In addition, the pyruvate that is formed from citrate (via oxaloacetate) would ordinarily be reduced to lactate. However, this reaction requires NADH, which is generated only via glycolysis. In its absence, pyruvate would accumulate inside the cell, potentially reaching inhibitory levels. By re-routing the pyruvate to α-acetolactate, this metabolic dilemma is resolved. Finally, α-acetolactate is unstable in the presence of oxygen and is non-enzymatically decarboxylated, forming diacetyl. This last step has practical implications, as noted below.

Following the addition of the culture, the mix is incubated at 20°C to 22°C for 12 to 16 hours. At the end of the fermentation, when the titratable acidity has reached 0.85% to 0.90% and the pH has decreased to about 4.5, the product is cooled to 4°C or less and agitated to break up the coagulum. Salt may be added, and, if desired, butter granules or flakes are added. The finished product should be viscous and pourable. The product is pumped into containers and distributed.

Factors affecting diacetyl formation in cultured buttermilk

Even if citrate-fermenting strains are included in the starter culture, diacetyl formation does not always occur in amounts necessary to impart the desired flavor. Several reasons may account for reduced citrate fermentation and diacetyl synthesis. First, there may simply not be enough citrate in the milk. Although milk contains, on average, about 0.15% citrate, this amount varies, depending largely on the diet of the cow. Therefore, sodium citrate is frequently added to the milk to provide a consistent source of substrate. If the temperature during incubation is too high (>24°C), growth of the homofermentative lactococci will be favored, too much acid will be produced, and the citrate-fermentors may be inhibited. Not only will the product have a "lacks flavor" defect, but it will instead have a harsh acid flavor.

In contrast, if the incubation temperature is too low (below 20°C), insufficient acid may be produced and the flavor will be flat or "lacks acid." Acid production is also necessary for diacetyl formation, since the citrate transport system is not activated unless the pH is below 5.5. In fact, maximum diacetyl synthesis occurs between pH 5.0 and 5.5. Finally, once diacetyl is made, low pH inhibits the reduction reactions that convert diacetyl to acetoin and 2,3-butanediol.

Another factor that is critical for synthesis of diacetyl is oxygen, which can stimulate diacetyl formation by as much as 30-fold. Several mechanisms are responsible for the oxygen effect. As noted above, pyruvate, the metabolic precursor of diacetyl, can serve as the substrate for several alternative enzyme reactions, including lactate dehydrogenase. In the presence of high atmospheric oxygen, lactate dehydrogenase activity is reduced, and the oxidative decarboxylation reaction responsible for diacetyl synthesis is enhanced. In addition, by oxidizing NADH, oxygen also slows the rate at which diacetyl is reduced to acetoin or 2,3-butanediol. For these reasons, it may be useful to stir air into the final product during the agitation step. In fact, maximum diacetyl production occurs about 72 hours after manufacture, so a fresh product may not have as good a flavor as one that is slightly "aged".

Defects

The flavor and texture defects that occur in cultured buttermilk are, in general, similar to those in yogurt. The most common flavor defect is "lacks flavor", caused by insufficient diacetyl formation due to reasons outlined above. Excess acidity is also objectionable to consumers. Texture defects include wheying off, too thin, or too viscous. The latter may be

caused by body-forming bacteria that over-produce EPS. Milk-borne defects, such as yeasty flavor, unclean, and rancidity, are caused by poor quality milk, especially milk that had been contaminated with psychrotrophic microorganisms.

SOUR CREAM

Despite the apparent differences in the appearance and texture, sour cream is actually quite similar to cultured buttermilk in several respects (Figure 5.7). The sour cream culture, for example, is the same as that used for buttermilk. The incubation conditions and the flavor compounds produced by the culture are also similar for both products. There are, however, several notable differences. For sour cream manufacture, cream (containing varying levels of milk fat) is used instead of low-fat or skim milk. The cream is pasteurized, but not quite at the severe conditions used for buttermilk or yogurt. This is because for sour cream, denaturation of protein is not as crucial, since the milk fat will impart the desired creaminess, thickness, and body. The cream is homogenized, which also promotes a desirable heavy body.

Sour cream manufacture

In the United States the starting material for sour cream manufacture is cream at 18% to 20% milk fat. Lower-fat versions, such as sour half-and-half (10% milk fat), are produced in some regions, as are higher-fat products (containing as much as 50% milk fat). The latter are generally produced as an ingredient for dips and other sour cream products. There is no need to add additional nonfat dry milk, as for yogurt and buttermilk, since achieving a firm gel and

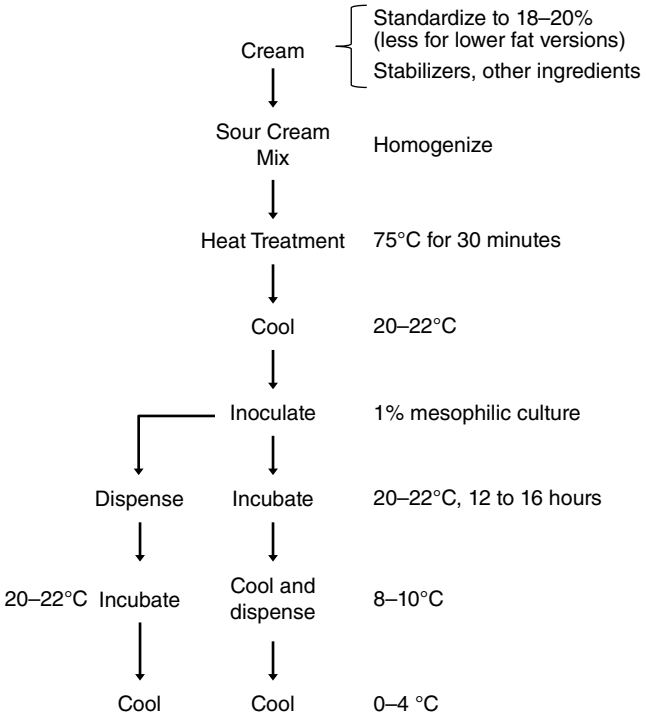


Figure 5.7 Manufacture of sour cream.

thick body is not an issue for these high-solids products. For the same reason, the cream is pasteurized at more typical time and temperature conditions (85°C for 25 seconds). However, the cream must be homogenized (usually twice) to produce a smooth-textured product with good viscosity. After the mix is cooled, the sour cream culture is added. The culture, containing mesophilic acid-producing, flavor-producing, and body-forming strains, is often the same as that is used for cultured buttermilk. The mix is then either filled into cups and incubated or incubated directly in vats (analogous to the two styles of yogurt). Incubation is at 20°C to 25°C for 10 to 16 hours. When the pH reaches about 4.4 to 4.6 (about 0.7% to 0.9% lactic acid), the sour cream is cooled, either by moving the cup-fermented product into coolers or, in the case of vat-fermented product, by stirring the product in jacket-cooled vats. The product is then pumped into containers. Sour cream should have a similar flavor profile as cultured buttermilk, with lactic acid and diacetyl predominating. Body characteristics are especially important, and various gums and other stabilizing agents are frequently added to the mix. Some manufacturers even add a small amount of chymosin to provide additional firmness. When defects do occur, they are often due to poor quality ingredients and post-pasteurization contamination by acid-tolerant yeasts and molds and psychrotrophic bacteria.

KEFIR

Kefir is a fermented dairy product that is described in many dairy and fermented foods reference texts. In addition, it has long been of academic interest to microbiologists interested in the unique microecology that exists in the kefir grains that are used for traditional kefir manufacture. Despite this interest, however, only in the past several years has kefir reached mainstream markets. Several brands are now available in North America, and it has become one of the more popular products in the cultured milk category. Despite its new-found fame in the West, kefir has a long history of consumption throughout a large part of the world. In the Middle East, Eastern Europe, and Central Asia, especially Turkey, Russia, Ukraine, Poland, and the Czech Republic, kefir is one of the most widely consumed cultured dairy products. It is interesting that yogurt manufacturers have begun to introduce fluid or pourable yogurt-like products that are only slightly different from traditional kefir.

Kefir originated in the Caucasus Mountain in Russia. The traditional kefir manufacturing process, which is still widely practiced, relies on a mixed assortment of bacteria and yeast to initiate the fermentation (Figure 5.8). The kefir fermentation is unique among all other dairy fermentations in that the culture organisms are added to the milk in the form of insoluble particles called kefir grains (Box 5.4). Moreover, once the fermentation is complete, the kefir grains can be retrieved from the fermented milk by filtration and reused again and again.

The flavor of plain kefir is primarily due to lactic and acetic acids, diacetyl, and acetaldehyde, produced by homofermentative and heterofermentative lactic acid bacteria. However, because kefir grains also contain yeast, in addition to lactic acid bacteria, other end-products are formed that make the finished product quite different from other cultured dairy products. This is because ethanol is produced when the yeasts ferment lactose, such that kefir can contain as much as 2% ethanol (accounting, perhaps, for its appeal). In the United States, this much ethanol would trigger action from the regulatory agencies (i.e., Alcohol and Tobacco Tax and Trade Bureau). Thus, if yeasts are present in the culture (most kefir products made in the United States claim yeasts on their labels), they must be low- or non-ethanol producers. Kefir grains can also contain acetic acid-producing bacteria, such as *Acetobacter aceti.*

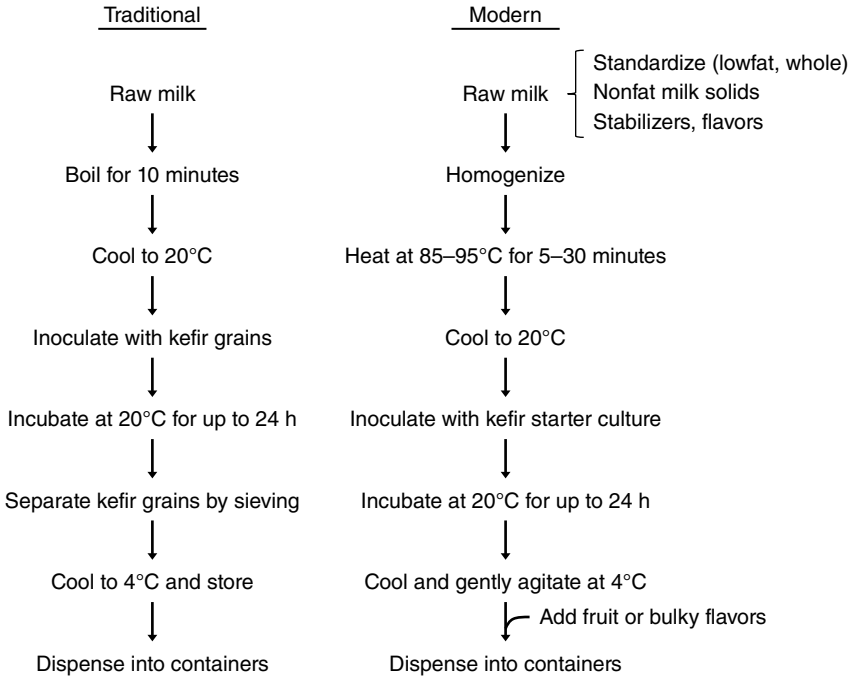


Figure 5.8 Traditional and modern manufacturing process for kefir.

Box 5.4 The microbial diversity of kefir grains

Throughout much of Europe and the Middle East, kefir has long been one of the most popular fermented dairy products. In Russia, for example, per capita kefir consumption is more than 7 kg per person, nearly double that of yogurt (Ptukha and Merzlyakova, 2017). The suggested health benefits, as well as the sensory properties of kefir have certainly contributed to its popularity (Bourrie et al., 2016). Microbiologists have their own reasons for devoting so much attention to kefir. In particular, kefir, and kefir grains specifically, represent not only an amazingly diverse ecosystem, they also provide an excellent opportunity for applying modern molecular techniques to characterize kefir micro-ecology.

A single kefir grain harbors more than 10^6 cells (or about 10^{10} per gram). A diverse range of both bacteria (Gram negative and Gram positive) and yeast and other fungi are present (Table 5.4.1). However, although several genera and species are common to kefir grains, there are strains unique to the geographical or climatic regions from which the grains had originated (Marsh et al., 2013). The physical intricacies of the grains, with different interior and exterior environments, confer additional ecological complexity. On a practical basis, kefir grains can be viewed as a natural immobilized cell bioreactor, with each grain serving as an inert support material for the cells. Remarkably, efforts to form or reconstitute kefir grains, *de novo* (i.e., from free-living cells), has not been reported (Wang et al., 2012). Thus, only grains can beget grains!

Table 5.4.1 Bacterial, yeast, and fungal species associated with kefir grains.

Lactobacillus			
L. kefir	*L. kefiranofaciens*	*L. delbrueckii*	*L. helveticus*
L. casei	*L. kefiri*	*L. brevis*	*L. paracasei*
L. parakefir	*L. plantarum*	*L. satsumensis*	*L. curvatis*
L. fermentum	*L. viridescens*	*L. acidophilus*	*L. gasseri*
L. kefirgranum	*L. parakefiri*	*L. parabuchneri*	*L. garvieae*
L. buchneri	*L. sunkii*	*L. crispatus*	*L. otakiensis*
L. instestinalis	*L. amylovorus*	*L. pentosus*	*L. salivarius*
L. johnsonii	*L. rhamnosus*	*L. rossiae*	*L. sakei*
L. reuteri	*L. kalixensis*	*L. rapi*	*L. diolivorans*
L. parafarraginis	*L. gallinarum*		
Pediococcus			
P. claussenii, P. damnosus, P. halophilus, P. pentosaceus, P. lolii			
Lactococcus			
La. lactis subsp. *lactis, La. lactis* subsp. *cremoris, La. garvieae, La. lactis* subsp. *lactis* biovar *diacetylactis*			
Streptococcous			
S. thermophilus			
Leuconostoc/Oenococcus			
Leuc. mesenteroides subsp. *mesenteroides, Leuc. mesenteroides* subsp. *cremoris, Leuc. pseudomesenteroides, Oenococcus oeni*			
Acetobacter			
A. sicerae, A. orientalis, A. lovaniensis			
Bifidobacterium			
B. breve, B. choerinum, B. longum, B. pseudolongum			
Yeast and fungal species			
Candida kefyr, C. holmii, C. friedrichii, C. inconspicua, C. maris, C. sake			
Saccharomyces cerevisiae, S. unisporus, S. turicensis, S. cariocanus, S. humaticus			
Kluyveromyces lactis, Kl. marxianus, Kl. marxianus var. *lactis*			
Kazachstania servazzii, Ka. solicola, Ka. aerobia, Ka. exigua			
Pichia fermentans, Issatchenkia orientalis, Dekkera anomala, Geotricum candidum			
Yarrowia lipolytica, Torulaspora delbrueckii			

Adapted from Bourrie et al., 2016.

Once the grains are dispersed into milk, some of the cells contained within the grains are released, whereas other remain attached to the grain. Attachment is mediated via one of several different types of exopolysaccharides (EPS) that are produced by the resident organisms. Kefiran, a water-soluble heteropolysaccharide, is probably the best studied (Gradova et al., 2015; Hamet et al., 2015). It consists of equimolar glucose and galactose and is produced by *Lactobacillus kefiranofaciens* (Hamet et al., 2013). As noted above, kefir organisms are not randomly distributed. Rather, some are contained mainly within the core of the grains, whereas others are located primarily in

the exterior. Given the microbial diversity and stability of kefir grains, symbiotic and synergistic relationships likely exist between different organisms, but these relationships have been difficult to establish.

Although the chemical composition of kefir grains varies depending on the source, they generally contain about 80% to 90% water, 2% to 5% protein, and 8% to 10% carbohydrate (Abraham and De Antoni, 1999). Most of the protein faction is in the form of casein, whereas most of the carbohydrate portion is polysaccharide. The irregular, cauliflower-shaped grains range in size from 3 mm to more than 3 cm in diameter. In their dry form, they are stable for many months. Although kefir grains are available commercially, many modern manufacturers now use pure lyophylized cultures containing many of the strains ordinarily found in the grains.

Until recently, the microbial composition of kefir grains relied on culture-based methods. However, high-throughput community sequencing is now being used to identify kefir organisms and to estimate their relative abundancies. Initially, these culture-independent methods were based on rRNA, and more recently, whole genome metagenomics approaches have been used (Marsh et al., 2013; Nalbantoglu et al., 2014).

In general, the kefir grain microbiota consists of genera from two main bacterial phyla, Firmicutes and Proteobacteria (Figure 5.4.1, Table 5.4.1). The predominant organisms in kefir grains are lactic acid bacteria, with homo- and heterofermentative species of *Lactobacillus* accounting for more than 80% of the total (Korsak et al., 2015; Marsh et al., 2013). Among the main species are *Lactobacillus kefiranofaciens,*

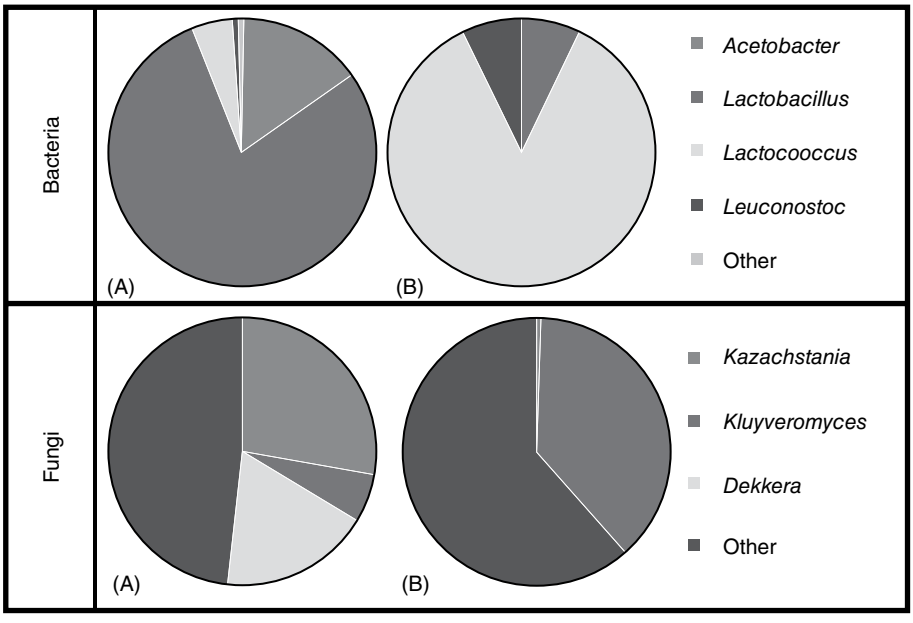


Figure 5.4.1 Microbiota changes (bacteria and fungi) in kefir, from grain (A) to fermented milk (B). From Bourrie et al., 2016.

Lactobacillus kefiri, Lactobacillus parakefir, Lactobacillus plantarum and Lactobacillus delbrueckii. Other dairy lactobacilli may also be present.

As noted above, cells also become detached from the kefir grains during the fermentation. Within the free-living kefir population are *Lactococcus lactis*, *Leuconostoc mesenteroides*, and *Streptococcus thermophilus*, in addition to the lactobacilli. This is consistent with the observation that lactobacilli are located in the interior of the grain and the other lactic acid bacteria associated with the exterior (Wang et al., 2012). Although *Acetobacter* and other acetic acid-producing bacteria (in the phylum, Proteobacteria) are less abundant, they are also consistently present in both kefir grains and liquid kefir.

Yeasts are integral to the kefir fermentation, and several species are well-represented in kefir and kefir grains. Based on recent sequenced-based analyses, the main genera include *Naumovozyma*, *Kluyveromyces*, and *Kazachastania* (Korsak et al., 2015; Marsh et al., 2013). These yeast ferment lactose via glycolysis and produce ethanol and CO_2 as end products. Thus, kefir can accumulate as much as 0.5% or more ethanol, which is sufficient to make kefir, in the view of government officials and (tax collectors) an alcoholic product. Therefore, the fermentation must be suitably controlled or non-ethanol- producing yeasts must be used to limit ethanol formation.

References

Abraham, A.G., and G.L. De Antoni. 1999. Characterization of milky kefir grains grown in milk and in soy milk. J. Dairy Res. 66:327–333.

Bourrie, B.C., B.P. Willing, and P.D. Cotter. 2016. The microbiota and health promoting characteristics of the fermented beverage kefir. Front. Microbiol. 7:647.

Gradova, N.B., A.A. Khokhlacheva, E.D. Murzina, and V.V. Myasoyedova. 2015. Microbial components of kefir grains as exopolysaccharide kefiran producers. Appl. Biochem. Microbiol. 51:873–880.

Hamet, M.F., J.A. Piermaria, and A.G. Abraham. 2015. Selection of EPS-producing *Lactobacillus* strains isolated from kefir grains and rheological characterization of the fermented milks. LWT-Food Sci. Technol. 63:129–135.

Hamet, M.F., A. Londero, M. Medrano, E. Vercammen, K. Van Hoorde, G.L. Garrote, G. Huys, P. Vandamme, and A.G. Abraham. 2013. Application of culture-dependent and culture-independent methods for the identification of *Lactobacillus kefiranofaciens* in microbial consortia present in kefir grains. Food Microbiol. 36:327–334.

Korsak, N., B. Taminiau, M. Leclercq, C. Nezer, S. Crevecoeur, C. Ferauche, E. Detry, V. Delcenserie, and G. Daube. 2015. Short communication: evaluation of the microbiota of kefir samples using metagenetic analysis targeting the 16S and 26S ribosomal DNA fragments. J. Dairy Sci. 98:3684–3689.

Marsh, A.J., O. O'Sullivan, C. Hill, R.P. Ross, and P.D. Cotter. 2013. Sequencing-based analysis of the bacterial and fungal composition of kefir grains and milks from multiple sources. PloS One, 8:e69371.

Nalbantoglu, U., A. Cakar, H. Dogan, A. Neslihan, D. Ustek, K. Sayood, and H. Can. 2014. Metagenomic analysis of the microbial community in kefir grains. Food Microbiol. 41:42–51.

Ptukha, A. and T. Merzlyakova. 2017. Review of the Russian Market of Fermented Milk Products. Russian Food and Drinks Market Magazine. (No. 6).

Wang, S.-Y. K.-N. Chen, Y.-M. Lo, M.-L. Chiang, H.-C. Chen, J.-R. Liu, and M.-J. Chen. 2012. Investigation of microorganisms involved in biosynthesis of the kefir grain. Food Microbiol. 32:274–285.

Kefir manufacture

In the United States, kefir is usually made with low-fat or whole milk (Figure 5.8). The milk is pasteurized at 85°C to 90°C for up to 30 minutes (like yogurt), then cooled to 20°C to 22°C. Kefir grains are generally not used in the United States. Instead, the milk is inoculated with commercially-available kefir starter cultures containing assorted lactic acid bacteria, including *Lactobacillus causasicus* and *Lactobacillus kefir*. The mixture is incubated for 16 to 24 hours or until a pH of 4.2 to 4.6 is reached. The coagulum is then gently stirred, flavoring ingredients (usually fruit) are added, and the product is then dispensed. Kefir should contain 0.8% to 1.0% lactic acid along with other heterofermentative end-products (acetic acid, ethanol, and CO_2). Acetaldehyde and diacetyl are also usually formed. The kefir will have a tart flavor and a smooth, viscous body. Production of CO_2 by the starter culture also provides effervescence and mouth feel.

OTHER CULTURED DAIRY PRODUCTS

The emphasis of this chapter has been on those products produced and consumed in the United States. However, there are hundreds of other products produced around the world that, although manufactured via similar processes, have unique and interesting features (Table 5.2).

Crème fraiche is a French or European style of sour cream, made with similar cultures and having similar flavor and aroma properties. It is made, however, with cream containing about 40% milk fat (in contrast to US sour cream at 18–20% fat), and, therefore, has a very rich body and smooth mouthfeel. It may also be less acidic, with a final pH of about 6.0 (although lower pHs are possible). The formation of diacetyl and other flavor compounds may be responsible for the somewhat less sour flavor.

Another popular cultured dairy product is villi. It is widely consumed in Finland and similar products are also produced in other Scandinavian countries. Villi is known for its high viscosity and musty flavor and aroma. The ropy texture is due to capsular EPS production by

Table 5.2 Cultured dairy products from around the world.

Product	Origin	Culture organisms	Unique features
Villi	Finland	*Lactococcus lactis* *Leuconostoc* spp. *Geotrichum candidum*	Ropy texture Musty flavor
Skyr	Iceland	*Lactobacillus delbrueckii subsp. bulgaricus* *Streptococcus thermophilus*	Concentrated, high protein content
Dahi	India	*Lactobacillus delbrueckii* subsp. *bulgaricus* *Streptococcus thermophilus* *Leuconostoc* spp.	Yogurt-like
Koumiss	Russia	*Lactobacillus delbrueckii* subsp. *bulgaricus* *Lactobacillus acidophilus* *Kluyveromyces* spp.	Mare's milk >1% ethanol
Bulgarian milk	Bulgaria	*Lactobacillus delbrueckii* subsp. *bulgaricus*	High acid (>2% lactic acid)

L. lactis subsp. *lactis* and *L. lactis* subsp. *cremoris*, and the musty flavor is caused by growth of the fungal organism *Geotrichum candidum*.

Koumiss, a cultured dairy product from Eastern Europe, was traditionally produced from mare's milk. It is now available in a cow's milk version. Koumiss is similar to kefir in that lactic acid and ethanol are both present, although liquid cultures, rather than grains, are used as the inoculum. As for many of these products, koumiss owes much of its popularity to its putative therapeutic properties.

Finally, the yogurt-like products dahi and laban are among the most widely-consumed cultured dairy products in India and the Middle East, respectively. Ordinarily, dahi and laban cultures will include mesophilic lactococci and lactobacilli, along with thermophilic *S. thermophilus* and *L. bulgaricus*. Incubation temperatures are usually longer (up to 15 h) at slightly lower temperatures (37–42°C). For dahi, sweeteners and flavoring agents are commonly added.

BIBLIOGRAPHY

Chaves, A.C.S.D., M. Fernandez, A.L.S. Lerayer, I. Mierau, M. Kleerebezem, and J. Hugenholtz. 2002. Metabolic engineering of acetaldehyde production by *Streptococcus thermophilus*. Appl. Environ. Microbiol. 68:5656–5662.

Fox, P.F., T. Uniacke-Lowe, P.L.H. McSweeney, and J.A. O'Mahony. 2015. Chemistry and biochemistry of fermented milk products. In: Dairy Chemistry and Biochemistry, 2nd edn, Springer, New York, pp. 547–567.

German, J.B. 2014. The future of yogurt: scientific and regulatory needs. Am. J. Clin. Nutr. 99 (Suppl):1271S–1278S.

Goh, Y.J. C. Goin, S. O'Flaherty, E. Altermann, and R. Hutkins. 2011. Specialized adaptation of a lactic acid bacterium to the milk environment: the comparative genomics of *Streptococcus thermophilus* LMD-9. Microb. Cell Fact. 10 (Suppl. 1)S22.

Liu, M., R.J. Siezen, and A. Nauta. 2009. In silico prediction of horizontal gene transfer events in *Lactobacillus bulgaricus* and *Streptococcus thermophilus* reveals protocooperation in yogurt manufacturing. Appl. Environ. Microbiol. 75:4120–4129.

Meunier-Goddik. L. 2012. Sour cream and crème fraiche. In Hui, Y.H., ed. Handbook of Animal-Based Fermented Food and Beverage Technology. CRC Press, Boca Raton, FL, pp. 235–246.

Routray, W., and H.N. Mishra. 2011. Scientific and technical aspects of yogurt aroma and taste: a review. Comp. Rev. Food Sci. Food Saf. 10:208–220.

Shah, N.P. 2017. Yogurt in Health and Disease Prevention. Academic Press, Cambridge, MA.

Shiby, V.K. and H.N. Mishra. 2013. Fermented milks and milk products as functional foods – a review. Crit. Rev. Food Sci. Nutr. 53:482–496.

Smid, E.J. and M. Kleerebezem. 2014. Production of aroma compounds in lactic fermentations. Annu. Rev. Food Sci. Technol. 5:313–326.

Tamime, A.Y., M. Hickey, and D.D. Muir. 2014. Strained fermented milks – A review of existing legislative provisions, survey of nutritional labelling of commercial products in selected markets and terminology of products in some selected countries. Int. J. Dairy Technol. 67:305–333.

Wu, Q., C. Cheung, and N. Shah. 2015. Towards galactose accumulation in dairy foods fermented by conventional starter cultures: Challenges and strategies. Trends Food Sci. Technol. 41:24–36.

Yildiz, F. 2010. Development and Manufacture of Yogurt and Other Functional Dairy Products. CRC Press, New York, NY.

6 Cheese

> We are still, however, far from having arrived at a complete elucidation of all the questions involved. It is particularly difficult to understand how various sorts of hard cheese, apparently containing the same microflora, should each have its own characteristic taste and smell. There can hardly be any doubt that these sorts of cheese in reality contain different species of bacteria, only we are unable to distinguish them by the methods hitherto available.
>
> From *The Lactic Acid Bacteria* by Orla-Jensen, published 100 years ago in 1918.

> Blessed are the cheese makers.
>
> From the 1979 film, *Monty Python's Life of Brian*

INTRODUCTION

Perhaps no other fermented food starts out with such simple raw materials and then ends up with products having such an incredible diversity of color, flavor, texture, and appearance as does the making of cheese. It is even more remarkable that milk, pale in color and bland in flavor, can be transformed into literally hundreds of different types of flavorful, colorful cheeses by manipulating just a few critical steps. How so many cheeses evolved from this simple process undoubtedly involved part trial and error, part luck, and plenty of art and skill. Moreover, despite empirical knowledge about the process, until very recently, cheese makers had little or no actual awareness of the science involved in cheese manufacture. The role of microorganisms, in particular, was only recognized at the beginning of the twentieth century. Now, however, it is likely that few fermented foods require such a combination of science, technology, and craftsmanship as does the making of cheese.

CHEESE PRODUCTION AND CONSUMPTION

On a volume basis, the cheese industry is one of the largest of all those involved in fermented foods manufacture. Of the 95 billion kg (209 billion pounds) of milk produced in the United States in 2015, half was used in the manufacture of 5.4 billion kg (11.8 billion pounds) of cheese. About a fourth of that cheese was used to make various types of processed cheese (discussed later).

Microbiology and Technology of Fermented Foods, Second Edition. Robert W. Hutkins.

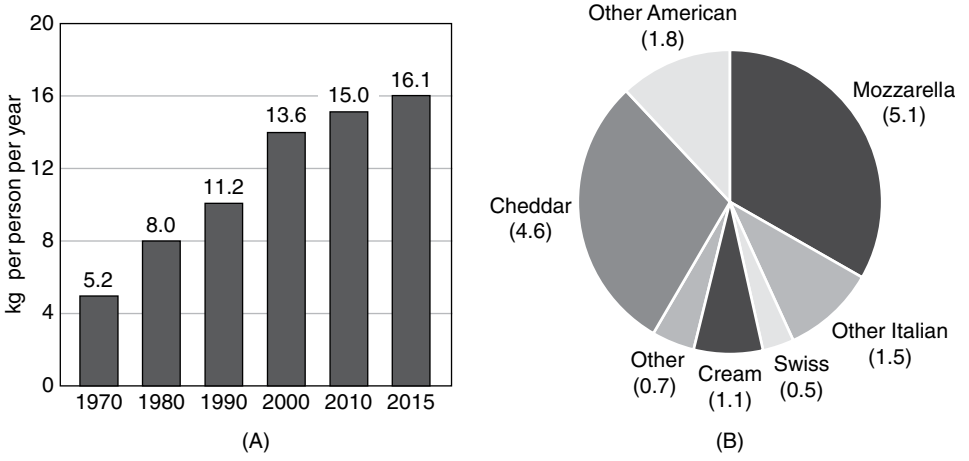


Figure 6.1 Cheese consumption in the US since 1970 (A) and by type of cheese (in kg) for 2015 (B). *Source:* USDA.

On a per capita basis, cheese consumption in the United States has increased dramatically in the past 35 years from 5 kg in 1970 to more than 16 kg (35 pounds) per person per year in 2015 (Figure 6.1A). The most popular cheeses have been the American style (e.g., Cheddar, Colby) and Italian style (e.g., Mozzarella, Parmesan) cheeses, accounting for 40% and 41%, respectively, of all cheeses consumed in the United States (Figure 6.1B). In addition, another 0.75 kg of imported cheese is consumed per person per year. Worldwide, France and Finland (27 kg per person per year) are the leading consumers of cheese, with Denmark (26 kg), and other European countries not too far behind (Figure 6.2).

On the production side, American and Italian-type cheeses are, by far, the main cheeses produced in the United States. For as long as production data had been available, the Cheddar- or American-type cheeses have always accounted for most of the cheese produced in the US. In the 1970s, two-thirds of all cheese produced in the United States belonged to this group (Cheddar, Colby, stirred curd). However, over the past 40 years, the Italian-type cheeses, and Mozzarella, in particular, have gained in popularity. These cheeses now exceed that of American-type cheese (Figure 6.3).

The US cheese industry began in the mid-1800s, with factories opening first in New York (1851) and Wisconsin (1868). Prior to that time, cheese was mainly produced directly on farms and sold locally. By the late 1800s, about 4000 cheese factories accounted for the nearly 100 million kg (217 million pounds) of cheese. Most cheese factories were located in the dairy-producing states of Wisconsin, Minnesota, Pennsylvania, and New York. In the past 30 years, California has emerged as the leading producer of milk, followed by Wisconsin and Idaho. These three states are also the leading manufacturers of cheese (but with Wisconsin first and California second), producing more than half of all cheese made in the US. However, like other segments of the food industry, more and more product is made by fewer and fewer plants. In 2015, there were half as many production facilities making American and Italian style cheese as there were in 1970, while, at the same time, production capacity has increased by 500%.

Obviously, France, Germany, Italy, the United Kingdom, and other European countries have been major producers of cheese for many centuries. Half of all cheese is produced in

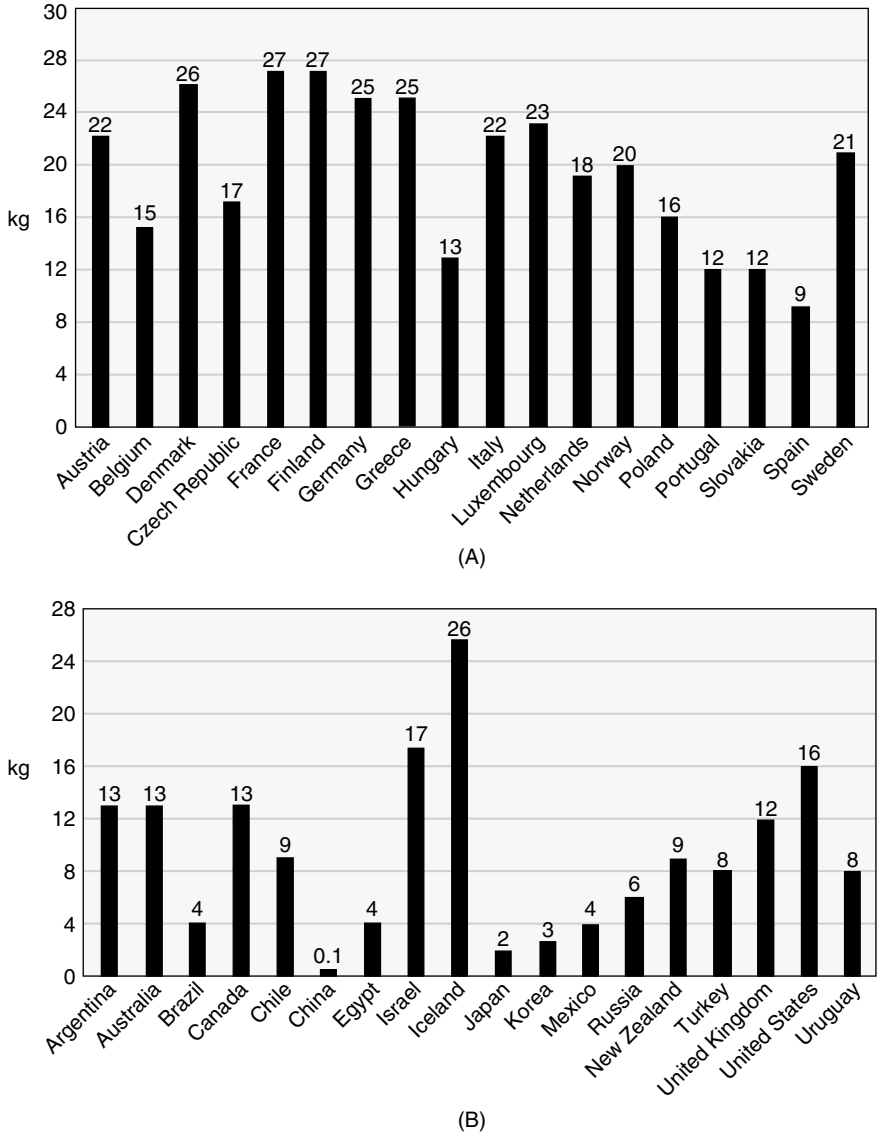


Figure 6.2 Cheese consumption for 2015 (per person per year) for Europe (Al) and all other regions (B). *Source:* International Dairy Foods Association and Canada Dairy Information Centre.

Europe. Canada, Australia, New Zealand, Brazil and Argentina have also become major producers.

The basic cheese manufacturing principles will be outlined in this chapter, emphasizing the key variables at each step that account for the myriad types of cheeses that exist throughout the world. Manufacturing details for specific cheeses representing the main cheese groups or families will then be described. Along the way, an understanding of dairy chemistry and the milk milieu and the role of the starter culture will be examined. Finally, how flavor and texture development occurs during cheese ripening, current problems and challenges faced by the cheese industry, and other issues will be addressed.

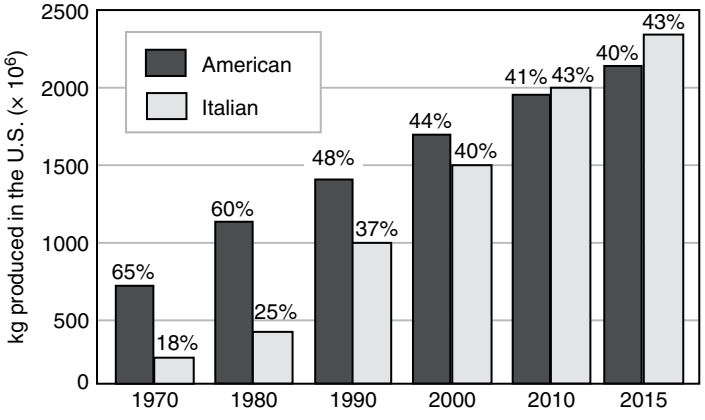


Figure 6.3 Production of American and Italian type cheeses in kg and as a percent of total cheese produced from 1970 through 2015. *Source:* USDA.

MANUFACTURING PRINCIPLES

Like so many fermented foods, the first cheese made by human beings was almost certainly a result of an accident. Some wandering nomad, as the legend goes, filled up a pouch made from the stomach of a calf or cow with a liter or two of fresh milk. After a few hours, the milk somehow had turned into a semi-solid like material. When the would-be cheese maker gave the container a bit of a shake, a watery-like fluid quickly separated from the creamy white curd. When tasted, this curd would have had a pleasant, moderately acidic flavor as well as a smooth texture. It would also have been observed that the curds had a longer shelf-life than the fresh milk from which it was made. Archeological evidence suggests that humans of the Neolithic Period (about 5000 BCE) had devised pottery and other vessels for making cheese. Remarkably, despite the rather crude production scheme, the product made those thousands of years ago was not much different than many of the cheeses currently produced and consumed even today.

Just what happened to cause the milk to become transformed into a product with such a decidedly different appearance, texture, and flavor? To answer that question, it is first necessary to compare the composition of the starting material, milk, to that of the product, the finished cheese (Figure 6.4). Cow's milk consists of, in descending order (and in general concentrations), water (87%), lactose (5%), fat (3.5%–4%), protein (3.2%–3.4%), and minerals (<1%), mainly calcium. The pH is about 6.6–6.8. In contrast, a typical cheese, such as Cheddar cheese, contains 36%–39% water, 30%–32% fat, 26%–28% protein, 2%–2.5% salt, 1% mineral (mostly calcium), and <1% lactose. The pH would be about 5.2.

Several major differences are evident. Cheese contains less water and more milk solids, in the form of fat and protein, than the milk from which it was made. Thus, cheese making can simply be viewed as a concentration process, in which the water portion, or whey, is removed and the solids are concentrated, in most cases by more than four-fold. In fact, as we shall see later, most of the steps involved in cheese making are performed for the singular purpose of removing water. Doing so not only concentrates and solidifies the solid matter, but also decreases microbial and enzymatic activities such that cheese is much better preserved than milk. As noted in the previous chapter, buttermilk, yogurt, sour cream, and other cultured dairy products are different from cheese in several respects, but chief among these differences is that these products do not involve a water removal step and they are almost always more acidic.

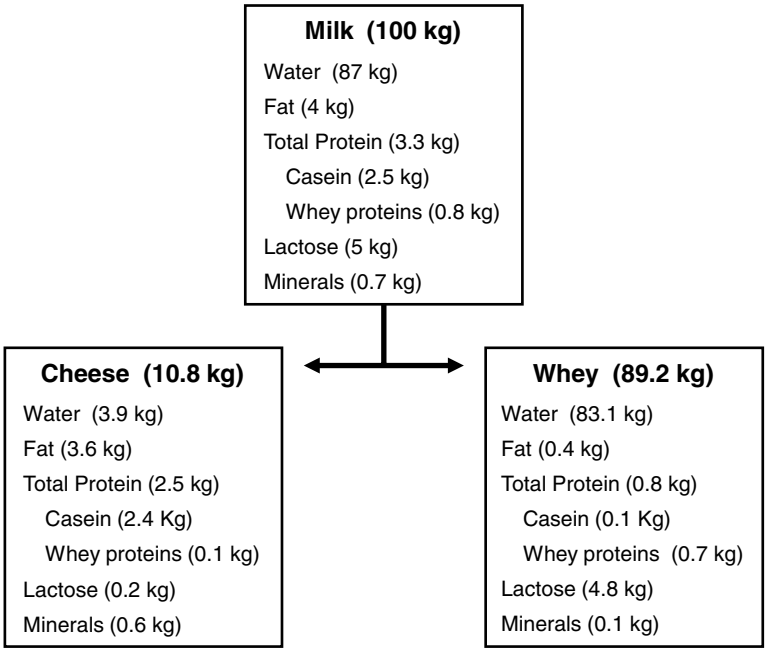


Figure 6.4 Partition of milk into cheese and whey.

Converting a liquid into a solid

It is fair to say that the means by which milk and curds are manipulated to concentrate milks solids accounts, in large part, for the hundreds of varieties of cheese produced around the world. Water removal and the concentration of protein and fat occur via a combination of biochemical, biological, and physical-chemical events. Many of these events happen at nearly the same time and often have complex effects on one another. For example, exposure of cheese curds to both high temperature and low pH enhances removal of water from the curd (a phenomenon known as syneresis). But if the curd-cooking temperature is too high, the microorganisms that produce acid and that lower the pH will be inactivated, resulting in poor syneresis (and poor quality cheese). If, on the other hand, the curd-cooking temperature is at the culture optimum, then too much acid may be produced, and the pH will be too low. This may result in significant mineral loss (specifically calcium), giving the cheese a "short" or crumbly texture. While this may or may not be a good thing, depending on the cheese characteristics one intends to achieve, it is critical to plan accordingly.

To further complicate the situation, removing water from the curd also removes the solutes dissolved in the curd. In particular, the amount of lactose in the curd, at least in the early steps of cheese manufacture, is proportionate with the moisture content. As the curds become dryer by cooking, less lactose remains. Ultimately, less substrate will be available later on for the culture to ferment, and less lactic acid will be formed. The point is that each process step has consequences (hopefully, intended) not only on other cheese making activities, but also on the overall properties and quality of the finished cheese.

The actual conversion of liquid milk into a solid mass of cheese can be accomplished in three general ways, as described below. In all cases, this step involves coagulation (or precipitation) of milk protein. Milk, as noted above, contains about 3.3% protein. Of the protein

fraction, about 80% is casein (or 2.5% of the milk). The remaining proteins, about 20% of the total or 0.7–0.8% of the milk, are known collectively as whey proteins. For most cheeses (with a few important exceptions), the protein portion consists almost entirely of casein. When milk coagulates and the coagulated material is separated, the soluble whey proteins are released into the water or whey fraction. The casein matrix not only contains some water (and whatever solutes are dissolved or suspended in the water phase), but also a large portion of the lipid fraction that was originally present in the milk, depending on how coagulation occurs.

There are three ways the initial coagulation step is accomplished. First, milk can be coagulated by acids produced by lactic acid bacteria, based on the same principle used for yogurt and other cultured dairy products (Chapter 4). When the milk pH reaches 4.6, casein is at its isoelectric point and its minimum solubility, and therefore it precipitates. However, unlike the process used for cultured products, the coagulum or gel is not left intact. Instead, the coagulated material is gently cut into die-sized curds. These curds are slowly heated and stirred and the whey is separated and drained. At the end, these acid-precipitated curds are comprised almost entirely of casein and water. In fact, pure casein and caseinate salts (made by neutralizing acid casein), both of which are of considerable commercial importance, are made via the acid precipitation method. In cheese making, the curds are then further processed, resulting in products such as cottage cheese and farmers' cheese. It is important to realize that casein coagulates at pH 4.6 whether acidification occurs via fermentation-generated acids or simply by addition of food-grade acids direct into the milk. In fact, the latter process is preferred by some producers of cottage, cream, and other acid-coagulated cheeses, due in part to the ease of manufacture and the elimination of starter cultures as an ingredient. However, because theses products are not fermented (and, therefore, they must be labeled as acid-set), their manufacture will not be considered further in this text.

The second and most common way to effect coagulation is by the addition of the enzyme chymosin. This enzyme hydrolyzes a specific peptide bond located between amino acid residues 105 (a phenylalanine) and 106 (a methionine) in κ-casein. The hydrolysis of this bond is sufficient to cause a part of κ-casein (the glycomacropeptide fraction) to dissociate from the casein micelle, exposing the anionic phosphates of β-casein. Thus, the remaining casein micelle becomes sensitive to calcium-mediated precipitation. In contrast to acid-precipitated casein, the coagulated casein network formed by chymosin treatment traps nearly all of the milkfat within the curd. Most of the cheeses manufactured around the world rely on chymosin coagulation. It is worth emphasizing that even though chymosin, alone, is sufficient to coagulate milk, lactic starter cultures are absolutely essential for successful manufacture of most hard cheeses. The lactic acid bacteria not only produce acid and reduce the pH, they also contribute to the relevant flavor, texture, and rheological properties of cheese, as described later.

Until relatively recently, most chymosin was obtained from its natural source, the stomachs of suckling calves after slaughter, via a salt extraction process. The relatively high cost of this enzyme along with increasing demand and sporadic supply problems have long driven cheese ingredient suppliers to develop less expensive, alternative enzyme products that could perform the same function as chymosin. A genetically engineered form of chymosin was approved by the FDA in 1990, and several such products were later approved that have since captured much of the American chymosin market (Box 6.1).

Finally, the third way to initiate coagulation is via a combination of moderate acid addition (pH 6.0), plus high heat (>85°C). Under these conditions, the whey proteins are denatured, thus the precipitate that form consists not only of casein, but also whey proteins.

Box 6.1 Making calf chymosin (sans the calf)

Chymosin, the enzyme that causes milk to coagulate, is an essential ingredient in the cheese making process. Until the 1990s, calf chymosin (the major source) had been the most expensive ingredient (other than milk) used in cheese manufacture, adding about $0.03–0.05 to each kg of cheese. This was because the chymosin supply depended on veal production, which was subject to considerable market variations, as well as production and purification costs. As the cheese industry grew worldwide, but especially in the United States in the 1960s, 1970s, and 1980s, the substantial increase in demand for calf chymosin created supply problems and led to even higher prices (Jacob et al., 2011). Thus, cheese makers sought other, less expensive sources of coagulant.

Among the early non-calf, animal rennet substitutes were bovine and porcine pepsin. These enzymes coagulate milk; however, they are somewhat less specific and generally produce cheese of lower quality. In the 1960s, fungal enzymes (sometimes referred to as microbial rennets) derived from *Mucor miehei* and *Endothia parasitica* (later reclassified as *Rhizomucor miehei* and *Cryphonectria parasitica*, respectively) were isolated and commercialized. Although considerably less expensive than calf chymosin, these enzymes were far from perfect. They had more non-specific casein hydrolysis activity, resulting in yield loss and flavor and texture defects. Some were heat stable and residual activity could be detected in the whey, limiting the application of whey as an ingredient in other products. Still, price and other considerations (e.g., kosher and vegetarian status) led these products to gain a substantial portion of the coagulant market.

The search for enzymes with properties more like calf chymosin shifted to an entirely new direction in the mid- to late 1980s when recombinant DNA technology was developed. During this time, several new biotechnology companies began projects to identify the gene coding for chymosin in calf abomasum mucosal cells and expressing the gene in suitable host cells. Although this genetic manipulation is facile in 2016, it was quite a genetic feat in the 1980s. This is in part because the actual gene of interest (the coding part or exon) also contained eight intragenic regions of non-coding DNA (or introns). Thus, it was the transcribed and edited mRNA that scientists had to isolate, and that could then be made into complementary DNA (cDNA) and cloned into a suitable expression system. Among the initial expression organisms were yeast, filamentous fungi, and bacteria (including *Kluyveromyces, Aspergillus,* and *E. coli).*

The genetic engineered chymosin was approved by the FDA in the early 1990s, and it soon gained wide acceptance in the US marketplace (Flamm, 1991). Subsequently, improved expression systems were developed and production conditions were optimized, leading to higher yields and levels of purity. Engineered chymosin is currently marketed worldwide by several major companies. In general, each use their own specific production strain having optimized activities.

Aside from the obvious cost advantage of the genetically engineered chymosins, other benefits were also realized (Badiefar, et al., 2011). These products are derived from a fermentor, rather than calf stomachs, and are not subject to supply problems or price fluctuations. They can be classified as vegetarian and can more easily obtain

kosher status. Importantly, the chymosin is relatively easy to purify and can be produced free of other proteases that are sometimes present in calf chymosin preparations. In fact, manufacturers claim that the engineered chymosin is close to 100% pure, has high specific activity, and is highly standardized. According to some experts, cheese made using these chymosin products has organoleptic properties (and yields) as good as (if not better than) cheese made with calf chymosin (Jacob et al., 2011). However, this view is not universal, and in some circles, calf chymosin is still considered the best for cheese quality (Fox et al., 2017).

Almost from the outset in the early 1990s, engineered chymosin sold for considerably less than the calf chymosin price. Even though calf chymosin prices have dropped in recent years, the engineered products currently (2017) sell for nearly half as much (based on International Milk Clotting Units). Thus, it is not surprising that engineered chymosin has displaced much of the calf chymosin market in the United States. Calf chymosin has essentially become a niche product, with less than 10% of the coagulant market. However, in Europe, where many cheese manufacturers prefer or are required to produce cheese by traditional techniques (i.e., according to "Protected Designation of Origin" requirements), calf chymosin is widely used. It also contains a small amount of bovine pepsin that may enhance flavor and texture development.

It is worth noting that unlike other foods produced via biotechnology, when engineered chymosin was introduced, there was little public concern or protest. Perhaps this was because the notion of a product produced by microorganisms in fermentors was more appealing to consumers than the image of calf stomach extracts. In addition, the FDA determined that no special labels would be required for cheese made with engineered chymosin, which they considered to be "not significantly different" from calf chymosin.

References

Flamm, E.L. 1991. How FDA approved chymosin: a case history. Bio/Technology 9:349–351.

Badiefar, L., M. Khodabandeh, and G. Ahmadian. 2011. A comparison of the production efficiencies of full-length and truncated forms of prochymosin. Int. J. Dairy Technol. 64: 288–293.

Fox, P.F., T.P. Guinee, T.M. Cogan, and P.L.H. McSweeney. 2017. Factors that affect cheese quality. In: Fundamentals of Cheese Science, 2nd edn. Fox, P.F., T.P. Guinee, T.M. Cogan, and P.L.H. McSweeney, eds. Springer, New York, pp. 533–542.

Jacob, M., D. Jaros, and H. Rohm. 2011. Recent advances in milk clotting enzymes. Int. J. Dairy Technol 64:14–33.

Fat may also be retained. Even in solutions where casein is absent (such as the whey), this process can still result in enough precipitated whey protein to form a cheese. Examples of precipitated cheeses include Ricotta cheese, the Hispanic-style cheese, queso blanco, paneer and Gjetost, a whey-derived cheese popular in Norway. In Italy, for example, it is common to devote a specific section of a cheese plant for production of Ricotta using the whey obtained from that cheese.

Squeezing out water

Once milk is transformed from a liquid into a gel, the next goal is to remove water. This is accomplished by cutting the single large gel mass into literally billions of smaller cubes of curd. The net effect of this cutting step is to significantly increase the surface area of the curds. Thus, for every 1 m^3 of a cheese gel that is cut into approximately 1 cm^3 cubes, 10^6 such curd particles will be formed. Because the distance the water molecules must travel (from the interior of the gel to the outside environment) decreases as the curd size is reduced, this step has the net effect of substantially increasing the rate of syneresis (defined previously as the separation of water from the coagulated milk). Additionally, when the curds are stirred and then heated, they shrink even more and syneresis is further enhanced.

Another important factor that contributes to the concentration of milk solids is acidification. As discussed previously in Chapter 5, as the pH approaches 4.6 (the isoelectric point of casein), the water-holding capacity decreases, and syneresis and whey expulsion increase. In cheese manufacture, as for cultured milk products, acidification is due to fermentation of lactose by the starter culture. Of course, in cheese manufacture, the culture performs other critical functions. In particular, culture bacteria are also responsible, in part, for desirable flavor and texture properties. But it is the ability to convert lactose to lactic acid and to reduce the cheese pH that makes the culture essential.

Finally, is possible to use chemicals, in the form of salt, or to apply physical forces, in the form of pressure, to squeeze out even more water. When dry salt is added to Cheddar cheese curds, for example, water diffuses from the curd by osmotic force. Similarly, salt-brined cheese also loses moisture and become dryer (as occurs for Parmesan). Pressure can be applied several ways, either by stacking cheese and adding weights to the cheese, by hydraulic presses, or simply by gravity.

GENERAL STEPS IN CHEESE MAKING

On a worldwide basis, there are probably thousands of different types of cheeses produced and consumed. Indeed, there are hundreds of cheese varieties produced in France alone (246, to be exact, at least according to the famous quote by former French president Charles de Gaulle)[1] Anyone who has visited a fromageri in Paris or a formaggio in Milan (or perhaps the National Cheese Emporium, made famous by a Monty Python sketch), can certainly appreciate the incredible number and variety of cheeses that are available. How could there be so many? And all made from such a bland-tasting starting material. Are the procedures for making cheese so complex as to allow for all the cheeses produced?

In reality, the basic manufacturing steps for all cheeses are surprisingly similar. However, it is the almost unlimited number of variables that exist at each of these steps that ultimately account for the myriad number of different cheeses. Described below are the various ingredients and manufacturing steps that are used in cheese making, with an emphasis on those variables that distinguish one type of cheese from another.

[1] "How can you govern a country which has 246 varieties of cheese?"

Milk

As shown in Figure 6.5, the first variable starts with the milk itself. Although most cheeses are made from cow's milk, many cheeses, including some of the world's most famous cheeses, are made using milk from other sources. For example, Feta and Chevre are ordinarily made from goat milk, Roquefort and Romano are made from sheep milk, and Mozzarella is often made from the milk of water buffaloes. The milk from these non-bovine animals is dramatically different in terms of gross composition and physical properties than milk from cows. For example, they all contain more fat than bovine milk; indeed, water buffalo and goat milk contain nearly twice as much. However, even for milk from the same species of animal, the composition can vary according to the breed, the nature of the feed, and even when the milk was obtained (i.e., morning versus evening).

Moreover, in cheese making, not only does the gross composition affect cheese properties, but so does the specific composition of each of the milk constituents. The lipid portion of goat milk, for example, contains a higher percentage of volatile, short- and medium chain fatty acids, such as caproic (C-6), caprylic (C-8), and capric acids C-10), as compared to bovine milk. Thus, goaty and rancid flavors are often evident in goats' milk cheeses, especially when hydrolysis of triglycerides by lipases is encouraged. The fat content also has

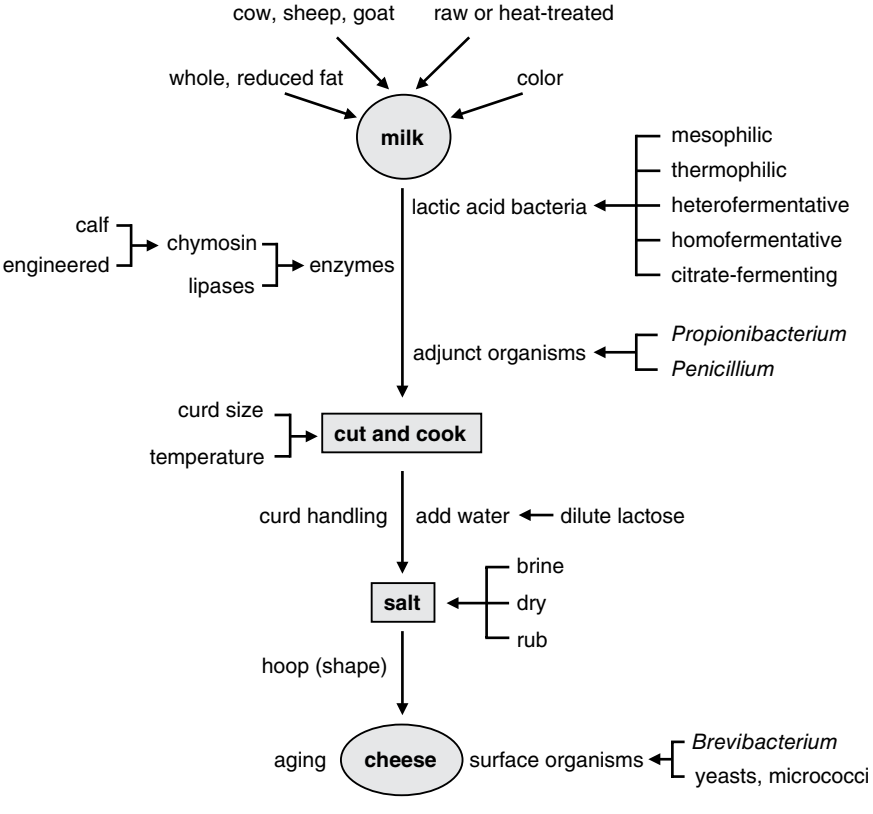


Figure 6.5 General steps for manufacture of cheese.

a profound influence of other properties of cheese. In particular, fat contributes to the body, texture, and smoothness of cheese, and also provide satiety. Milk fat also serves as a substrate for important flavor-generating reactions performed by starter culture bacteria as well as adjunct microorganisms. Finally, many of the flavor constituents derived from non-lipid substrates that form during cheese ripening are soluble in the lipid phase. For example, hydrophobic peptides derived from casein hydrolysis (many of which are bitter) are found in the fat portion of the cheese.

For some cheeses, the milk may be used as delivered from the farm or is standardized to give a fat content that is optimum for a given cheese. For example, Cheddar type cheeses are made with whole milk, containing 3.5 to 4.5% milkfat. This will produce a cheese containing at least 50% fat (on a dry basis), which is a typical requirement for many cheeses. However, other cheeses are made with milk adjusted to 3% fat (e.g., Swiss cheese) or less (part-skim Mozzarella). In part, this is because the body characteristics of certain hard cheeses, for example, Swiss and Parmesan, require less fat and a higher casein-to-fat ratio. In contrast, some cheeses are made from milk that is enriched with cream, such that the fat content of the cheese (so-called double cream cheese) will be 60% (or even 72% for triple cream cheese).

Another key variable involves the handling of the milk, and in particular, whether the milk has been heated or not. In the United States, all un-aged cheese (aged less than 60 days) must be made from pasteurized milk, whereas only aged cheese (held for more than 60 days at a temperature not less than 1.7°C) can be made from raw milk. While the scientific basis for this aging regimen for raw milk cheeses has been challenged, the rationale for the pasteurization requirement is clear–to ensure that non-aged cheese is safe. Given the concern about food safety in the United States, there has been a trend among large US manufacturers to use pasteurized milk for all cheeses, even those that will still be aged. Indeed, the US FDA has been reviewing this issue for several years. Ultimately, they may decide to leave the rule as currently stated, extend the pasteurization requirement to all cheeses, or perhaps modify the aging requirements. The debate about the safety of raw milk cheeses is explored in more detail in Box 6.2, and control measures are also described later in this chapter.

In contrast to the situation in the US, in France, Italy, and other major cheese-producing countries, there are no such pasteurization requirements. Even fresh cheeses in some coun-

Box 6.2 Safety of cheese made from raw milk

Raw milk cheese has been a way of life for cheese manufacturers and cheese consumers throughout much of the world and for most of human cheese-eating history. Only in the past century has cheese been made from anything other than raw milk (or occasionally slightly heated) milk. Nonetheless, the use of pasteurized milk for cheese is now so common in the United States that most of the cheese manufactured in the US originates from pasteurized milk. Indeed, if the cheese is not made from pasteurized milk, regulations exists that require that such cheeses be aged for at least 60 days at a minimum temperature of 1.7°C (35°F). The rationale for this policy, established in the 1930s, was based on very limited research from that era suggesting that pathogenic bacteria, if present in the raw milk, would die during the 60-day ripening period. Factors considered to contribute to cell death included low pH, low water activity, low

Eh, high salt-in-moisture levels, the presence of organic acids and a competitive microbiota, and the absence of fermentable sugars. In other words, the collective effect of these inhospitable conditions were thought to create an environment that would result in the eventual lysis and death of any pathogenic bacteria that may have been present in the original cheese.

In reality, the 60-day requirement was actually nothing more than an approximation based on a small number of studies. Moreover, these early studies were conducted long before *Listeria monocytogenes*, *Escherichia coli* O157:H7, and other contemporary pathogenic organisms were recognized as potential contaminants of raw milk. That these more recent (i.e., post 1930s) pathogens could be inherently more resistant to environmental extremes or that they could induce stress resistance defense systems were scenarios that could hardly have been imagined.

It is now well-known that some of these pathogens can tolerate all sorts of inhibitory conditions and challenges. For example, *L. monocytogenes* is particularly capable of surviving, even growing, at acid pH and in the presence of high salt concentrations. Furthermore, when exposed to various shocks, such as high, sub-lethal temperatures, this organism synthesizes a set of proteins that enable it to tolerate other subsequent stresses. These observations led researchers to challenge the conclusions made 80 years ago, i.e., that a 60-day aging process would result in raw milk cheese that is free of pathogens. Research now indicates that these "newly" recognized pathogens could survive aging and, if present at sufficiently high numbers in the raw milk, could be present in the aged cheese. For example, in the 1980s and 1990s, research publications from the University of Wisconsin and elsewhere showed that *L. monocytogenes* could survive more than one year in Cheddar cheese and that levels greater than 10^7 cfu/g could be reached in Camembert cheese (summarized by Ryser, 1999).

In response to these findings, the FDA announced in 2002 that this policy would be reviewed, and they left open the possibility that all cheese, whether aged or not, would be required to be made from pasteurized milk. Although manufacturers of aged cheese were undoubtedly concerned about this announcement, the greatest furor of opposition came from cheese-loving consumers. Their position, widely expressed on various websites and in the popular press, was that cheese made from raw milk is superior in flavor, texture, nutrition, and overall sensory appeal to that of pasteurized milk, and that any perceived food safety risks were exaggerated or non-existent (Paxson, 2008; West, 2008). Moreover, despite the research showing that pathogens might, at least in theory, survive 60 days of aging, the food safety record for aged cheese is extraordinarily good. There are few published accounts of *properly aged* (emphasis added) raw milk cheese having been responsible for foodborne disease, and in those cases where the cheese was implicated, other extenuating circumstances were often involved (e.g., post-aging contamination by workers or equipment).

Despite the excellent safety record for aged raw milk cheese, the exception is for fresh (not aged) raw milk cheeses. These cheeses have been the cause of many of the recent cheese-borne disease outbreaks (Table 6.2.1). In particular, Queso fresco, a popular Hispanic-style cheese, has been implicated in many of the US outbreaks (Yoon et al., 2016). In the majority of these outbreaks, poor handling of the milk or cheese or poor sanitation and hygiene were responsible. It is worth noting that there

Table 6.2.1 Safety record of cheese.

Year	Organism	Cases	Cheese	Country
2000	*Listeria monocytogenes*	3	Mexican-style	USA
2000s	*Salmonella* Newport	85	Cortia	2000s
2000s	*Escherichia coli* O157:H7	38	Gouda	USA
2001	*Salmonella* Enteritidis	215	Cantel	France
2000s	*Salmonella* Newport	27	NA	USA
2002	*Escherichia coli* O157:H7	13	Gouda	Canada
2004	*Escherichia coli* O157:H7	3	NA	Canada
2004	*Escherichia coli* O157	3	Brie	France
2005	*Escherichia coli* O26	6	Brie	France
2005	*Escherichia coli* O26:H11	NA	NA	France
2005	*Listeria monocytogenes*	10	Tomme	Switzerland
2006	*Escherichia coli* O157:H7	3	Goat cheese	France
2006	*Salmonella* Typhimurium	NA	NA	Netherlands
2006	*Salmonella* Newport	85	Mexican-style	USA
2006	*Salmonella* Newport	96	Cotija	USA
2007	*Salmonella* Typhimurium	29	NA	USA
2008	*Salmonella* Muenster	25	Goat cheese	France
2008	*Escherichia coli* O157:H7	16	NA	Canada
2010	*Escherichia coli* O157:H7	8	Soft	USA
2010	*Escherichia coli* O157:H7	15	NA	USA
2010	*Escherichia coli* O157:H7	38	Gouda	USA
2014	*Staphylococcus aureus*	14	Tomme	Switzerland

Only data since 2000 is shown. NA, not available. Adapted from Yoon et al., 2016.

are also reports of cheeses made from pasteurized milk implicated in food poisoning outbreaks (Yoon et al., 2016). Post-pasteurization contamination of milk, in particular, is a frequent cause of these outbreaks.

Fresh or non-aged cheeses made from raw milk pose a particularly significant risk to certain consumers. Those include the very young and very old, pregnant women, and other immuno-compromised populations. Brie, Camembert, and other surface-ripened- by-mold cheeses, for example, have been on the "do not eat" list for pregnant women and HIV-positive individuals, due to the potential occurrence of *L. monocytogenes* in these cheeses. For this very reason, the safety and risks of *L. monocytogenes* in soft Camembert-like cheeses made from pasteurized and unpasteurized milk was assessed by the FDA (2012). Although risks for this cheese were higher when made from unpasteurized milk, it was mainly susceptible populations that were at the greatest risk from consuming these cheeses. In general, these and other studies suggest that while raw milk cheese may have higher risk potential than pasteurized milk cheeses, the more relevant criteria could be the type of cheese and the moisture and aging properties (Choi et al., 2016). Various surveys of raw milk cheeses support such a view (Brooks et al., 2012; Verraes et al., 2015).

Recently, the risks of illness from consuming raw milk or raw milk cheese was estimated to be more than 800 times higher than for consumers of pasteurized milk and cheese (Costard et al., 2017). However, the outbreak data from which the estimated risks was based had combined the raw milk and raw milk cheese consumption. Also, there was no data provided on the type of cheese (aged or fresh). Finally, another very recent analysis concluded that there was no difference in prevalence of *L. monocytogenes* in European cheese made from raw and pasteurized milk (Martinez-Rios and Dalgaard, 2018).

References

Brooks, J.C., B. Martinez, J. Stratton, A. Bianchini, R. Krokstrom, and R. Hutkins. 2012. Survey of raw milk cheeses for microbiological quality and prevalence of foodborne pathogens. Food Microbiol. 31:154–158.

Choi, K.H., H. Lee, S. Lee, S. Kim, and Y. Yoon. 2016. Cheese microbial risk assessments – a review. Asian Australas. J. Anim. Sci. 29:307–314.

Costard, S., L. Espejo, H. Groenendaal, and F.J. Zagmutt. 2017. Outbreak-related disease burden associated with consumption of unpasteurized cow's milk and cheese, United States, 2009–2014. Emerg. Infect. Dis. 23:957.

Martinez-Rios, V., and P. Dalgaard. 2018. Prevalence *of Listeria monocytogenes* in European cheeses: A systematic review and meta-analysis. Food Control 84:205–214.

Paxson, H. 2008. Post-pasteurian cultures: the microbiopolitics of raw-milk cheese in the United States. Cult. Anthropol. 23:15–47.

Ryser, E.T. 1999. Incidence and behavior of *Listeria* in fermented dairy products. In: E.H. Marth and E.T. Ryser, eds. Listeria, Listeriosis, and Food Safety, 2nd edn. Marcel Dekker, New York.

Verraes, C., G. Vlaemynck, S. Van Weyenberg, L. De Zutter, G. Daube, M. Sindic, M. Uyttendaele, and L. Herman. 2015. A review of the microbiological hazards of dairy products made from raw milk. Int. Dairy J. 50:32–44.

West, H.G. 2008. Food fears and raw-milk cheese. Appetite. 51:25–29.

Yoon, Y., S. Lee, and K.-H. Choi. 2016. Microbial benefits and risks of raw milk cheese. Food Control 63:201–210.

tries can be made from raw milk (unless they are to be exported to the United States; then they must conform to US requirements).

Milk for some cheeses is not pasteurized, but nonetheless receives a heat-treatment (i.e., below pasteurization conditions). This practice is often used for Swiss cheese and other aged cheeses. Whether pasteurized or heat-treated, the reason is to inactivate microorganisms. The difference is that pasteurization targets both pathogenic and spoilage microorganisms, whereas sub-pasteurization treatments usually target only spoilage organisms. Pasteurization, however, not only kills pathogens and undesirable spoilage bacteria, but it also inactivates various autochthonous members of the microbiota ordinarily present in raw milk. Many enzymes, either present in the raw milk or produced by microorganisms are also inactivated. Since these microorganisms and enzymes are major contributors to the overall flavor and texture properties of the finished cheese, quality differences between cheeses made from raw or heat-treated milk can be significant. These differences may be noticeable in both aged and non-aged cheese.

It should be emphasized that apart from whether or not the milk is raw or pasteurized, other factors are also important. Milk should of course be of high microbiological quality,

free of antibiotics, and within the compositional standards specified by government regulations. In particular, some European cheeses must conform to the "Protected Designation of Origin" (PDO) requirements for how and when the milk is collected and handled.

Finally, one of the most obvious ways to treat milk to distinguish one cheese from another is by the color. Milk, and the finished cheese, can be made more yellow by adding the natural coloring agent, annatto, or made whiter by adding bleaching-type agents. Although the color has no effect on flavor or texture, manufacturers have learned that visual appeal can have a pronounced effect on acceptability and preference. In the United States, for example, consumers from the Midwest, in general, prefer orange-colored Cheddar cheese, whereas Northeasterners favor white Cheddar.

Starter cultures

The composition of the starter culture depends on the intentions of the cheese maker. In general, if the cheese making procedure includes a step where the curds will be exposed to high cooking temperatures, such as during the manufacture of Swiss, Parmesan, and Mozzarella cheese, then a thermophilic lactic acid bacterial culture able to withstand those temperature must be used. If a particular flavor compound is desired, such as diacetyl in Gouda cheese, then again, the culture must contain specific organisms capable of producing those flavor compounds. In fact, culture technology is now so advanced that each strain present in the starter culture can be selected on the basis of the specific performance attributes desired by the customer. Thus, the culture can contribute to considerable variation in the finished cheese, such that even modest changes in the culture composition or amount can result in dramatic differences in the cheese.

As described in Chapter 4, cultures used for cheese fermentations consist of several genera and species of lactic acid bacteria. Apart from selecting a culture that provides for the desired rate and extent of acid development and that produces the desired flavor and texture, the main distinction for culture selection is based on temperature. Mesophilic cultures, containing strains of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*, grow within a wide temperature range of about 10°C to 40°C. Growth rates at the extreme ends of this range are usually low and temperatures much above the upper limit for growth can be lethal. Thus, the optimum temperature for growth of mesophilic cultures is about 28°C to 32°C (depending on the strain), which is generally very near the temperature range that the milk or cheese is held during manufacture (i.e., when fermentation is expected to occur). The mesophilic cultures have long been the workhorses of the cheese industry, and are used for the majority of cheeses.

Thermophilic cultures, mainly *Lactobacillus helveticus* and *Streptococcus thermophilus*, were, for many years, of secondary importance compared to the mesophiles. That it no longer the case, and now these cultures are widely used. They have a temperature optima around 42°C to 45°C and are able to grow at temperatures as high as 52°C. Like the mesophiles, however, temperatures just a few degrees higher than their maximum growth temperature may result in thermal inactivation. This means that when curds are heated, the temperature must not exceed that which the culture can tolerate. Otherwise, the culture will be inactivated or injured and the subsequent fermentation will either be slow or not occur. However, it should be noted that thermal effects on microorganisms obey first-order kinetics, such that inactivation or killing occurs at a logarithmic rate. Thus, even when the temperature reaches a lethal level, some cells will survive (depending on the exposure time) and be able to grow (albeit slowly), once a compatible temperature is established.

Most cheese starter culture species, whether mesophilic or thermophilic, are homofermentative. However, heterofermentative lactic acid bacteria may be included in mesophilic cultures that are used for particular cheeses. Specific heterofermentative species include *Leuconostoc mesenteroides* subsp. *cremoris* and *Leuconostoc lactis*. It is not coincidence that these organisms also ferment citrate and produce diacetyl. Thus, the famous Dutch cheeses such as Gouda and Edam, have a buttery aroma, as well as a few small eyes, due to CO_2 formation. Finally, cultures may also contain non-lactic acid bacteria for Swiss-type and surface-ripened cheeses, as well as fungal spores for mold-ripened cheeses, as will be discussed later.

Until about 40 years ago, bulk cultures grown in whey or milk without pH control were the dominant form in which cultures were used (reviewed in Chapter 3). From these bulk cultures, inoculum levels of about 1% (w/w) would have been used for Cheddar and related types of cheese. This 1% inoculum would have given an initial cell concentration of about 5×10^6 cells per ml of milk. Other cheeses are made using more culture (e.g., Mozzarella is normally made with a 2% starter) or less culture (e.g., Swiss is made with 0.5% culture). Although these traditional bulk cultures are still occasionally used, bulk culture tanks began to be commonly equipped with external pH control systems in the 1980s. This simple technology made it possible for operators to partially neutralize the medium as the cells grew, and, therefore, maintained the pH at a level that was more to the liking of the starter culture organisms.

Specialized culture media with enhanced buffering capacity was also introduced that provided "internal" pH control. With the widespread use of these bulk culture systems that had pH controls, culture viability and cell density (i.e., cells per gram) have been significantly enhanced. By obtaining greater cell densities in the bulk tank, the inoculum volumes described above no longer applied. Indeed, less culture is necessary, perhaps half or more as much on a weight or volume basis. In addition, highly concentrated, direct-to-vat cultures are also now widely used. These products may contain ten or more times as many cells as traditional bulk cultures. One 500 ml can, for example, is sufficient to inoculate 5,000 L of milk (i.e., the equivalent of a 0.01% inoculum).

Despite the generally consistent nature of modern cultures, their activity and cell density may still be subject to variation. Thus, it is important for cheese manufactures to adjust inoculum levels to satisfy production schedules and performance expectations. In the case of Cheddar cheese, if too much culture is added, acid development might occur too rapidly, resulting in early loss of calcium and demineralization of the cheese (discussed later). In addition, a large culture inoculum may lead to excessive production of proteolytic enzymes that can eventually affect cheese yield, flavor, and texture. In contrast, if not enough culture was added, fermentation and acid development is delayed, causing production schedule headaches as well as opportunities for spoilage or pathogenic organisms to grow.

It is important to note that, despite the availability of defined and consistent starter cultures, there are some cheeses made using more traditional starter cultures. Examples would include the famous Parmigiano Reggiano cheeses that conform to the PDO (or DOP in Italy). This is the only cheese allowed to be called Parmesan in Europe and can be manufactured only in designated provinces of Italy. It is made using whey as starter culture, a form of backslopping. Likewise, many of the Dutch-type cheeses (e.g., Gouda and Edam) are also still made using undefined strains maintained simply as a mixed culture.

Regardless of the form of the culture (e.g., defined or undefined, bulk or direct-to-vat) the milk for most cheeses is ordinarily raised to a temperature between 30°C and 40°C when

using mesophilic cultures or 35 to 45 when using thermophilic cultures. If culture growth is to be encouraged early (i.e., before chymosin addition), the milk may be held for a period of time within this temperature range. However, despite being held at favorable temperatures, culture activity and acid development is modest, with only a modest decrease in pH. This is because the cells either are coming out of a somewhat dormant phase (in the case of frozen or lyophilized direct-vat-set cultures), or are adapting from a rich, almost ideal bulk culture medium to a milk medium that requires induction of at least some new biochemical pathways, such as those involving protein hydrolysis and amino acid use. Depending on the culture media and culture preparation conditions, however, it is possible for cultures to emerge from the lag phase rather quickly.

Sooner or later, depending on the cheese, growth of the culture, fermentation of lactose, and production of end-products becomes critical. This may commence early, such as during or immediately after the cooking/stirring step. Alternatively, fermentation may not occur until several hours after the cheese has left the vat. Regardless of exactly when fermentation occurs, delays due to culture inhibition can cause serious problems for the manufacturer. Large cheese factories that process a million or more kg of milk per day require high throughput (i.e., the rate that milk is converted to finished cheese products). A bottleneck at the fermentation step may place upstream operations on hold, disrupting production schedules and perhaps causing employees to work extra hours. Of course, if the fermentation is slow, sluggish, or fails altogether, cheese quality will often be poor.

There are several possible causes of starter culture inhibition. Although food laws and regulations vary according to country, most jurisdictions require that milk be free of antibiotics. If present these residues could inhibit the lactic culture. However, due to the ease of testing, it is now relatively rare that cheese milk would contain undetected antibiotics. Milk may also contain natural immunoglobulins that bind to culture bacteria, forming clumps that eventually settle in the vat. This is also a mostly rare phenomenon. Another potential inhibitor is formed via the lactoperoxidase reaction. This reaction occurs when the enzyme lactoperoxidase oxidizes thiocyanate in the presence of hydrogen peroxide to form hypothiocyanate. This reactive compound can be inhibitory to some starter culture lactic acid bacteria. Lactoperoxidase and thiocyanate are both naturally present in milk, and hydrogen peroxide can be produced by the endogenous microbiota. Finally, chemical agents used to sanitize cheese vats can occasionally inhibit cultures if residues are not adequately rinsed.

By far, however, the main cause of culture inhibition is bacteriophage infection. Bacteriophage (or simply phage) are viruses that infect bacteria. In cheese and dairy environments, the main targets or hosts for these phages are the lactic acid bacteria used as starter cultures. Their detrimental role in the cheese making process, and the means by which phage problems can be controlled, will be discussed later in this chapter.

Coagulation

In many cheese factories, chymosin is added to the milk immediately after or nearly at the same time as the culture is added. However, some cheese makers add the culture and then allow for a "pre-ripening" period before adding the chymosin. This practice gives the culture a brief opportunity to produce a small amount of acid and a slight lowering of the milk pH. Since chymosin is an acid protease (its optimum activity on κ-casein occurs at pH 5.5), it will be more active as milk pH decreases. The solubility of calcium also increases as the pH decreases. Thus, with pre-ripening, less chymosin can be used to give the same curd firmness.

For similar reasons, it is also common for cheese manufacturers to add calcium chloride to the milk to promote coagulation (and perhaps yield).

In any event, the amount of chymosin added, and the length of the setting period prior to cutting depends on the cheese being made and the curd firmness desired. Usually, about 200 ml of single-strength chymosin per 1000 kilograms of milk will give a suitable coagulation within about 30 minutes. For many large, automated manufacturers, the point at which the curd is sufficiently firm and ready for cutting is based strictly on the clock (but also on *a priori* knowledge of what times give the best cheese). Although specialized instruments are available that can determine if the curd is ready for cutting, curd firmness is more often than not determined on a subjective basis, i.e., when the operator deems it ready based on a simple cutting method.

Cutting and cooking

The coagulated mass is next cut using harp-like, wired knives that cut the curds into die-sized particles. The knives are constructed such that the curds can vary in size. Since this step is performed to enhance syneresis, the size, or more importantly, the surface area of the curd particles, has a major influence on the rate of water removal from the curd. Hard, low-moisture cheeses like Parmesan and Swiss are typically cut into kernel or wheat berries-size curds, whereas soft, high-moisture cheeses are cut into larger pieces. The size of the curd also influences fat loss – more fat is retained in large curds than in small ones. Regardless of the size of the curd at cutting, the actual composition varies relatively little. For the most part, the curd is constructed of a calcium–casein complex that contains entrapped fat and bacteria (including starter culture organisms). The main constituent, however, is water, which contains various water-soluble components including whey proteins, enzymes, vitamins, minerals, and lactose.

Syneresis begins as soon as the curds are cut, and increases during the ensuing minutes when the curds are gently stirred. The initial rate of syneresis depends on the starting pH of the curd, because prior acid development greatly enhances syneresis (such that the lower the pH, the greater is the rate of syneresis). However, the cooking and stirring step is the primary means of enhancing syneresis. All other factors being equal, the higher the temperature and the longer the curds are cooked and stirred, the dryer will be the finished cheese. Thus, the cooking step is one of the major variables that cheese manufacturers can manipulate to produce different types of cheese.

Although each of the cheese making steps so far discussed might appear to have distinct functions and outcomes, the reality is that these events have interrelated effects. This is especially true for the cooking step. As noted previously, the more the curds are cooked, the more water is removed from the curd. Since lactose is dissolved in the water phase, it follows that as the curd becomes dryer, less lactose remains in the curd. Less lactose in the curd means less lactose will subsequently be available for fermentation. Cheese manufacturers can, therefore, influence acid production and cheese pH by modulating the cooking time and temperature conditions. The reader is reminded, however, that whatever the cooking temperature, the culture must be able to withstand that temperature. Otherwise, the cells will be injured, attenuated, or worse yet, inactivated.

When heat is applied during the cooking step, it must be done gradually via a step-wise progression. Heating too rapidly may cause the exterior of the curds to harden, a phenomenon known as case-hardening. This shell-like "skin" that surrounds the curd particles prevents water migration and effectively reduces syneresis. The cooking and stirring step has so much

of an effect on the finished cheese that some experienced cheese makers can tell how dry the cheese will be simply by touch and the feel of the curd. Of course, some soft cheeses, like Brie, are not cooked at all, but rather are stirred at the setting temperature. Finally, although the actual heating step usually occurs via indirect heat transfer through jacketed vats, it is also possible to inject steam directly into the whey-curd mixture.

Curd handling

Perhaps the most influential and certainly variable step during the cheese making process involves the means by which the curd is handled during and after the cooking and stirring steps. In general, the whey is removed when the desired acidity is reached, when the curd has been cooked for a sufficient length of time, or when it is sufficiently firm or dry. There are several means by which the curd is separated and the whey is removed. In traditional Cheddar cheese manufacture, the curds are simply pushed to the sides of the cheese vat and the whey is drained down from the center, with screens in place at the drain end to prevent curd loss. Alternatively, the curds can be collected in cheese cloth and hoisted above the whey, as in traditional Swiss and Parmesan cheese manufacture. In more modern, large production factories, where cheese vats must be cleaned and re-filled, it may be more efficient to pump the curds and whey to draining tables or Cheddaring machines, where whey separation occurs. Similarly, the curd-whey mixture can be added or pumped directly into perforated cheese hoops, where the whey drainage step is completed.

Although there are many ways to manipulate the curd to alter the properties of the cheese, one simple and common twist in the separation step is worth special mention. Specifically, the goal of this step is to reduce the lactose content in the curd while maintaining the moisture. The trick involves removing part of the whey and then stopping the draining process while water is added back to the curds. Since the lactose concentrations in the curd and in the whey are in equilibrium, when whey is removed and replaced by water, lactose will diffuse from curd to water. This leaves the curd with markedly less lactose available for subsequent fermentation by the starter culture. This is the principle for many so-called "sweet" or low-acid cheeses, which may be from 0.2–0.4 pH units higher than normal. Thus, the initial pH of Colby, Gouda, Havarti, and Edam cheeses are generally in the range of 5.2 to 5.4.

Even when this lactose dilution step is applied, there is yet another variable that can have a profound effect on the finished cheese. If the added water is warm (i.e., about 35°C), then the cooking process will continue and syneresis will also proceed. This is how Gouda, Edam, and Havarti are produced. However, if the water is cold (about 15°C), the moisture content of the cheese may increase, as is the case for Colby and related types. A similar washing step is also used for Mozzarella, in part for pH control, but more so to remove lactose and galactose from the curd (discussed later).

Once the curds are separated from the whey, several things begin to occur. First, the starter culture finds itself at a temperature conducive for growth, and soon the fermentation of lactose to lactic acid occurs. A subsequent decrease in curd pH and an increase in the titratable acidity (expressed as percent lactic acid) of the expressed whey is evident. The curds, almost immediately after whey is removed, mat or stick together. This step can occur in the cheese hoops or forms (as noted above, when the curds are pumped directly from the vat) or the matted curds can be left undisturbed in the vat. The matted curd can be cut into slaps and those slabs piled on top of one another while fermentation is occurring, a process known as Cheddaring. Alternatively, the dry curds after draining can be stirred to facilitate additional whey removal and to lower the moisture in the finished cheese.

Salting

Salt is an essential ingredient that provides flavor, enhances syneresis, and contributes to the preservation of most cheeses. Even the simple step of salting, however, represents an important variable during cheese manufacture. Salt can be applied directly (i.e., in dry form) to the milled curds, as in the case of Cheddar, or salt can be rubbed onto the surface of hooped cheese, as in the case of some blue cheese varieties (e.g., Gorgonzola and Roquefort). Alternatively, some cheeses, such as Swiss, Mozzarella, and Parmesan, can be placed into brines. When salting occurs via brining methods, the amount of salt that ends up in the cheese is a function of the diffusion rate into the cheese, as well as the geometry of the cheese block, the duration of brining, and brine strength. If the cheese is shaped or cut into small units and left in the brine, as with Feta cheese, salt concentrations can be very high (>3%). In contrast, large blocks or wheels of brined Swiss cheese typically contain less salt (<1%), especially in the interior sections. Brined cheeses that are then allowed to air dry develop natural rinds, due to surface dehydration.

Aging

The last step in the cheese making process has as much influence as any previous step with regard to the properties and qualities of the finished cheese. As noted in Chapter 1, it is a fine line that separates spoiled from fermented. This is especially true for the production of a perfectly flavored, three-year old Cheddar cheese and a bitter, rancid, gassy, sour Cheddar cheese that is quickly rejected by any discerning consumer. Although the distinctly different properties of both of these two cheeses are the result of microbial and enzymatic activities, there is one clear distinguishing factor. The key difference is that the gourmet cheese is produced when aging occurs under controlled conditions, whereas the rejected cheese occurs when control is absent or lost. Control, in this context, refers not only to post-manufacturing steps, but all of the steps starting at the outset. Thus, as a general rule, any cheese that is intended for aging must be manufactured, from the very start, differently than an unaged cheese. The handling of the milk, the cheese pH, the moisture and salt content, the temperature and humidity regimen during aging all are important determinants that influence aged cheese quality.

In addition to its impact on the finished cheese, aging or ripening is also one of the most complex and most variable of all cheese making steps. This is due to the complexity of the enzymes and the diversity of microorganisms that are responsible for the flavor and texture changes that occur during ripening. The enzymes in cheese may occur naturally in the milk or be added directly in the form of rennet, chymosin, or lipase extracts. Enzymes are also derived from starter culture bacteria, adjunct organisms, or endogenous milk-borne organisms. Furthermore, the availability of substrates and the pH and Eh conditions in the cheese influence the activity of these enzymes and the types and amounts of products that are formed. Similarly, microorganisms in cheese obviously include the starter culture, but other organisms may originate from the milk, equipment, or the environment. Although the temperature in aging rooms is generally low (usually around 3°C to 7°C, but sometimes much higher), and the cheese milieu is not necessarily conducive for growth (as noted above), metabolism of the various substrates in cheese by intact organisms still occurs. A ripening cheese represents a rather vibrant ecosystem.

TYPES OF CHEESE

Given the hundreds of cheeses produced worldwide, it is not possible to discuss each particular one. There are so many different types of cheese that even categorizing them into manageable groups or families is not easy. For example, cheeses can be grouped on the basis of their level of hardness (e.g., from soft to hard), moisture content (e.g., low, semi- or high), or cooking temperature. Another common way to classify cheeses is based on the organisms responsible for ripening and flavor development (e.g., external mold, internal mold, or surface ripened by bacteria). The extent of aging is also used to classify cheeses. Finally, cheeses can be grouped simply on the basis of provenance – Dutch, American, Italian, etc.

For the purpose of this discussion, cheeses will be organized by a hybrid mixture of classification schemes based on the primary properties of those cheeses and their distinguishing characteristics (Table 6.1), as noted above. For the most part, only the most well-known and widely consumed cheeses will be discussed and only general manufacturing procedures are described. The role of microorganisms involved in their production will be emphasized. The reader seeking detailed procedures is advised to consult other excellent sources for specific manufacturing details (see Bibliography).

Acid-coagulated cheeses

In the United States, the most popular of the acid-precipitated cheeses are cottage cheese and cream cheese. Similar cheeses are also produced throughout the world. Although per capita consumption of cottage cheese (all varieties) has declined in the past twenty-five years (1990–2015) by more than 30% (from 1.5 kg to 1.0 kg per person per year), cream cheese per capita consumption has increased by nearly the same amount (from 0.8 kg to 1.2 kg per person per year). This increase in cream cheese consumption is undoubtedly due to an equal increase in the popularity of bagels (and perhaps cheesecakes). The availability of flavored, whipped, and low- and reduced- fat cream cheese products has also contributed to this increase. Other cheeses in this category, including bakers' cheese and farmers' cheese, have only a small share of the market.

These cheeses all rely on a common theme. Specifically, the starter culture ferments lactose in the milk to lactic acid, such that a pH of 4.6 or below is reached. In cottage cheese manufacture, the starting material is simply skim milk. Often, non-fat dry milk is added to increase the throughput, or the amount of cheese produced per vat, and to improve body. The milk is always pasteurized, as required by law, since this is a fresh or non-aged product. A mesophilic lactic starter culture, containing strains of *Lactococcus*, is then added at a rate or amount that depends on the production schedule preferences of the manufacturer. For a fast make, high inoculum rates are used. The inoculated milk is mixed, then allowed to incubate quiescently in the vat at 30°C to 32°C, the optimum temperature for the culture. At high inoculum levels and at this temperature, an active culture can coagulate the milk in five hours or less.

In contrast, if a lower inoculum is added and the incubation temperature is set at 20°C to 22°C, well below the optimum for growth (essentially, room temperature), coagulation may take as long as 12 to 16 hours. Thus, a busy cheese maker could, under the first scenario, produce multiple batches of cheese each day out of the same vat. However, it is also possible, in the alternative procedure, to inoculate or set the milk at 5:00 pm, go home, sleep, and return to work early the next morning and have the cheese ready for the next step. Of course, inoculum and temperature regimens in between the long-set and short-set make

Table 6.1 Properties of major cheese groups.

Type	Cheese	Starter culture	Other organisms	Salt	Moisture	pH
Cheddar type	Cheddar	mesophilic[1]		1.5	37	5.5
	Cheshire	mesophilic		1.7	38	4.8
	Colby	mesophilic		1.5	39	5.5
Dutch type	Gouda	mesophilic	*Leuconostoc* spp.	2.0	41	5.8
	Edam	mesophilic	*Leuconostoc* spp.	2.0	42	5.7
Cheese with eyes	Emmenthal	thermophilic[2]	*Propionibacterium*	0.7	35	5.6
	Gruyere	thermophilic	*Propionibacterium*	1.1	33	5.7
Grating type	Parmesan	thermophilic		2.6	31	5.4
	Romano	thermophilic		5.5	23	5.4
Pasta filata	Mozzarella	thermophilic		1.2	53	5.2
	Provolone	thermophilic		3.0	42	5.4
Blue mold	Roquefort[3]	mesophilic	*Penicillium roqueforti*	3.5	40	6.4
	Gorgonzola	mesophilic	*Penicillium roqueforti*	2.5	45	6.2
	Stilton	mesophilic	*Penicillium roqueforti*	2.3	39	6.2
External mold	Brie[4]	mesophilic	*Penicillium camemberti*	1.6	52	6.9
	Camembert[4]	mesophilic	*Penicillium camemberti*	2.5	49	6.9
Surface ripened	Havarti	mesophilic		1.9	43	6.4
	Munster[5]	mesophilic	*Brevibacterium linens*	1.6	42	6.4
	Limburger	mesophilic	*Brevibacterium linens*	2.0	45	6.8
Brined	Feta	mesophilic		3.0	53	4.5

[1] Mesophilic cultures = *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*.
[2] Thermophilic cultures = *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. bulgaricus, and/or *Lactobacillus helveticus*.
[3] Citate-fermenting *Leuconostoc* or *Lactococcus* sp. may be added.
[4] *Streptococcus thermophilus* may be added.
[5] For Limburger and other surface-ripened cheeses, species of *Arthrobacter*, *Micrococcus*, and yeasts may also be present.
Data from Guinee and Fox, 2004; Johnson and Law, 2010; and other sources.

times are also possible. Finally, although not required for coagulation, a small amount of chymosin (1 to 2 ml per 1000 kg) is sometimes added to promote a more firm coagulum (usually for large curd cottage cheese).

Once a pH of about 4.7 is obtained, a soft curded gel mass is formed. The gel is then cut using a pair of cheese knives that resemble square or horizontal-shaped harps. One knife contains vertical wires, and the other horizontal wires. The distance between the wires determines the curd size, which in turn depends on the product being made (e.g., large, medium, or small curd cottage cheese products are available). Following the appropriate passes through the gel, die-shaped cubes are formed.

Almost immediately, whey becomes apparent and the curds begin to separate. The curds after cutting are very soft and fragile, and must be handled carefully to avoid shattering or fracture. Not only do fractured curds result in product defects, but, importantly, the small, broken curds or "fines" are lost when the whey is drained, resulting in loss of yield. Thus, the cut curds are initially left undisturbed for about fifteen minutes, and then are stirred gently.

Stirring continues as the curds are heated by gradually raising the temperature in the jacketed vats. Ultimately, the curd-whey mixture is heated to about 52°C to 56°C, usually within 1.5 hours (but according to some procedures, as long as three hours), with constant stirring.

Heating not only accelerates the rate of syneresis, but temperatures above 45°C also arrest the fermentation. In fact, inactivating the culture at this step is the only way to keep the pH from decreasing too low. If the coagulated cheese is not cut until the pH has already reached 4.6 or below, then by the time the cook temperature is sufficiently high enough to inactivate the culture, the curds may already be too acidic. Cooking also inactivates coliforms and other heat-sensitive microorganisms (especially psychrotrophs, such as *Pseudomonas*).

After cooking has been completed, the whey is drained and cold (4°C) water is applied to the curds to quickly reduce the temperature. The curd usually is washed two or three times. The water used for this washing step must be slightly acidic so that the precipitated casein is not re-solubilized. Also, the wash water is often chlorinated to ensure that spoilage microorganisms are not inadvertently added back to the curds. The water is drained, leaving behind what is called dry curd cottage cheese. This product has a bland, acidic flavor (pH about 4.6 to 4.8), and contains mostly protein (20%) and water (nearly 80%). It is used mostly as an ingredient in lasagna, blintzes, and other prepared products. However, it is far more common to add a cream-based dressing to the dry curds, producing the familiar creamed cottage cheese products of varying fat levels (generally ranging from 0% to 4%). The cream dressings typically contain gums and thickening agents, salt, emulsifiers, anti-mycotic preservatives (e.g., natamycin, sorbates, and other organic acids), and flavoring agents. Of the latter, diacetyl distillates may be added to impart buttery-like flavor notes.

The packaged dry or creamed cottage cheese is a perishable product and has a shelf-life of only two to three weeks under refrigeration conditions. However, the addition of new generation preservatives, including bacteriocin-containing products, and the application of modified atmosphere (using CO_2) and aseptic packaging, may increase shelf-life to as long as 45 days. Cottage cheese is nonetheless susceptible to spoilage for several reasons. The cheese vats are often open (although most modern vats are enclosed), and exposure to air and environmental microorganisms can be significant. Because the final pH of creamed products can be as high as 5.4, and the water activity is also high (0.98 to 0.99), *Pseudomonas* and other psychrotrophic Gram-negative bacteria can grow and produce fruity, rancid, and bitter off-flavors. Yeast and molds are the other main spoilage organisms, causing appearance as well as flavor and aroma defects. Flavor defects, such as high acidity and bitterness, are the result of excess growth by the starter culture. Other quality defects relate to texture and include shattered, gummy, or soft curd. They are most often caused by manufacturing and curd-handling flaws.

Manufacture of cream cheese (and its lower fat version, Neufchatel) is similar in principle to cottage cheese, but the starting material, the manufacturing steps, and the finished product are quite different. First, cream cheese is made from pasteurized and homogenized milk containing as much as 12% fat. Most other cheeses are made using non-homogenized milk

because homogenization results in a soft curd, which, in the case of cream cheese, is desirable. A mesophilic culture is added, followed by either a long or short set incubation. The acid-induced coagulum that forms at pH 4.7 is stirred (not cut) while the temperature is raised to as high as 73°C. The curds are then separated from the whey by special centrifuge-type devices or ultrafiltration systems. Although the resulting cheese material can be packaged as is, most cream cheese is mixed with other ingredients, including cream and gums, and then homogenized or mixed. The temperature is maintained above 72°C throughout the process. Packaging is done under aseptic conditions (i.e., in rooms under positive pressure with high efficiency air filtration systems in place), giving this product a long shelf-life (>45 days).

Cheddar family

Cheddar cheese had long been the most popular cheese consumed in the United States, but has slipped to second place, behind Mozzarella (see below). According to USDA sources for 2015, more than 1.5 billion kg of Cheddar cheese was produced in 2015. Another 0.6 billion kg of Cheddar-related types (Colby, Stirred Curd, and Monterey Jack) were also produced. The latter are collectively referred to as American style cheeses. However, this designation can be confusing since American cheese is often considered synonymous with processed American cheese, a totally different product (Box 6.3). On a per capita basis, 6.4 kg (14 pounds) of Cheddar and Cheddar-type cheese were consumed in 2015.

Box 6.3 "American" cheese

It may be ridiculed, disparaged, and misunderstood, but American cheese remains among the most popular of cheeses consumed in North America. Ask any kid raised in the US what kind of cheese he or she prefers, and the likely answer will be "American" or perhaps a popular brand name. This appellation has less to do with provenance and more to do with the particular style of cheese. Indeed, American cheese refers to one of several types of process cheese products. In fact, more than half of the cheese now purchased at retail (i.e., from supermarkets) is process, aka American cheese. Importantly, the term "American" has no legal meaning, but is simply an adjective that describes those natural cheeses belonging to the Cheddar-style family. Nonetheless, the US FDA allows various forms of process cheese to bear this name, as described below.

The process cheese category consists of three general types of products, each having a specific standard of identity that describes in detail the types of ingredients that are allowed, how it is to be manufactured, and the gross composition of the finished product. Pasteurized processed cheese is made from a single type of cheese and is labeled accordingly. Thus, Pasteurized processed American cheese is made from Cheddar or other American-type cheese. It contains a maximum of 43% moisture and a minimum of 27% milkfat. Pasteurized processed cheese food may contain additional optional ingredients, and more water and less fat are allowed (44% and 43%, respectively). Pasteurized processed cheese spread contains even more water

(44% to 60%), less fat (20%), and stabilizers are allowed. Many of the loaf-type products fit this description.

Not only do these products provide consumers with convenience and functionality, they can be considered an excellent example of value-added food technology. Cheese is, after all, a commodity whose dollar value is rather modest. Aging can certainly increase its value, but wholesale block and barrel prices for Cheddar cheese in the United States during 2016 averaged less than $1.60 per pound. Considering that it may cost the cheese manufacturer nearly $1.40 just for the milk that goes into a pound of cheese, process cheese is a great way to add versatility, functionality, shelf-life, and other value.

Cheddar cheese was first made in the village of Cheddar, England. It is different from most cheeses due to a specific curd handling technique called Cheddaring. During Cheddaring, the curds are separated from the whey and allowed to mat, after which the matted curd slabs are flipped and stacked. The Cheddaring practice, like many great discoveries, probably occurred as a result of an accident. Perhaps the cheese maker had drained the whey, and instead of stirring the curds prior to filling forms or hoops, he or she was delayed or distracted. The curds then matted, staying warmer than usual, and then began to stretch and become plastic. The cheese that was then produced had a unique texture and body that led to the adoption of the Cheddaring process. The manufacture of Cheddar cheese has evolved from a traditional, strictly batch operation to a more modern process that is more automated and mechanized.

The early steps in Cheddar cheese manufacture are rather straightforward and similar to the generic steps described above. Whole milk, either pasteurized or raw, is brought to a temperature of 30°C to 32°C and is inoculated with a mesophilic lactic culture containing strains of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris.* The culture, or rather the specific strains present in that culture, is selected based on the desired properties expected in the finished cheese. If the cheese is intended for the process market, then speed is the main criteria, and fast-growing strains of *L. lactis* subsp. *lactis* are often used. If, however, the cheese is to be aged, then the ability to generate appropriate flavor and texture development will likely drive selection of the culture (usually containing *L. lactis* subsp. *cremoris*). Two physiological properties, however, are required: the strains must ferment lactose and they must be able to hydrolyze and use proteins as a nitrogen source. Of course, other properties, including phage resistance, as well as the form of the culture (whether bulk set or direct-to-vat set, frozen or lyophilized, mixed or defined) are also important, and are discussed in later in this chapter.

After culture addition, chymosin is added, coagulation occurs, and the curd is cut using medium sized knives (giving curds about 0.6 to 0.8 cm in diameter). Following a short five- to ten-minute resting or "healing" period to allow the curds to form, the curds are gently stirred. Heat is then applied gradually, about 0.3 degrees per minute, to a final curd-whey temperature of about 38°C to 39°C. As the curds are cooked, the stirring speed is increased to promote heat transfer, and stirring is maintained for an additional 45 minutes. Although the starter culture bacteria are contained within the curd, as a general rule, little fermentation should occur during the cooking or stir-out steps. A decrease of only 0.1 to 0.2 pH units is normal. Although *L. lactis* subsp. *lactis* has a slightly higher temperature range for growth compared to *L. lactis* subsp. *cremoris*, both grow slowly at the upper end of common

cooking temperatures. If however, lower cooking temperatures are used, growth of the culture can occur, resulting in fermentation and lactic acid formation. Although fermentation during this step may be desirable for some cheeses, for Cheddar it generally is not. This is because acidification is accompanied by demineralization of casein as the calcium becomes solubilized. When the whey is drained, the remaining curds contain less calcium, resulting in cheese that holds fat poorly and is less elastic, with a short, brittle texture. The importance of calcium during later stages of the Cheddar process is discussed below.

For traditional Cheddar manufacture, once most of the whey is drained, the curds are gathered or pushed to both sides of the vat, where they quickly mat or stick together. By the time the last of the whey is removed, the curds will have turned into a cohesive mass. A knife is then used to cut the matted curds into approximately 20 cm × 80 cm slabs. Depending on the cheese maker's preferences, the slabs are then either rotated or flipped or flipped and stacked, a process known as Cheddaring. Because the temperature of the Cheddar slabs is maintained at 28°C to 32°C, the culture can begin to grow in earnest, increasing from about 10^6/g to 10^8/g of curd within the Cheddaring period. As the lactic culture grows, it performs two critical functions. First, it ferments lactose in the curd, in homolactic fashion, to lactic acid. Second, it begins to hydrolyze casein via a cell wall-anchored proteinase (as described in detail in Chapter 4), forming a variety of variously sized peptides. The consequences of both of these activities will be discussed below.

During the Cheddaring process, several biological, chemical, and physical changes occur that are collectively responsible for the characteristic properties of this cheese. However, without an active starter culture, none of these changes will occur. As the culture produces lactic acid and as pH becomes more acidic, calcium that was initially associated with the negatively-charged amino acids residues of casein (e.g., serine phosphate) is displaced by protons. In this state, the casein is more soluble, smooth, and elastic, as is evident as the Cheddar slabs stretch and become plastic-like. Eventually, however, if the pH of the cheese becomes too acidic (i.e., less than 5.0), the cheese will become short and crumbly. Fermentation during Cheddaring, what Cheddar masters refer to as dry acid, is much preferred over acid formation during the cooking step, or wet acid development. In addition to the changes in casein structure that occur as a result of acid formation, gravitational forces from stacking the Cheddar slabs on top of one another also affect cheese structure. This facilitates linearization and stretching of the casein and allows the curds to knit when pressed (see below).

The longer the cheese is Cheddared, the more the culture will grow and with it, the greater will be the acidity. The extent of Cheddaring, and with it the extent of acid formation, depends, therefore, on the desired properties of the finished cheese. Lactic acid levels of 0.4% to 0.6% are normally achieved by the end of Cheddaring (about one and a half to two hours after whey separation), resulting in a finished cheese pH of 5.0 to 5.2. In addition, longer Cheddaring times also result in more cell mass produced. Since the lactococci in the starter culture produce enzymes that degrade milk proteins, higher concentrations in the curd at this stage likely results in greater proteolysis at later stages, i.e., during ripening.

Although the traditional Cheddar process is still practiced in the United States in small cheese factories, most mid-sized and large operations use different procedures and produce a somewhat different type of product. This is because large cheese factories require much greater throughput than can be obtained by the more manual and slower traditional procedures. Thus, setting, coagulation, and cutting steps are performed in enclosed vats, and the cut curds are then pumped to another location for further processing. This allows the manufacturer to clean, sanitize, and re-fill the original vat.

In addition, modern operations have eliminated the labor-intensive flipping and stacking Cheddaring steps by employing alternative methods. Cheese makers learned that a similar texture could be obtained if the dry curds, after draining, were simply stirred in the vat. An additional variation of this process involves adding cold water to the curds. As noted above, this step results in less lactose in the curd, and it also increases the moisture. The resulting cheese, known as Colby, is a less acidic, high moisture cheese, compared to traditional Cheddar. In some modern cheese factories, curds are continuously conveyed to the top of Cheddaring towers. By the time the curds have descended from these vertical towers and emerge out at the bottom, they have achieved a similar level of Cheddaring as traditional curd.

After Cheddaring, or when the pH is about 5.2 to 5.4, the plastic, elongated Cheddar slabs are chopped up in a special milling device (called, appropriately, a Cheddar mill) to reduce the slabs to uniform, thumb-sized pieces. These Cheddar curds are bland, with a squeaky, rubbery texture. Next, salt is applied, in an amount ranging from 2% to 3%, and in a manner that permits even salt distribution (i.e., while the curds are continuously being stirred).

Salting is a critical step, since it has a profound effect on the quality of the finished cheese. In addition to providing flavor, salt has a major influence on controlling microbial and enzymatic activities in the ripening cheese. Although 2% salt might not be expected to have much of an effect on microorganisms or enzymes in food, one must consider that cheese consists of two phases, a fat phase and a water phase. It is in the water phase that the salt is dissolved, and likewise, that is where the microorganisms live. Since Cheddar cheese contains no more than 39% water (at the end of manufacture), the relevant salt concentration, i.e., that with which the microorganisms must contend, will be more than 5% ($2/39 \times 100 = 5.1\%$). At this salt concentration, the growth of both the starter culture bacteria and many of the other adventitious microorganisms, as well as the activities of microbial and milk-derived enzymes are effectively controlled. This is not to imply that microbial and enzymatic activities are actually halted, because salt-tolerant organisms and enzymes remain active in the presence of high salt concentrations. Rather, the salt provides a means to check or contain those activities and to create a selective environment that aids in establishing a desired microbiota.

The active salt concentration is often referred to by cheese manufacturers as the salt-in-moisture or S/M ratio. For example, a cheese containing 2.0% salt and having a moisture content of 38% will have an S/M of 5.3. The S/M value is arguably the main determinant affecting cheese ripening. The higher the S/M level, the more inhibitory or restrictive that environment will be to microorganisms and enzymes. In contrast, for cheeses having low S/M values (i.e., below 4.5), the microbiota will not be effectively constrained or controlled. If the "wrong" organisms or enzymes are present, production of off-flavors and other spoilage defects may occur. High S/M levels can also be problematic. Thus, cheese ripening rates, which are also a function of microbial and enzymatic activities, will be inhibited if the S/M is too high. Thus, cheese manufacturers must carefully adjust salt concentrations and moisture levels (as well as pH) to achieve the desired ripened cheese properties. In general, an S/M between 4.5 and 5.5 is desirable.

The next step involves filling forms, hoops, or barrels with the salted curds. The size of these forms range from 9 kg to as high as 290 kg (20 pounds to 640 pounds). Obviously, the form also gives shape to the cheese, varying from rectangular blocks to barrels. Many of the cheese forms have collapsible ends, such that pressure can be applied to press the curds together. This enhances the transformation of the curds into a solid mass and also helps to squeeze out whey (through perforations in the hoops). Usually, about 138 kPa (20 psi) is applied for 12 to 16

hours. The cheese is then removed from the forms, and, for the 9 kg and 18 kg blocks, placed in oxygen-impermeable bags, vacuum is applied, and the bags are sealed. The cheese is moved into cold rooms, ranging in temperature from 4°C to 12°C for storage or aging.

Cheese with eyes

The intentional encouragement of CO_2 formation occurs in several cheeses. However, it is primarily the Swiss-type cheeses for which quality depends so much on proper development of the CO_2 bubbles known in cheese vernacular as "eyes". Indeed, Emmenthal (or Swiss) cheese, the most famous of the eye-containing cheeses, is often considered by experienced cheese makers to be the "easiest cheese to make but the hardest cheese to make well". This is because the manufacture of high quality Swiss cheese, and proper eye development, in particular, depends on two rather independent processes. First, Swiss cheese requires excellent curd handling technique, such that the conditions are correct for eyes to form. Second, there must be precise control over the microorganisms that are involved in the fermentation and that produce the CO_2 gas that ultimately results in eye formation. It is entirely possible to produce a cheese that tastes like Swiss cheese, but that has small eyes, large eyes, irregularly-shaped eyes, too many eyes, too few eyes, or no eyes. The goal, of course, is a cheese with the correct flavor and body characteristics *and* that has just the right number of uniformly distributed, evenly-shaped round eyes, all within the desired size range. The trick, therefore, is to manage the early cheese making steps such that the curd is within the correct pH and lactic acid levels and has the right texture and elasticity necessary to accommodate the carbon dioxide that is produced later in the process.

The manufacture of Swiss cheese might seem easy because the steps involved in curd making appear rather simple and straightforward. However, care must be taken from the outset. First, the milk is usually standardized to 3% fat. If too much or not enough fat is present in the milk, the body will be too soft or too hard, leading to poor eye development. Raw milk may be used, but it is not uncommon to give the milk a modest heat treatment, about 50–56°C for 15 seconds. This is below that used for pasteurization but it is still effective at inactivating many of the milk-borne bacteria that could otherwise cause problems during the ripening period. Finally, the quality of the cheese also depends heavily on the performance of the bacteria used in the mixed species starter culture, as well as the ratio of those organisms.

To achieve the texture and body characteristics necessary for proper eye development, Swiss cheese must have the correct pH and moisture content and retain (or lose) the right amount of calcium. The curds are cut to about rice-sized particles and then are cooked to a much higher temperature than for Cheddar-type cheeses. Typically, the temperature will be raised over a 40-minute period from 32°C to 35°C (the temperature at setting) to as high as 55°C, and then is held at that temperature during the stir-out step for another 45 minutes or until the curd is sufficiently dry. Thermophilic cultures that are tolerant of high temperature, therefore, are used for most types of Swiss cheese (see below). There are, however, some versions that include mesophilic *L. lactis* subsp. *lactis* and use lower cooking temperatures.

It is important to note that reducing the moisture in the curd will also result in less lactose in the curd. As noted above, controlling the amount of lactose in the curd is an effective way to limit the amount of acid that will ultimately be produced during fermentation, an important consideration that will be further discussed later. High cooking temperatures also slow acid development and prevent solubilization of calcium phosphate into the whey. The whey

is drained at the end of cooking, while the pH is still high (about 6.3). This keeps phosphate in the curd, which promotes buffering and keeps the pH from becoming too low later during the fermentation.

In the traditional process, when the curds are sufficiently firm, they are collected or dipped into cheese cloth, which is then placed into a large round form. In a modified version used by many US manufacturers, the curds are allowed to settle under the whey, sometimes with weights applied, then the whey is drained, the matted curd is cut into block-sized sections, and placed into forms. In larger operations, curd-whey mixtures are pumped into draining vats and then the curd is filled into large forms. For all of the processes, the cheese blocks are then pressed and held for up 16 hours at near ambient temperature.

As described above, for Cheddar cheese the fermentation occurs in the vat or on draining tables. In contrast, for Swiss cheese there has been no opportunity, up this point, for fermentation. Rather, the fermentation does not begin until after the cheese is out of the vat and filled into forms. In addition, the fermentation takes a much longer time, as much as 24 hours. The cheese, however, is still warm following the cooking step, so the internal temperature may be as high as 35°C for several hours. The actual fermentation is quite different from that which occurs during Cheddar cheese manufacture. In Cheddar cheese, the culture contains mesophilic lactococci, and although more than one species may be present (e.g., *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*), they are obviously closely related and serve an almost identical function. In contrast, the Swiss cheese culture contains three different organisms, from three different genera. Included are two lactic acid bacteria, *S. thermophilus* and *L. helveticus*, and one non-lactic, adjunct organism, *Propionibacterium freudenreichii* subsp. *shermani*. All three are essential, and all are responsible for the fermentation pattern that is unique to Swiss cheese.

The culture that is initially added to the milk contains different proportions of each organism. The *S. thermophilus* generally outnumbers the *L. helveticus* by as much as ten to one. Therefore, at the outset, growth of *S. thermophilus* occurs first, in part because it is present at a higher concentration, but also because its simple physiological requirements are more easily met, especially compared to the more fastidious *L. helveticus*. As noted previously, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* have a cooperative relationship, such that growth of one organism promotes growth of the other, and *S. thermophilus* and *L. helveticus* share a similar relationship. Thus, in Swiss cheese, *S. thermophilus* growth is stimulated by amino acids and peptides released from casein via *L. helveticus* proteinases. Likewise, growth of *L. helveticus* does not commence until the pH and Eh within the cheese are sufficiently reduced by *S. thermophilus*, a period that may take as long as twelve hours.

Another factor that influences the outcome of the Swiss cheese fermentation relates to manner in which these organisms ferment lactose. Specifically, when *S. thermophilus* ferments lactose, only the glucose portion is used and nearly all of the galactose is secreted into the curd. Active growth of *L. helveticus* does not begin until after about eight to twelve hours, and then this organism, along with *S. thermophilus,* consumes the remaining lactose. However, *L. helveticus* is also able to ferment the galactose left behind by *S. thermophilus*, such that after about 18 to 24 hours all of the carbohydrate in the curd has been fermented.

If the curd had contained just the right amount of lactose at the start of the fermentation (i.e., after cooking), and all of the lactose and its constituent monosaccharides were fermented to completion, then the final pH should be very near 5.2 ± 0.1. If there is too much lactose or too little lactose at the start, due to insufficient or excessive cooking, then the pH

can end up being less than 5.0 or above 5.4. Both of these situations could result in poor quality cheese, as described below.

After the primary fermentation period, the cheese blocks or wheels are placed into brines containing 20% salt for as long as three days. The blocks may be flipped and additional salt may be applied. Then the blocks are removed and allowed to air dry in coolers at 10°C to 15°C for five to ten days. As noted for Cheddar cheese, salt provides flavor and influences the activities of microorganisms and enzymes present in the cheese, although the average concentration in Swiss cheese is much lower (<1%). In the case of traditional Swiss cheese and other brined cheeses, salt also helps form a natural rind, due to dehydration at the surface of the cheese. The hard rind provides an excellent natural protective barrier or casing. Brining, in contrast to direct or dry salt methods, also creates a salt gradient, with the concentration decreasing from the surface toward the interior of the cheese. This also affects the development of the microbiota.

The next step is perhaps the most crucial. The dried blocks or wheels are moved into warm rooms where the temperature is maintained at 20°C to 25°C. It is during the ensuing three to four weeks that growth of *P. freudenreichii* subsp. *shermani* occurs. Although this bacterium is morphologically, physiologically, and genetically distant from the lactic acid bacteria, it is similar in that it prefers an anaerobic atmosphere and has a fermentative metabolism. Propionibacteria are neutrophiles and are salt-sensitive, so low pH and high salt conditions are inhibitory. They are added to the milk as part of the starter culture, but at a much lower rate – the inoculated milk contains only about 10^2 to 10^3 cells per ml. And although these bacteria are moderately resistant to high temperature, growth does not occur until the cheese is moved into the warm room. When these bacteria are grown on lactose or other fermentable carbohydrates, large amounts of propionic and acetic acids and lesser amounts of carbon dioxide are produced. However, as explained above, by the time Swiss cheese is placed in the warm room, there is little to no carbohydrate still in the curd and available for fermentation. This begs the question – what does *P. freudenreichii* subsp. *shermani* use as a substrate? The answer is somewhat surprising, in that this organism metabolizes the lactic acid that was originally produced by the lactic starter culture. The metabolic pathway used by *P. freudenreichii* subsp. *shermani* is called the propionate pathway (Figure 6.6). The pathway yields propionic acid, acetic acid, carbon dioxide and ATP. If, however, any lactose were still available, then more of these products will be formed, usually to the detriment of

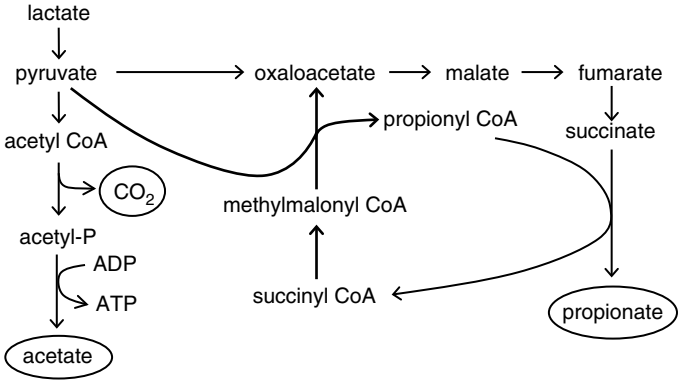


Figure 6.6 The propionic acid pathway of propionibacteria. Only the key intermediate compounds are shown. Adapted from Hutkins, 2001.

the cheese. This is because excess production of end-products, and carbon dioxide, in particular, can result in serious consequences for the final product.

As noted above, *P. freudenreichii* subsp. *shermani* is initially present at relatively low levels in the cheese. As it grows in the warm room, small micro-colonies within the curd matrix are formed. Likewise, the fermentation end products from those micro-colonies are evolved in that same vicinity and diffuse out into the neighboring region within the curd. Although the acids are readily dissolved, the carbon dioxide molecules diffuse through the curd only until they reach weak spots. There, they meet up with other CO_2 molecules made by other nearby micro-colonies. Eventually, enough CO_2 molecules will have accumulated, and *voila* – an eye is formed. However, this entire sequence of events depends on several hard-to-control variables.

First, CO_2 formation must be slow and steady. If too much CO_2 is produced all at once or the curd is too firm, the gas pressure can exceed the capacity of the curd to sustain the gas. If this occurs, large or even exploded holes are formed. If the body of the curd is too soft and the weak spots too numerous, then many small eyes will form. The hard rind produced as a result of the brining and drying steps also serves an important role; without a rind, the CO_2 could theoretically escape clear out of the cheese. Of course, CO_2-impermeable bags now provide an easy remedy for this problem (thus was born rindless Swiss, as described below). Obviously, time is a critical factor as well–too much or not enough incubation time will result in less than perfect eye development. Experienced cheese makers have adopted various professional tricks, from tapping the cheese and listening for just the right echo, to visually examining the expansion of the wheel or block.

Finally, when the cheese leaves the warm room, it is moved into a cooler at 2°C to 5°C for aging, which can vary from three months to two or more years. In addition to the acid products (i.e., propionic and acetic acids), *P. freudenreichii* subsp. *shermani* is also responsible for producing other important end-products. In particular, *P. freudenreichii* subsp. *shermani* produces specialized peptidases that release proline, which has a sweet-like flavor. Various other amino acids and peptides are also metabolized, generating nutty flavors that are characteristic of Swiss cheese.

Variations of the traditional Swiss or Emmenthal cheese (made in Switzerland) include Gruyere (France), Jarlsberg (Norway), and Samsoe (Denmark). All of these have a natural rind and varying levels of eye formation. In the United States, rindless versions are more common, with the cheese blocks wrapped in CO_2-impermeable plastic wrapping.

Mozzarella and Pasta Filata cheese

As a recently as the 1970s, Mozzarella was still considered a mostly ethnic cheese, used primarily as an ingredient in Italian cuisine. The popularity of this cheese – it is now the most popular cheese among American consumers – is due to one product: pizza. Of the more than 1.9 billion kg (4.2 billion pounds) of Mozzarella and related cheeses produced (USDA data for 2015), about 70% is used by the food service industry as an ingredient on pizza. According to USDA data for 2015, per capita consumption of Mozzarella cheese exceeds 5 kg per person per year. From 1980 to 2015, Mozzarella production has increased by a whopping 580% (16% annually). In fact, starting in the 1980s, many cheese factories that once made Cheddar converted their operation to Mozzarella manufacturing. And despite the apparent differences in flavor and appearance between Cheddar and Mozzarella cheese, both share several common manufacturing steps.

In the United States, all Mozzarella cheese is made from pasteurized milk. In large part, this is because Mozzarella is considered a fresh cheese that is never aged or ripened. Thus, legal requirements dictate that the milk be pasteurized. Although some Mozzarella cheese is made from whole milk, most is made from reduced fat or partially skimmed milk. Special considerations for the manufacture of reduced fat Mozzarella and other low- or reduced fat cheeses will be described later. Although almost all Mozzarella cheese made in the United States comes from cow's milk, it is worth noting that some traditional Italian Mozzarella is made from the milk of water buffalos.

After the milk is pasteurized and standardized, a thermophilic culture is added. Although the organisms used for Mozzarella, *S. thermophilus* and *L. helveticus,* are the same as those used for Swiss cheese, the specific strains and ratios are likely quite different. Strain selection for Mozzarella cheese is based, like all cultures, on the desired properties of the particular cheese. This is especially true for Mozzarella cheese, where the strains may have a profound effect on the functional properties of the finished cheese (Box 6.4).

Box 6.4 The serious science of making pizza

Given that the global pizza industry is a $128 billion industry (according to MPQ Pizza Magazine, December, 2016), that 350 slices of pizza are eaten every second of every day in the US alone, and that Mozzarella and pizza-type cheeses are the main ingredients on a pizza, it is not surprising that Mozzarella and other pizza cheese are now the most widely consumed cheeses. This also means that producers are under considerable pressure to produce cheese with the precise functional properties desired by customers. Thus, there is a lot at stake. And although the general manufacture of Mozzarella and other pizza cheeses is not complicated, making these cheeses, essentially to order, can be a tricky business.

Of course, pizza restaurants are the main users of Mozzarella, with several companies dominating the industry (including Pizza Hut, Dominos, Little Caesars, Papa Johns, and Godfathers). When considering the cheese, each pizzeria seems to have their own particular preference. Some pizza chains desire cheese that stretches a particular distance when a slice is moved from plate to mouth. Others want a cheese that remains white and resists browning even when the pizza is baked quickly at extremely high temperatures. Thus, Mozzarella production is now often customized to the exact needs of the customer, such that even before the vat is filled with milk, the manufacturer is ready to produce a cheese meeting not only compositional requirements, but functional specifications as well.

Because of these considerations, there is now much interest in understanding and predicting how a given cheese will perform during pizza manufacture and how manufacturing conditions can be manipulated to produce a cheese that has specific functional properties (Fox et al., 2016). Unlike most other cheeses, Mozzarella, at least when used on pizza, is prized less for its flavor and more for its physical and functional attributes. Mozzarella, after all is a bland, slightly acidic cheese, with no aged cheese flavor. However, it has an unusual ability to stretch, flow, retain fat, melt evenly, and provide a chewy mouth feel. These properties, however, are not necessarily automatic, and are influenced by the culture, the coagulant, the manufacturing conditions, and

post-manufacturing handling and storage. Ultimately, these factors affect pH, loss of calcium, proteolysis, and the overall composition of the finished cheese.

The metabolic activities of the thermophilic starter culture bacteria have a major impact on the finished product. After the curds are cooked and the whey is drained, the curds are either Cheddared or dry stirred. During this time, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (the main organisms used for Mozzarella) ferment lactose, producing lactic acid and causing the curd pH to decrease. However, only the glucose portion of lactose is metabolized and the galactose is released back into the curd.

When the pH reaches 5.2, the curds head off to the cooker-stretcher (operating at 85°C), which heats the curd to a temperature near 60°C. This process inactivates enzymes (although some residual chymosin might remain) and the starter culture (or at least reduces the cell concentration several fold). Thus, there is essentially no opportunity left for this galactose (and any remaining lactose) to ferment. Instead, it will remain intact and be present in the finished cheese.

When the cheese is exposed to high baking temperatures (which dry the cheese surface and reduce the water activity), the reducing sugars (i.e., galactose and lactose) react with amino acids, via the non-enzymatic Maillard reaction, forming brown pigments (Figure 6.4.1). Although moderate browning may be desirable, excessive browning or blistering is undesirable. Because most pizza manufacturers prefer white, non-browning cheese, steps must be taken to reduce the galactose concentration in the cheese. One common way to accomplish this is the "mechanic" approach, simply by washing the curds with water. Other sugar and moisture adjustment methods have also been devised to address this problem (Ma et al., 2013b; Ma et al., 2014; Moynihan et al., 2016).

The other method for managing lactose fermentations and minimizing galactose accumulation in the curd is the biological approach, specifically by using cultures that can ferment galactose (Mukherjee and Hutkins, 1994; de Vin et al., 2005; Wu et al., 2015). These cultures may consist of mesophilic or thermophilic bacteria, including *Lactococcus lactis* subsp. *lactis*, *Lactobacillus casei*, and *Lactobacillus rhamnosus* that generally have no problem fermenting galactose via tagatose or Leloir pathways (Wu et al., 2015). However, their use may require other changes in the manufacturing process (e.g., lower cooking temperature). Selected strains of *Lactobacillus helveticus* (that are thermophilic and galactose-fermenting) can also

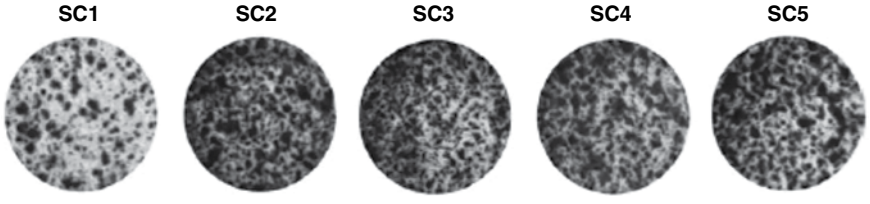


Figure 6.4.1 Mozzarella cheeses made with different strain combinations of *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus helveticus*, and *Streptococcus thermophilus* and baked at 232°C for 5 minutes. From Ma et al., 2013a, with permission. See the original figure for experimental details.

be used. Culture suppliers may also be able to provide selected galactose-fermenting variants of normal Mozzarella strains (i.e., *S. thermophilus* and/or *L. delbrueckii* subsp. *bulgaricus*).

The culture, and specifically, the proteolytic activity of the *Lactobacillus* (either *L. delbrueckii* subsp. *bulgaricus* or *L. helveticus*), also affects other important properties. The ability of Mozzarella cheese to stretch is largely a function of protein structure. If, however, the culture is proteolytic (or residual coagulant activity is present in the curd after cooking), protein hydrolysis will occur, and the shortened casein strands will lose their ability to stretch. This may or may not be a good thing, depending on the preference of the pizza manufacturer (Dave et al., 2003).

If a short, non-stretching cheese is desired, then greater proteolysis may be encouraged. However, manipulating any one variable to affect a given property will likely affect some other property. As noted previously, extensive proteolysis makes the cheese "soft" and "gummy", and less amenable to shredding. In addition, it is the protein network that traps fat in Mozzarella. During baking, the fat melts and liquefies, but is retained by the protein matrix. If proteolysis has occurred and the protein network is disrupted, the fat is no longer contained and leaks out. On a pizza, the fat forms unsightly pools of melted oil, a defect known as oiling-off. The ability of the shreds to remain visibly intact may also be important for consumers. Finally, since proteolysis increases the free amino acid concentration, the more proteolytic the culture (or the coagulant), the greater the rate of the Maillard reaction and the potential for browning problems.

Ordinarily, the amount of time between manufacture of Mozzarella and its use on a pizza is only a few weeks. Thus, proteolysis in Mozzarella is nowhere near as extensive as in an aged cheese. Still, even moderate proteolysis can affect cheese functionality. Many Mozzarella producers, therefore, freeze the cheese within a few days of manufacture, giving it just enough aging time to develop appropriate properties. Some manufacturers have even developed processes in which the cheese is frozen within hours of manufacture.

Manufacturing variables have also been modified to affect other functional properties. For example, most Mozzarella is eventually used in a shredded or diced form, so producing a cheese that is easy to shred, without gumming up the equipment, is important. These modifications have led some to question whether the cheese produced is actually Mozzarella, as defined by the standards of identity. Nonetheless, it seems likely that the demand for Mozzarella or Mozzarella-type pizza cheese with particular functional characteristics will continue to be a driving force for innovative research within the Mozzarella and starter culture industries. Economic considerations, where 14 inch diameter pizzas (1000 cm^2), with toppings, sell for as little as $10, will put considerable pressure on Mozzarella manufacturers to deliver all of those functional properties for rock bottom prices.

References

Mukherjee, K.K. and R.W. Hutkins. 1994. Isolation of galactose fermenting thermophilic cultures and their use in the manufacture of low-browning Mozzarella cheese. J. Dairy Sci. 77:2839–2849.

Dave, R.I., D.J. McMahon, C.J. Oberg, and J.R. Broadbent. 2003. Influence of coagulant level on proteolysis and functionality of mozzarella cheeses made using direct acidification. J. Dairy Sci. 86:114–126.
Fox, P.F., T.P. Guinee, T.M. Cogan, and P.L.H. McSweeney. 2017. Cheese as an Ingredient. In: Fundamentals of Cheese Science. Springer, New York, pp. 629–679.
Ma, X, M.O. Balaban, L. Zhang, E.A.C Emanuelsson-Patterson, and B. James. 2014. Quantification of pizza baking properties of different cheeses, and their correlation with cheese functionality. J. Food Sci. 79:E1528–E1534.
Ma, X., B. James, M.O. Balaban, L. Zhang, and E.A.C. Emanuelsson-Patterson. 2013a. Quantifying blistering and browning properties of mozzarella cheese. Part I: Cheese made with different starter cultures. Food Res. Int. 54:912–916.
Ma, X., B. James, M.O Balaban, L. Zhang, and E.A.C. Emanuelsson-Patterson. 2013b. Quantifying blistering and browning properties of mozzarella cheese. Part II: Cheese with different salt and moisture contents. 2013. Food Res. Int. 54:917–921.
Moynihan, A.C., S. Govindasamy-Lucey, M. Molitor, J.J. Jaeggi, M.E. Johnson, P.L.H. McSweeney, and J.A. Lucey. 2016. Effect of standardizing the lactose content of cheesemilk on the properties of low-moisture, part-skim Mozzarella cheese. J. Dairy Sci. 99:7791–7802.
Wu, Q., C.K.W. Cheung, and N.P. Shah. 2015. Towards galactose accumulation in dairy foods fermented by conventional starter cultures: challenges and strategies. Trends Food Sci. Technol. 41:24–36.
de Vin, F., P. Radstrom, L. Herman, and L. De Vuyst. 2005. Molecular and biochemical analysis of the galactose phenotype of dairy *Streptococcus thermophilus* strains reveals four different fermentation profiles. Appl. Environ. Microbiol. 71:3659–3667.

After rennet addition, coagulation, and cutting, the curd-whey mixture is pumped into draining tables, where the curds are gently stirred and then cooked to 56°C. The whey is drained and the curds are either allowed to mat, as far Cheddar, or are dry-stirred (as far stirred-curd Cheddar). Frequently, the curds will be washed after draining to reduce lactose levels. Mozzarella is a brine-salted cheese; however, salt may also be added directly to the curds prior to the cooking-stretching step to minimize the lengthy brine-salting time ordinarily required.

The fermentation of lactose in Mozzarella is similar to that which occurs in Swiss cheese. In both fermentations, galactose appears in the curd as a result of the inability of *S. thermophilus* to efficiently metabolize both of the monosaccharide moieties of lactose. In Swiss cheese, there is plenty of time for the companion strain (i.e., *L. helveticus*) to eventually ferment the galactose and remaining lactose. However, the situation is quite different for Mozzarella. This is because when the pH during Cheddaring or dry-stirring approaches 5.2, the curds are moved to a cooker-stretcher device that exposes the curds to temperatures as high as 85°C. Although the curd temperature may not reach such a high temperature (usually the curd is less than 60°C), this treatment is still sufficient to inactivate many of the starter culture bacteria present in the cheese. Thus, any galactose or lactose still in the curd at the cooking-stretching step will likely remain in the cheese for the duration of its manufacture and storage. The consequences of this are discussed in Box 6.4.

In addition to its effect on the culture and residual enzymes, the cooking-stretching step also has an important impact on the physical properties of the cheese. Prior to this step, Mozzarella curds are not much different than other rennet set curds. However, the cheese exiting the cooking-stretching machines has properties unlike any other cheese. The cooking-stretching machines use augers to knead, stretch, and convey the curds in an upward manner (about a 30 degree incline), all the while exposing the curds to a hot water-steam mixture at

85°C to 90°C. In the United States and Italy, almost all Mozzarella is stretched in high through-put continuous cooker-stretcher devices. Only a relatively few very small manufacturers rely on traditional techniques – manually kneading the curd in hot water is an arduous and time-consuming task.

Although what actually happens to the casein matrix during this step is not clearly understood, it appears that the casein network becomes linearized, giving the fibrous property that is characteristic of Mozzarella cheese and desired by consumers. It is critical that the cooking-stretching step occurs when the curd pH reaches 5.2, otherwise the cheese will be short-textured and will stretch poorly. Finally, the molten, plastic, and fluid curds are then dropped into a hopper and filled into molds of varying loaf sizes and shapes. The cheese sets up quickly in the molds and is then dropped into a chilled salt brine. After brining (about 16 hours, but much less if dry salt had been added to the curd), the cheese is dried and packaged. In Italy and in small US factories that employ traditional manufacturing practices, Mozzarella can be formed into small round shapes, which are then packaged in water or dilute brines.

Mozzarella cheese, as noted above, is not aged; however, even within just a few days, important functional properties can change. One of the first changes that occurs is the equilibration of calcium, that which exists as part of the casein complex and that in the form of calcium phosphate. Importantly, the loss of calcium from the casein complex improves the melting and stretching properties of the cheese.

The other main change in the functional properties of Mozzarella is due to protein hydrolysis. Proteolysis can occur as a result of residual proteinases released by the lactic starter culture, as well as by residual coagulant (which appears to be the main source of proteinase activity). Although flavor changes due to protein hydrolysis are usually minor, texture properties can be significant. As the long casein strands are hydrolyzed, the cheese has less stretching capacity. Also, the hydrolyzed casein is less able to hold or contain fat. The latter may result in a defect known as "oiling off" that is readily apparent when the cheese is used in pizza and other cooked products. Also, since most Mozzarella cheese is marketed in a shredded form, excessive proteolysis makes the cheese less shreddable. For these reasons, fresh Mozzarella is best used within two to three weeks. Alternatively, the cheese can be frozen shortly after manufacture or after shredding (the preferred form for most of the large pizza manufacturers), and although this will prevent proteolysis, freezing is not without its own set of effects.

Mozzarella is but one of several so-called pasta filata (Italian for stretched curd) types of cheese. Another well-known variety is Provolone cheese. This cheese is made essentially as for Mozzarella except that a lipase preparation is added to the milk to promote lipid hydrolysis and release of free fatty acids. Lipases can be either fungal-derived or are from animal tissues. A slightly rancid but not unpleasant flavor, described as "picante", develops during a short aging period. Provolone is often shaped into pear-shaped balls and is frequently smoked.

Hard Italian cheese

Given the popularity of Mozzarella cheese and Italian cuisine, in general, it is not surprising that production of other Italian cheeses, such as Parmesan, Romano, and other hard grating types, have increased by more almost 200% in the last 20 years. Although Parmesan is the prototype of the hard Italian grating type cheeses, there are many variations of this cheese, including Romano, Asiago, and Grana.

As recently as 2002, the US Food and Drug Administration's official Standard of Identity for Parmesan was changed to accommodate newer methods of manufacturing, such that less aging was required. In contrast, Parmesan made in Italy must conform to rigid manufacturing procedures if it is to be called Parmigiano Reggiano. The milk, for example, must be obtained from cows raised in specific regions and fed a specified diet. Only raw milk can be used, and it must be standardized in a specific manner (which includes a natural creaming step). Calf rennet is used for coagulation. No commercial starter culture can be used; instead, whey is the only allowed source of starter bacteria. The cheese is ultimately aged for at least 12 months, but usually longer (two to three years). Despite these considerable challenges, annual production of Parmigiano Reggiano is substantial and continues to grow (nearly 140 million kg in 2016). However, even in Italy it is expensive (often more than $25 per kg). Recently, the European Union ruled that only cheese made following these prescribed procedures could be called Parmesan. Other famous European cheeses, including Roquefort and Feta, have received similar EU protections (so called Designation of Protected Origin or DPO).

In the United States, where manufacturers are not bound by EU rules, Parmesan is made from cow milk standardized to about 2.4% to 2.8% fat. The milk is inoculated with a thermophilic culture and rennet is added. The curds are cut to give small curds, similar to Swiss, cooked to 45 to 48°C, and stirred for up to 45 minutes. Some acid development during the stir-out step occurs, and when the pH reaches about 5.9, the whey is drained. The curds are placed into forms and pressed for 12 to 16 hours, during which time the thermophilic culture ferments the lactose. Next, the cheese is brined for two weeks and dried for up to six weeks (to obtain a moisture content of 32%). Aging is essential for Parmesan cheese, and until recently, ten months of aging was required to satisfy the US Standards of Identity (and to produce good flavor).

In 1999, Kraft successfully petitioned the FDA to permit cheese aged for a minimum of six months, instead of the nine months, to be called Parmesan, provided the original characteristics and properties were still maintained. Apparently, the use of enzyme technologies, combined with a slight increase in ripening temperatures, can produce a cheese that, while not intended to be the quality equivalent of traditional, well-aged Parmesan, still has a clean and acceptable flavor that is quite suitable as a grating cheese. One of the more popular variations of Parmesan, Romano, is made in a similar manner as Parmesan, but it contains lipase and, at least in Italy, is made from sheep's milk. One of Italy's oldest cheeses, Pecorino Romano, has PDO status.

Dutch-type cheeses

Among the most pleasant-tasting, colorful, and microbiologically complex cheeses are the so-called Dutch-type cheeses, of which Edam and Gouda are the most well-known. In fact, there are comparable cheeses produced throughout the world, using similar manufacturing procedures. Although eyes are usually present in these cheeses, there are fewer of them and they are much smaller than in Swiss cheese. The texture and flavor is also completely different from Swiss – these cheeses are softer, with a sweet, mild, buttery flavor. They are also easily distinguished from other cheeses by the characteristic yellow, orange, or red wax that covers the round wheels.

Although the basic manufacturing procedures for these cheeses are similar to those already described for other cheeses, there are several unique steps. The starter culture contains, in addition to acid-forming lactococci, one or more flavor-forming lactic acid bacteria.

The latter include various species of *Leuconostoc*, including *Leuconostoc mesenteroides* subsp. *cremoris* and *Leuconostoc lactis.* Not only are these organisms heterofermentative, producing lactic acid, acetic acid, ethanol, and carbon dioxide, but they are also capable of fermenting citrate. Fermentation of citrate by these bacteria results in formation of diacetyl (see Chapter 3), which imparts the buttery, creamy flavor that characterizes these cheeses. In addition, the citrate fermentation pathway generates carbon dioxide, accounting for the eyes that are often present.

After culture and rennet addition, cutting, and cooking (at about 38°C), another unique manufacturing step occurs. A portion of the whey is drained, and then hot water is added (maintaining the temperature at 38°C) to very near the original volume. Stirring continues until the desired curd firmness is achieved, and the remaining whey-water mixture is drained. This washing step, as described previously, effectively reduces the lactose concentration in the curd, making less available for the lactic culture. However, by using hot water, the moisture content is not increased. Ultimately, the cheese will be sweeter, and less acidic, with a final pH between 5.4 and 5.6.

Surface-ripened by bacteria

Among the cheeses that are the most aromatic and flavorful, Limburger, Munster, and other surface-ripened cheeses are right at the top of the list. A well-ripened Munster can fill the room with the strong sulfury volatile compounds that are produced by *Brevibacterium linens*. This organism (and others that are generally present; see below) is neither added to the milk nor curds, but rather is applied to the cheese after its manufacture. As profound as is the aroma and flavor of this cheese, its orange-red appearance is nearly as dramatic. In fact, the natural pigment produced by *B. linens* that accounts for the color of these cheeses has recently found use as a coloring agent in other food products.

The reader is probably wondering why the Munster or Brick cheese common to US consumers lack the punch described above, despite having the expected appearance. This is because the early manufacturers of these cheeses, who typically had emigrated from European countries where these cheeses were popular, realized that US consumers preferred more mild-flavored cheeses. Thus, the manufacturing process was modified to accommodate the particular preferences of the new American customers. Thus was born the mild-flavored Munster and Brick cheeses that are so widely consumed today. Although the preference for the mild flavored versions continues even today among many consumers, the more authentic versions have made a come-back (see below).

Limburger, Munster, Brick, and related cheeses actually belong to a family of cheeses referred to as washed rind or smear-ripened. They are initially made according to rather standard procedures. Whole milk is inoculated with a mesophilic lactic culture (*L. lactis* subsp. *lactis*) and chymosin is added. The coagulated milk is cut and stirred, but the curds are cooked to more moderate temperatures, usually between 30°C and 34°C. The curds are collected into open-ended, brick-shaped forms, which are turned every three to five hours to promote whey drainage. After about twelve hours, the cheese is brined or dry salted at the surface and held in warm (20°C) and humid (90% relative humidity) rooms.

To inoculate the surface organisms, the fresh cheeses are washed with suspensions of organisms obtained from well-ripened cheeses, a form of backslopping. The endogenous microbiota present on the shelves in the ripening room can also be used to initiate surface ripening (i.e., by direct transfer of "smears" from one cheese to another). Under the warm, humid, and aerobic conditions, ripening does not take long, and there will be considerable

surface growth, color formation, and flavor development within just a couple of weeks. The formation of alkaline end-products can raise the pH to near-neutral. The cheese is packaged and moved into a 10°C cooler for another month or two. These cheeses can easily become over-ripened, so they must be kept at low temperature. The packaging materials usually include parchment, wax paper, and foil to minimize oxygen availability (and to contain volatile aroma).

The microbiota of these cheeses can be complex. Among the organisms present in these cheeses are *B. linens,* as well as micrococci, *Corynebacterium*, *Arthrobacter*, and various yeasts. The contribution of the latter organisms is not clear. Commercial cultures are also available. They typically contain *B. linens*, but other organisms including fungi from the genera *Debaryomyces* and *Geotrichum* may also be included. These cultures can be added to the brine, sprayed, or smeared onto the surface.

The strong aroma and flavor of Limburger, Munster, and related cheeses is due to production of several volatile compounds. In defense of these cheeses, it is fair to say that the bark is worse than the bite, in that the flavor is nowhere near as strong as the aroma might portend. In fact, these so-called washed-rind, surface-ripened cheeses have fast become popular in the United States. Many artisan or farmstead cheese makers have adopted this style, and are producing award-winning cheeses. Thus, domestic versions of Tilsiter, Raclette, Beaufort, Gruyere, and other similarly-produced cheeses are now more widely available. In 2016, a Wisconsin-made, Alpine-style cheese called Little Mountain received the Best in Show award at the American Cheese Society competition, besting more than 1800 other cheeses.

Mold-ripened cheese

Aside from the fact that the two main mold-ripened cheeses, the blue-type and the Brie-type, are both fungal fermentations, they share few common properties. The blue mold cheeses contain visible mold growth throughout the curd interior and relatively little at the surface. The mold responsible, *Penicillium roqueforti*, produces blue-green spores, in addition to a myriad of enzymes that ultimately generate typical blue cheese flavors. In contrast, the Brie-type cheeses are made using *Penicillium camemberti*, which produces white mycelia, and grows only at the surface. Blue cheese is acidic, salty, brittle and crumbly, while the Brie-type cheeses are satiny smooth, soft, and creamy, with a near neutral pH.

1. Blue mold-ripened cheese

Although blue mold-ripened cheeses are made throughout the world, three specific types have achieved a significant measure of fame to warrant their own name (and have PDO status). Roquefort, perhaps the most well-known of all blue cheeses, must be made according to a strict set of manufacturing requirements. For example, the milk must come from specially-bred sheep that have grazed in the Causses region of France. The milk is neither pasteurized, standardized, nor homogenized, and the cheese must ultimately be aged in caves within that same region. In Italy, the manufacture of Gorgonzola cheese is similarly restricted to the Po Valley region of Northern Italy and its manufacture is also subject to specified procedures. This cheese is made from cow's milk, but is otherwise very similar to Roquefort. Gorgonzola, however, is usually not quite as strongly flavored as Roquefort. Finally, the English representative to the blue-mold family is called Stilton.

It also is made from cow's milk and only in three counties of central England. Many other blue mold versions are produced in Europe and throughout the world and, despite the fame of the PDO cheeses are of excellent quality.

In general, the manufacture of blue mold-ripened cheese requires several specialized steps. In large part, the goal is to ensure that the aerobic mold can grow well within the interior of the cheese, where the Eh is ordinarily low. Although the blue mold organism, *P. roqueforti*, is capable of growing at relatively high CO_2 and low O_2 levels, air incorporation is still an important feature of the process. The coagulated milk is cut, when very firm, into larger than normal-sized curds (as much as 2.5 cm). As noted earlier, the larger the curds at cutting, the higher the moisture will be in the cheese. However, an additional effect is to create a more open or porous texture such that diffusion of air is increased.

Frequently, the mesophilic lactic starter culture, in addition to containing *L. lactis*, will also include heterofermentative *Leuconostoc* or citrate-fermenting lactic acid bacteria. These bacteria produce CO_2 that contributes to the open texture that enhances oxygen diffusion and gas exchange. Spores of *P. roquefortii* can be added to the curds or to the milk, along with the lactic starter culture, prior to setting. Later, once the cheese is hooped and the lactose fermentation is complete, the cheese wheels are brined or dry-salted or both. At this point, some manufacturers mechanically aerate the cheese. This is done using a set of large bore needles (diameter about 0.24 cm) to pierce the cheese and provide a mean for air to penetrate the inner portions. Later, when the cheese is cut vertically, one can see that mold growth followed the spike lines where oxygen was available.

Next, the cheese is aged for several weeks in a warm (10°C to 12°C), humid (90% to 95% relative humidity) environment that promotes growth of the *P. roqueforti* throughout the interior of the cheese. Lower ripening temperatures (4°C to 8°C) and longer times may also be used. Since the only way to arrest further mold growth is to cut off its supply of oxygen, the ripened cheeses are then wrapped in oxygen-impermeable foil. Although some consumers like blue cheese flavor, they may not be too fond of the moldy appearance. Thus, some manufacturers may limit growth of *P. roqueforti*, giving a somewhat whiter cheese with only a few streaks of blue. Even consumers who enjoy blue veined cheese may object to extensive growth at the surface, so the wheels are often cleaned prior to final packaging. Pimaricin may also be applied to control surface growth.

Although other organisms, including yeast and bacteria, are often present in these cheeses, especially at the surface, the flavor compounds characteristic of blue cheese clearly are generated primarily via mold growth and metabolism. Fungi, in general, are prolific producers of proteases, peptidases, and lipases, and *P. roqueforti* is no exception. Thus, the release and subsequent metabolism of protein hydrolysis and lipolysis products are important in blue cheese flavor. Of particular importance are ammonia and amines, derived from amino acid metabolism, and methyl ketones, derived from free fatty acids.

The free fatty acids, by themselves, may also contribute to cheese flavor, but their metabolism, via β-oxidation pathways to 2-heptanone, 2-nonanone, and other ketones, are primarily responsible for the flavor of blue cheese. As much as 20% of the triglycerides in the milk may be hydrolyzed. When blue cheese is made from raw milk, natural milk lipases may also contribute to formation of free fatty acids. These lipases are loosely associated with the surface of the casein micelles and are dislodged by agitation. In addition, lipase substrates (triglycerides) are exposed when the fat globule is disrupted. Therefore, one useful trick is to add a portion of homogenized raw cream to the cheese milk to accelerate flavor development.

Finally, it is important to note that mold growth during blue cheese ripening is accompanied not just by flavor development, but also by a marked increase in pH. This occurs because *P. roqueforti,* not having lactose on which to grow (all of the lactose is fermented by the starter culture), instead uses lactic acid as an energy source. Therefore, consumption of lactic acid causes the pH to rise from about 4.6 to above 6.0. Production of ammonia and other amines also contributes to an increase in pH. This increase in pH, in turn, promotes flavor production because many of the fungal decarboxylases and other enzymes involved in methyl ketone formation have neutral pH optima. However, as the pH approaches neutrality, there may be important food safety and preservation consequences. Specifically, acid-sensitive pathogens that are ordinarily kept in check or inhibited at low pH may be able to grow once the pH reaches non-inhibitory levels.

2. White mold-ripened cheese

The white mold-ripened cheeses, of which Camembert and Brie are the most well-known, are primarily made in France, where they are also among the most popular. These cheeses vary only slightly; Camembert is made principally in the Normandy region of France, whereas most Brie is produced in Melun and Meaux, just outside of Paris. Brie wheels are usually a bit larger, with bacteria on the surface contributing to flavor development.

Although similar versions of both Brie and Camembert are made in Germany, Switzerland, and other European countries, as well as South America and even the United States, manufacturing conditions are not that different. In general, whole cow's milk is used, and double or even triple cream versions exist in which the milk is supplemented with cream (75% fat, so not for the faint of heart). Since these cheeses are aged for as little as a few weeks, in the United States, the milk must be pasteurized. In France, however, raw milk is more frequently used. The milk is inoculated with a mesophilic starter culture, which is allowed to grow and produce acid before the chymosin is added. Spores of *P. camemberti* can also be added to the milk (or applied later to the surface of the hooped cheese). A related species, *Penicillium caseicolum* is also used, because it produces a somewhat whiter appearance.

As with blue cheese, the coagulum is firm when cut into large curd particles. The curds are gently stirred, but not cooked. Rather, they are almost immediately filled into forms. These steps decrease syneresis and result in cheese with high moisture levels. The hooped cheeses are flipped several times over an 18-hour period, during which time the lactose is fermented and the pH decreases to as low as 4.7. As with blue cheese, the cheese is then brined or dry-salted, and, if not already added to the milk, spores are applied, but only onto the surface.

The cheese wheels are then moved into ripening rooms (similar to those used for blue cheese, e.g., 10°C, high humidity). The cheese sits on shelves designed to promote contact with air, and are turned periodically. It takes only a week or two for a white mycelium mat to form across the entire surface. However, the extent of mold growth and subsequent flavor development depends on the target market, so the ripening time may be several weeks longer. In cheese made from raw milk, a diverse microbiota, consisting of yeasts, brevibacteria, enterococci and staphylococci, and coliforms, may emerge during extended ripening. Thus, not only will there be more flavor and texture development (see below) as ripening continues, but the surface mat may become less white and more pigmented. In the United States, a more mild-flavored, white-matted product is preferred, so ripening times are usually short. To control growth of mold, the cheese is wrapped and either held for more ripening or else stored at <4°C.

Not only do the Brie-type cheeses have a different appearance from the blue mold cheeses, but they also have a quite different flavor and texture. Despite these differences, the progression of flavor and texture development is similar. The proteinases and peptidases produced by *P. camemberti* are similar to those produced by *P. roqueforti*. Subsequent production of ammonia, methanethiol, and other sulfur compounds are derived from amino acid metabolism and are characteristic of Brie-type cheese. The scent of ammonia can be striking in well-aged Brie. Lipolysis of triglycerides and fatty acid metabolism by *P. camemberti* are also important, and methyl ketones can be abundant.

It is interesting that whereas blue cheese is crumbly and brittle, Brie-type cheeses are soft and creamy. The creamy, even fluid texture of these cheeses is now thought to occur as a result of protein hydrolysis, as well as the increase in pH due to ammonia. In particular, not only are fungal proteases important in texture development, but α_{s1} casein hydrolysis by chymosin and the natural milk protease, plasmin, is also involved.

For many of the cheeses discussed in this chapter, the geometry of the forms or hoops that give shape to the cheese have not been described in much detail. In many cases, whether the cheese is collected and shaped into rectangular blocks or round forms or whether the hoops or forms contain 5 kg or 200 kg has only modest bearing on the properties of the cheese. This is most definitely not the case for Brie-type cheese, where shape has a major impact on flavor and texture development. Since ripening depends on the fungi growing exclusively on the surface, the rate of flavor and texture development within the interior of the cheese is necessarily a function of the diffusion rate of enzymes and enzyme reactants and products from the exterior.

Although Camembert wheels usually have a smaller diameter (about 10 cm to 12 cm) than Brie (about 30 cm to 32 cm), their heights are essentially the same (about 3.5 cm) and the geometric core of these cheeses is never more than 2 cm from the surface. Thus, the enzymes and reactants (e.g., ammonia) produced at the surface have only a short distance to travel before reaching the center. If Brie cheese were to be made in larger or thicker wheels, like those used for blue cheese (e.g., 15 cm to 20 cm diameter), it would obviously take much longer for the enzymes and reactants to diffuse into the center region. During that period, the exterior portions would have been exposed to those enzymes far too long. The cheese might not ever ripen in the center, while becoming over-ripened at the surface.

Finally, as with *P. roqueforti,* growth of *P. camemberti* in the manufacture of Brie-type cheeses depends on lactic acid as an energy source. The subsequent rise in pH (from 4.6 to as high as pH 7.0 at the surface) is similarly due to lactate consumption and ammonia production. This return to a neutral pH also contributes to the diversity of microorganisms that grow on the surface of these cheeses (see above) and, more importantly, poses a special food safety risk due to the possible presence of *Listeria monocytogenes*.

Brined cheese

High-salt, high-acid cheeses were likely among the first cheeses intentionally produced by humans. They would have had a much longer shelf-life than other similarly produced soft cheeses, but without the high salt content. These cheeses have long been popular in Greece, Turkey, Egypt, Israel, and other Middle Eastern countries, as well as the Balkan region of Eastern Europe. In the past thirty years, production and consumption of these cheeses has spread throughout Europe, the United Kingdom, the United States, New Zealand, and Australia. The most popular cheese in this category is undoubtedly Feta cheese, which has its origins in Greece and which also has PDO status. Other similar types exist and vary based

mostly on salting method (whether applied before or after curd formation). Although they are often packaged dry in the United States, it is common for retail Feta-type cheeses to be packaged in tubs containing salt brine.

The general manufacturing procedures for Feta-type cheese start with a pasteurization step, since the cheese is usually consumed fresh (aged for as little as a few weeks). In Greece and other European countries, Feta (and Feta-type) cheese is made from raw milk. The milk itself can be obtained from cows, sheep, or goats (only the latter is used for PDO Feta). In some modern facilities, ultrafiltered milk is used. The milk is inoculated with a mesophilic starter containing *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*. The culture is added at the rate of 2% and allowed to ripen in the milk for as long as two hours prior to addition of chymosin. The large inoculum and long ripening time results in considerable acid development, demineralization, and a decrease in pH even before chymosin is added.

A rennet paste, a crude preparation containing a mixture of enzymes, is used in traditional Feta manufacture. Other proteases as well as lipases are among the enzymes present in this paste. Lipases can also be added separately, in the form of a lipase extract obtained from animal or microbial sources. The coagulation time can be an hour or longer. The coagulum will be very firm at cutting. The large curds (2 cm) are stirred for only a short time before being dipped and filled into hoops, which are flipped and turned for several hours. Fermentation occurs quickly and the pH will reach 4.7 or lower within eight hours. The cheese is then either placed into a salt brine (18–20%) for 12 to 24 hours, or dry salted for one to three days. The salted cheeses are then placed in a lower salt (10%) brine and held until packaging and distribution.

The Feta-type cheeses have a very short, crumbly texture, due, in part, to low pH (<4.7), demineralization, and high salt. As the pH approaches the isoelectric point of casein (pH 4.6), less water is retained. Also, high brine concentrations cause the casein network to shrink, releasing even more water from the cheese. Both of these factors make Feta crumbly. The main flavors in Feta are due to lactic acid and salt, but, depending on the presence of lipases (exogenous or from milk), there can also be considerable hydrolysis of triglycerides and formation of free fatty acids (ranging from 2 carbon all the way to 18 carbons). However, it is the release of short and medium chain fatty acids, including acetic (C2), propionic (C3), butyric (C4), caproic (C6), and caprylic (C8) acids, that account for the rancid-like flavor notes characteristic of these cheeses.

Process and cold pack cheese

Although cheese is the main ingredient, process cheese (or what many consumers mistakenly call American cheese) itself is not a fermented food. However, because process cheese is so popular in the United States and there is so much confusion regarding the differences between natural cheese and process cheese, it is worthwhile to describe the manufacture of process cheese.

Briefly, process cheese is made by adding emulsifying salts to natural cheese, along with water and other dairy and non-dairy ingredients. The mixture is then agitated while being heated to about 70°C or higher. The emulsification that occurs in process cheese is different than other true food emulsions. In the case of process cheese, sodium or potassium polyphosphate and citrate emulsifying salts raise the pH and the monovalent cations displace the divalent calcium ions from the casein complex. This results in more soluble sodium (or potassium) caseinate that contains both lipophilic and hydrophilic regions. These casein species can then form emulsion-like mixtures with the lipid portion of the cheese.

After heating, the cooled mixture is then formed into slices or loaves or filled into jars or cans. The finished products have excellent functionality and convenience features. Due to the heat treatment and other inherent conditions (pH, water activity, antimycotic agents), these products also have long shelf-lives, even at ambient temperature.

The quality of the cheese used as the starting material is among the factors that influence process cheese manufacture. A typical blend consists of 15% aged and 85% young or so-called "current" cheese. These ratios can vary considerably depending on the type of cheese that is available. In general, the aged cheese provides flavor; the young cheese provides elasticity, body, and emulsifying properties. Too much of the former may cause a soft, soupy body, too much of the latter may lead to hard body and brown pigment formation.

Cold pack cheese, in contrast, is made by mixing or grinding different types of natural cheese in the absence of heat. Various optional ingredients, including color, spices, and other flavoring agents, as well as antimycotic agents, can also be added. These products have excellent flavor, but typically lack the functionality of process cheeses.

CHEESE RIPENING

Freshly made cheese has essentially none of the flavor, aroma, rheological, or appearance properties of aged or ripened cheese. Rather, the metamorphosis from a bland, pale, rubbery mass of protein and fat into a flavorful, textured fusion of complex substances takes time and requires patience. Although efforts to reduce aging time and accelerate the ripening process have been somewhat successful (see below), for the most part, cheese ripening is a sequential process, with each step relying on a preceding step. In other words, a particular flavor compound may be present in a cheese only as a result of several preceding metabolic steps.

A good example of sequentially-produced flavor compounds is the methyl ketones. These are a class of lipid-derived products, which as noted above, contribute to the characteristic flavor of blue cheese. They are synthesized by *P. roqueforti* from free fatty acids. Thus, methyl ketone formation depends on release of these acids from triglycerides via lipolytic enzymes. The latter are secreted by *P. roqueforti* and other microorganisms or are naturally present in the milk. Similarly, hydrogen sulfide, which is an important flavor note in aged Cheddar cheese (or may also be a defect; see below), is derived from sulfur-containing amino acids that form via protein and peptide hydrolysis.

Of course, not only is ripening a sequential process, but it also is subject, on occasion, to potential chaos and disarray, such that the cheese may ripen poorly or unexpectedly. In some cases, over-production of an otherwise desirable compound occurs, resulting in serious flavor defects. For example, as noted above, hydrogen sulfide, at concentrations in the parts per billion range, imparts a pleasant sulfury aroma in Cheddar cheese, but when present at parts per million levels, the cheese is nearly inedible. In contrast, while blue cheese may contain 90 ppm to 100 ppm of methyl ketones, a small amount (1 ppm to 2 ppm) is perfectly fine in Cheddar cheese. Controlling the ripening process, then, is key to successful and consistent production of aged and well-ripened cheese.

Many factors contribute to the ripening process, including live microorganisms, dead microorganisms, enzymes, and chemical and physical reactions. Manipulating and controlling these activities and reactions depends on characteristics intrinsic to the cheese, such as moisture, pH, salt, and Eh, as well as those extrinsic factors that are influenced by

the cheese manufacturer. The latter include the source and handling of the milk, the temperature and humidity of the ripening room or environment, and other manipulations performed by the manufacturer. It is the collective result of these events that dictate the properties of the ripened cheese. Although development of cheese flavor, texture, and appearance is complicated, so say the least, research on the cheese microbiome has provided insights into the role of starter and non-starter microbes function together during cheese ripening (Box 6.5).

Box 6.5 Cheese and their microbiomes

A visit to one's local fromagerie provides ample evidence that cheeses vary widely in appearance, flavor and aroma, and texture. Likewise, one might expect to observe similar differences in the microbiomes of different cheeses. This turns out to be partially true – Brie cheese certainly has a different microbiota from Cheddar. But, as described below, the microbiotas that form on similarly-produced cheeses are highly reproducible.

First, it's relevant to remember that most modern cheese fermentations are initiated using defined cultures, comprised of microorganisms having predictable phenotypes. Often, the milk is first heat treated, reducing microbial diversity. Aseptic cheese manufacture limits exposure and access, further affecting the range and number of microbial members introduced from the environment.

The exception to this scenario is for cheese made by traditional or artisanal procedures. Cheese produced under these conditions may experience considerable cultural, temporal, and spatial changes during the course of the fermentation (Bokulich and Mills, 2013). That these changes can be measured both quantitatively and qualitatively makes these environments an ideal model system to study microbial communities and their organization. Of course, cheese microbiologists are highly motivated to understand how the microbiota contributes to sensory attributes, functional properties, and preservation (Irlinger and Mounier, 2009).

The availability of high throughput molecular sequencing methods provides a basis for assessing the structure and composition of microbial communities in cheese. As elegantly described by Wolfe et al., 2014, the ecosystem that forms on the rind of artisanal cheeses contains a diverse community of microbes, including prokaryotic and eukaryotic organisms. Remarkably, these microbial communities shared similarity even when the cheeses were from disparate locations, suggesting that these communities were organized in a repeatable or tractable fashion. Recently, these rinds communities were also reported to be suitable venue for horizontal gene transfer among its bacterial members (Bonham et al., 2017). These and other studies clearly demonstrate that in rind-formed cheeses, the diversity of both fungi and bacteria cannot be overstated (Figure 6.5.1).

Cheese rind, it should be noted, is of considerable practical importance. For cheeses inhabited by a surface microbiota, the rind contributes important flavor, aroma, texture, and visual characteristics to the cheese. More broadly, the presence of the surface material, whether formed by dehydration (like for Parmesan), wax (like for Gouda and Edam), or by surface growth of microorganisms (like for Brie or Munster) protects the cheese from external influences (e.g., moisture, air, insects, vermin). Indeed, complex

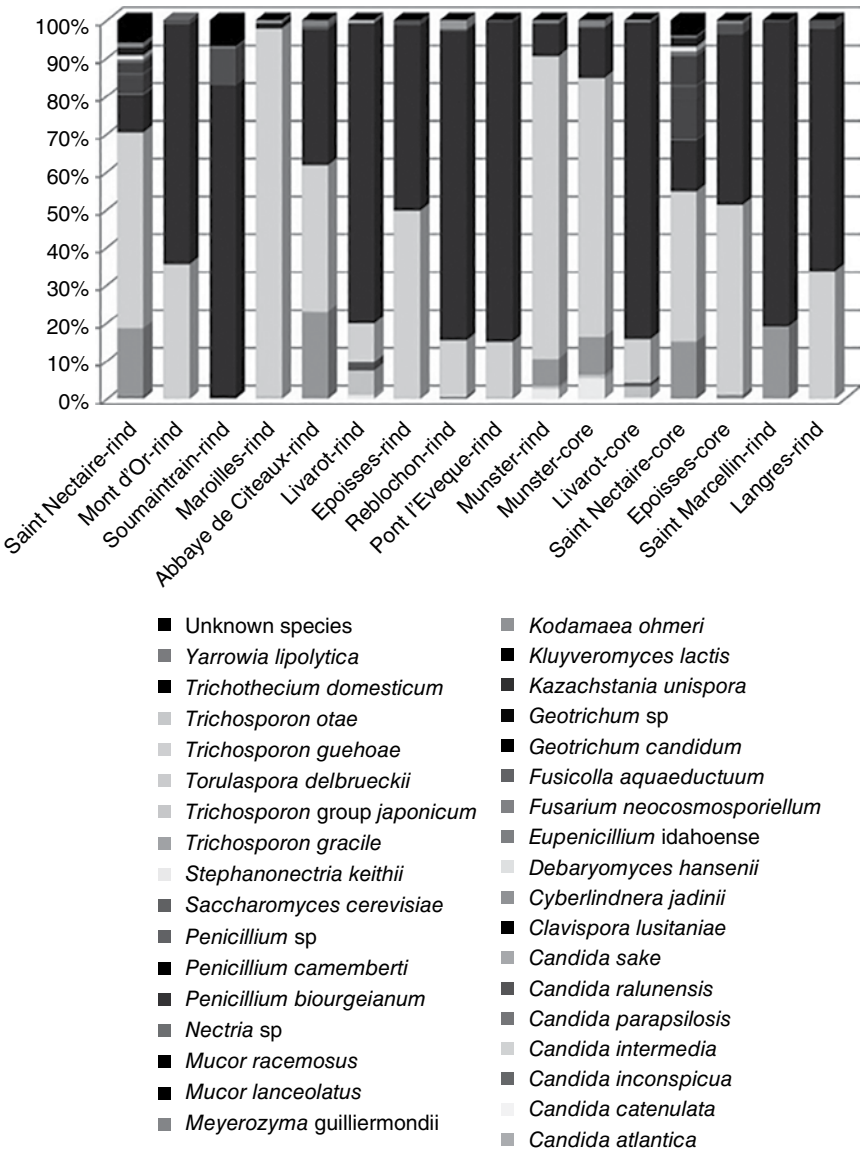


Figure 6.5.1 Relative abundance of sequences corresponding to fungi in 12 French surfaced-ripened cheeses. Adapted from Dugat-Bony et al., 2016, with permission. See original text for details.

microbial consortia, such as the rind communities described in this and other reports, may also exclude pathogenic bacteria and contribute to self-protection (Callon et al., 2014).

Numerous studies have been reported that describe microbiomes in a range of different cheeses, including those made using defined as well as natural cultures. Several general themes have emerged from these studies. First, raw milk can be an important

contributor of microbial contaminants (Alessandria et al., 2016). However, at least in aged cheese like Grana, the starter culture may provide a competitive barrier and potentially limit the expansion of those contaminants during ripening. The influence of the lactic starter cultures was also observed for a semi-hard sheep's milk cheese (Ramezan et al., 2017). Lactic acid bacteria dominated the curd during ripening and presumably inhibited potential spoilage organisms.

In cheeses that were surface-ripened with a defined culture, growth of specific organisms followed succession patterns that were correlated by changes in pH (Dugat-Bony et al., 2015). This point was further confirmed by a metatranscriptomic analysis that showed that pathways involved in lactose metabolism and lactic acid formation were followed by lactate consumption and amino acid degradation pathways.

Transcriptome analysis of a ripened, stretched-curd cheese aged at different temperatures demonstrated that expression of genes involved in flavor and texture formation (e.g., protein and lipid metabolism) was increased at higher temperatures (De Filippis et al., 2016). Additionally, the transcriptomes were consistent with the formation of flavor compounds present in these cheeses.

Finally, cheese microbiome studies have revealed unexpected variables may affect microbial composition and cheese quality. To this point, even the time of day when the cheese was made may influence the microbiota that merges (O'Sullivan et al., 2015)!

References

Alessandria, V., I. Ferrocino, F. De Filippis, M. Fontana, K. Rantsiou, D. Ercolini, and L. Cocolin. 2016. Microbiota of an Italian Grana-like cheese during manufacture and ripening, unraveled by 16S rRNA-Based approaches. Appl. Environ. Microbiol. 82:3988–3995.

Bonham, K.S., B.E. Wolfe, and R.J. Dutton. 2017. Extensive horizontal gene transfer in cheese-associated bacteria. eLife 6:1–31.

Bokulich, N.A., and D.A. Mills. 2013. Facility-specific "house" microbiome drives microbial landscapes of artisan cheesemaking plants. Appl. Environ. Microbiol. 79:5214–5223.

Callon, C., E. Retureau, R. Didienne, and M.C. Montel. 2014. Microbial biodiversity in cheese consortia and relative *Listeria* growth on surfaces of uncooked pressed cheeses. Int. J. Food Microbiol.174:98–109.

De Filippis, F., A. Genovese, P. Ferranti, J.A. Gilbert, and D. Ercolini. 2016. Metatranscriptomics reveals temperature-driven functional changes in microbiome impacting cheese maturation rate. Sci. Rep. 6:21871.

Dugat-Bony, E., C. Straub, A. Teissandier, D. Onésime, et al. 2015. Overview of a surface-ripened cheese community functioning by meta-omics analyses. PLoS ONE 10:e0124360.

Dugat-Bony, E., L. Garnier, J. Denonfoux, S. Ferreira, A.-S. Sarthou, P. Bonnarme, and F. Irlinger. 2016. Highlighting the microbial diversity of 12 French cheese varieties. Int. J. Food Microbiol. 238:265–273.

Irlinger, F. and J. Mounier, 2009. Microbial interactions in cheese: implications for cheese quality and safety. Curr. Opin. Biotechnol., 20:142–148.

O'Sullivan, D.J., P.D. Cotter, O. O'Sullivan, L. Giblin, P.L.H. McSweeney, and J.J. Sheehan. 2015. Temporal and spatial differences in microbial composition during the manufacture of a continental-type cheese. Appl. Environ. Microbiol .81:2525–2533.

Ramezani, M., S.M. Hosseini, I. Ferrocino, M.A. Amoozegar, and L. Cocolin. 2017. Molecular investigation of bacterial communities during the manufacturing and ripening of semi-hard Iranian Liqvan cheese. Food Microbiol. 66:64–71.

Wolfe, B.E., J.E. Button, M. Santarelli, and R.J. Dutton. 2014. Cheese rind communities provide tractable systems for *in situ* and *in vitro* studies of microbial diversity. Cell 158:422–433.

As noted above, cheese contains mostly water, protein, and fat as it leaves the vat and is moved into the ripening coolers. Although some protein hydrolysis certainly occurs during the lactic fermentation by starter culture bacteria and by the action of milk proteases and the coagulant, the main casein subunits, α_{s1}, α_{s2}, and β-casein, are still intact. Similarly, the triglycerides in milk are also mostly unaffected by the early cheese making steps. However, within just a few days, enzymes begin to attack these substrates and initiate the ripening process.

PROTEOLYSIS IN CHEESE

It was long argued that milkfat was the primary constituent responsible for cheese flavor. While it is certainly true that many cheese flavors are either evolved from the lipid fraction or are soluble in the lipid phase, the general view (with several notable exceptions) is that the protein fraction makes perhaps an even more important overall contribution (Table 6.2). Cheeses made under experimentally-controlled conditions in which proteolysis does not occur develop neither the flavor nor texture of a normal cheese. The recognition that proteolysis has such a profound influence on cheese flavor and ripening has led to a detailed understanding of many of the specific steps involved in the degradation of milk proteins and the metabolism of the hydrolysis products (Figure 6.7).

For the most part, the relevant organisms responsible for these activities are lactic acid bacteria, either those added as part of the starter culture or those present as part of the ordinary microbiota. Lactic acid bacteria cannot synthesize amino acids from ammonia and instead require pre-formed amino acids for protein biosynthesis and cell growth. However, milk contains only a small amount of free amino acids (<300 mg/L), which are quickly assimilated. Thus, growth of lactic acid bacteria in milk depends on the ability of these cells to metabolize proteins in milk. The means by which large casein molecules are broken down by these bacteria into their component amino acids (and beyond) consists of four main steps: (1) hydrolysis of casein; (2) transport of casein-derived amino acids, peptides, and oligopeptides into the cytoplasm of starter and non-starter lactic acid bacteria; (3) intracellular hydrolysis of accumulated peptides; and (4) metabolism of amino acids.

In Cheddar-type cheeses, starter culture lactic acid bacteria, notably, *L. lactis* subsp. *lactis*, produce a cell-wall anchored proteinase that hydrolyzes specific casein subunits,

Table 6.2 Sources of flavor compounds in cheese[1].

Protein (casein)	Carbohydrate	Lipid
peptides	lactate	fatty acids
amino acids	acetate	keto acids
sulfur compounds	pyruvate	esters
ammonia and amines	ethanol	methyl ketones
pyruvate	diacetyl	lactones
acetate	acetoin	thioesters
aldehydes	2,3-butanediol	
alcohols	acetaldehyde	
keto acids		

[1] Adapted from Law and Tamine, 2010; McSweeney, 2004; and Singh et al., 2003.

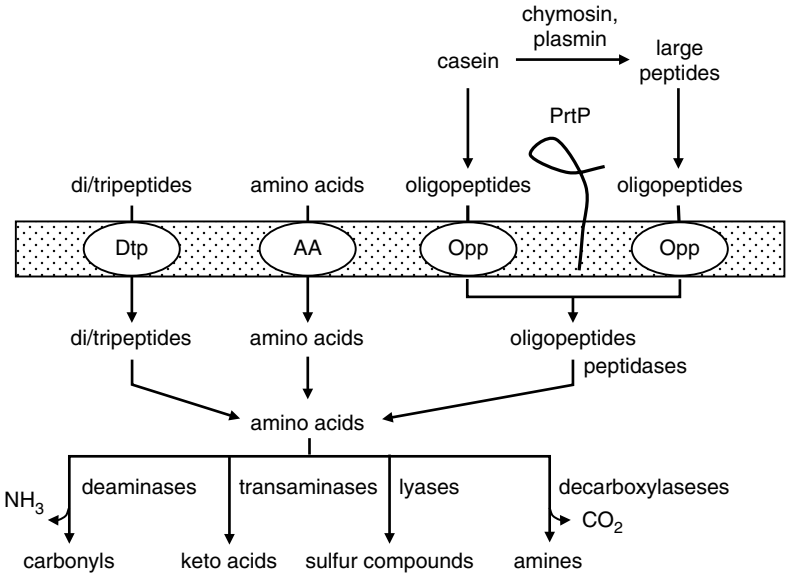


Figure 6.7 Proteolysis during cheese ripening. The proteolytic system in lactococci starts with the hydrolysis of casein by PrtP. The main products are oligopeptides, which are then transported across the cell membrane by the Opp transport system. Any free amino acids and di- and tripeptides in the milk are similarly transported by amino acid (AA) and di- tripeptide transporters (DtpT, DtpP), respectively. Once inside the cell, the oligopeptides are hydrolyzed by various peptidases, including PepA, PepC, PepN, and PepX. The di- and tripeptides are also hydrolyzed. The free amino acids are then metabolized or used for protein biosynthesis.

producing as many as 100 or more different peptides varying in length from four to thirty amino acid residues. Although proteinases are produced by several strains of *L. lactis* subsp. *lactis* and by other lactic acid that bacteria produce, the enzymes are structurally quite similar (in terms of their DNA and amino acids sequences). However, the specific casein substrates and hydrolysis products of the different PrtP enzymes can vary considerably.

For example, some proteinases hydrolyze α_{S1}-, β-, and κ-caseins, whereas others have preference for β-casein and relatively little activity for α_{S1}-and κ-caseins. It should be noted that residual chymosin and milk proteinases (mainly plasmin) may also contribute to the peptide pool, especially during the early stages of cheese ripening. However, it is the starter and non-starter lactic acid bacteria that are responsible for most of the subsequent casein hydrolysis. In fact, when cheese is made with starter strains that do not produce cell wall proteinase, casein degradation occurs to a very limited extent and cheese flavor and texture do not develop. Likewise, cheese made in the absence of non-starter lactic acid bacteria is similarly bland.

Hydrolysis of the casein-derived peptides was previously thought to occur by extracellular peptidases produced by starter or non-starter lactic acid bacteria. It now is generally accepted that peptide hydrolysis does not occur outside the cells, but rather the peptides are transported via various peptide transport systems and are subsequently hydrolyzed by intracellular peptidases. The main transporter, called the oligopeptide transport system (Opp), is present in most starter lactic acid bacteria and transports oligopeptides across the cytoplasmic membrane and into the cell. Mutants defective in Opp, like mutants defective in proteinase production, grow poorly in milk because they are unable to use casein as a

nitrogen source. Starter lactococci also have amino acid as well as di- and tri-peptide transport systems; however, they are somewhat less important since most of the peptides present in cheese are larger than three amino acid residues.

Once inside the cells, the peptides can be hydrolyzed by a variety of peptidases, depending on their size and amino acid content. Aminopeptidases hydrolyze peptides at the amino end (i.e., the peptide bond located between the amino terminal amino acid residue and the penultimate residue), whereas carboxypeptides act at the carboxy end. Endopeptidases also exist that hydrolyze peptide bonds within a peptide. Lactococcal peptidases also have specificity with regard to the amino acids and their position within a given peptide bond. For example, aminopeptidase P (PepP) from *L. lactis* subsp. *lactis* hydrolyzes peptides containing proline at the penultimate residue.

Protein hydrolysis also has a major impact on texture. The solubility of coagulated casein is limited, and is even less at low pH, making young cheese plastic and rubbery. However, as cheese ages and casein is degraded, the peptides are more soluble, and elasticity increases. Eventually, however, when the casein and casein-derived peptides are more extensively degraded, the cheese develops a crumbly, short texture.

As noted above, cheese flavor and texture development is largely a function of the proteolytic capacity of the bacteria present in the ripening cheese. However, for the most part, the starter culture bacteria are not maintained very long after the first few weeks of aging. That is not to say that their impact is not significant. In fact, as the starter bacteria lyse (due to salt, low water activity, lack of fermentable substrates, and other factors), their entire portfolio of intracellular enzymes are released into the curd matrix. Thus, the various peptidases important for peptide degradation are free to hydrolyze available substrates. Likewise, enzymes that act directly on amino acids and triglycerides are also released, as are the substrates themselves.

Finally, non-starter lactic acid bacteria, as well as other indigenous bacteria, play an important (and depending on the cheese, perhaps the most important) role in cheese ripening. In particular, several *Lactobacillus* spp., including *Lactobacillus casei*, *Lactobacillus paracasei*, and *Lactobacillus plantarum,* increase in number by several orders of magnitude (from 10^2 per gram to more than 10^7) during aging, and produce peptidases and other enzymes that generate aged cheese flavor. There are even commercial products containing selected strains that can be added to cheese to boost or accelerate flavor development (see below). Of course, some of these adventitious bacteria can also produce off-flavors. Heterofermentative lactobacilli are especially of concern due to their ability to produce acetic acid and CO_2 from residual sugars.

CURRENT ISSUES IN CHEESE TECHNOLOGY

Given the important role of microorganisms in the manufacture of cheese, it should come as no surprise that among the most important issues faced by the cheese industry, most are microbiological in nature (not withstanding yield, costs, and other economic issues). The key to making any high quality fermented food, including cheese, is to control the activities of the microbial starter culture and other organisms. This means ensuring that they grow when they are supposed to, that agents that would otherwise interfere with their growth are controlled, and that they produce the correct amount and types of enzymes, flavors, and other products.

Although consumer trends often influence and raise specific sorts of problems, several long-term issues continue to challenge cheese technologists. These questions include: (1) how to improve and accelerate cheese flavor development and reduce bitterness, especially in low-fat cheese; (2) how to control bacteriophages that infect, inhibit, and sometimes inactivate starter cultures; (3) how to enhance shelf-life and prevent growth of spoilage microorganisms and ensure that cheese is free of potential pathogenic microorganisms; and (4) how to increase the value of whey using microorganisms.

1. Bitterness and accelerated ripening

For most cheeses, production of good aged cheese flavor and texture requires that significant hydrolysis of casein occurs and that the appropriate hydrolysis products are formed. At the same time, the main flavor defect in cheese is bitterness, which is also caused by casein hydrolysis and subsequent formation of bitter peptides. Excessive proteolysis also causes texture defects. Thus, aging cheese it like walking a microbial tightrope – it requires a fine sense of preparation, balance, timing, and proportion. A single misstep may lead to an unacceptable outcome. The practice of accelerated ripening–obtaining good cheese flavor and texture in half or even a third of the time, is to continue the analogy, like running along the tightrope. There is tremendous economic incentive, however, to decrease aging time, because the aging process adds about one cent per kg per month to the cost of the cheese. Refrigeration and storage account for much of this cost, but one of the main downsides to long aging periods is simply that the product is sitting and not selling (i.e., inventory cost). Reduced fat cheese poses another set of flavor problems. Not surprisingly, considerable research has been aimed at developing accelerated cheese aging programs.

As described above, the first step in the use of casein by the lactic starter culture is hydrolysis of casein by the cell wall anchored proteinase (PrtP). Of the 100 or more peptides that are produced, most contribute little to overall cheese flavor. However, several of these peptides have a bitter flavor. If the starter culture cannot degrade these bitter peptides, the cheese will be bitter. Starter culture strains that produce PrtP (or related proteinases), but that are otherwise unable to degrade the bitter peptides so produced, are referred to as bitter strains.

In general, bitter peptides are hydrophobic and contain proline, and are hydrolyzed only by specific intracellular aminopeptidases, including PepN and PepX. Bitter strains lack the ability to produce these enzymes. It is interesting that most commercial strains of *L. lactis* subsp. *lactis*, the most widely used organism in the cheese industry, are bitter. In contrast, most of the *L. lactis* subsp. *cremoris* strains are non-bitter. However, *L. lactis* subsp. *lactis* grows faster and, at least in unripened cheese (e.g., cheese destined for the process kettle), its inability to degrade bitter peptides is not an issue. Even cheese made with non-bitter strains, if aged long enough, may eventually accumulate enough bitter peptides to develop bitter flavor. Bitter peptide production is further increased at higher ripening temperatures (i.e., >12°C), which is perhaps the most common way to accelerate cheese ripening (see below).

The main approach of most accelerated aging strategies is to promote proteolytic and other enzymatic activities. Several options exist. The easiest and perhaps most common method is simply to raise the temperature during the aging period. Some cheese manufacturers have developed specific regimens whereby the temperature is ramped up (or down) for various periods of time in order to optimize ripening.

The addition of adjunct cultures, or strains specifically associated with flavor development, has also been promoted as a means of accelerating the aging process (Box 6.6). Adjunct

Box 6.6 Adding flavor with adjuncts

The development of cheese flavor and texture is a complicated process. Cheese flavor, especially aged cheese flavor, requires many enzymes, acting in concert on a range of substrates, usually over a long period of time. The enzymes involved in these reactions originate from three main sources: the coagulant, the milk, and bacteria. The latter are either normally present in milk due to unavoidable contamination or are deliberately added. And although it is certainly possible to make cheese without bacteria (i.e., aseptic cheese), the finished product would contain few, if any of the flavor or texture characteristics one might expect. This would be especially true if the cheese were aged, since aged flavor and texture would simply not develop.

While the coagulant and natural milk enzymes certainly contribute to cheese flavor and texture development, their roles are considered to be rather limited, whereas the presence of bacteria is essential. Moreover, it is not just the lactic starter culture bacteria that are responsible for the desirable changes that occur during cheese ripening - bacteria present as part of the natural milk flora also serve as a vital reservoir of enzymes that are necessary for proper cheese maturation. If, however, the milk is pasteurized or if it is produced and handled under strict hygienic conditions, the natural microbiota will be rather limited, both in number and diversity. The finished cheese may ripen slowly and, in some cases, never achieve the desired quality expectations.

The realization that the natural milk microbiota is important for cheese ripening led investigators first to identify the relevant organisms, and then to consider the possibility of adding them directly to the milk to boost, enhance, or accelerate flavor development (Reiter et al., 1967). These studies revealed that the bacteria that were most frequently associated with good cheese flavor were lactic acid bacteria, but not the same species or strains used as the starter culture. Therefore, as a group, they are referred to as non-starter lactic acid bacteria (NSLAB). Accordingly, cheese flavor is enhanced when appropriate NSLAB are added to cheese milk. Although a rather diverse range of NSLAB have been considered as adjunct cultures, most consist of species of *Lactobacillus* (Gobbetti et al., 2015). It also appears that the presence of these NSLAB may out-compete "wild" NSLAB that might otherwise cause flavor defects. They are now commercially available in the form of adjunct cultures (Bockelmann, 2010). Such products are widely used for accelerated ripening programs as well as for reduced fat cheese, which often suffer from flavor problems.

When adjunct cultures were first considered for modifying cheese flavor, cells attenuated by freeze or heat shocking, physical-chemical treatment, or genetic means were used (El Soda et al., 2000). This was done to make the cells nonviable and to minimize subsequent acid production and metabolism. However, as noted above, adjunct cultures are now commercially available and are added directly to the cheese milk, albeit at much lower inoculation rates compared to the starter culture (Fenelon et al., 2002).

Among the adjunct species currently used commercially are strains of *Lactobacillus helveticus*, *Lactobacillus casei*, and *Lactobacillus paracasei*. In particular, the specific NSLAB used as adjunct cultures are selected based largely on several criteria, including, their ability to: (1) survive cheese making and ripening conditions;

Table 6.6.1 Non-starter lactic acid bacteria used as culture adjuncts in cheese.

Lactobacillus helveticus	*Enterococcus faecium*
Lactobacillus paracasei subsp. *paracasei*	*Pediococcus pentosaceus*
Lactobacillus delbrueckii subsp. *lactis*	*Pediococcus acidilactici*
Lactobacillus plantarum	*Staphylococcus xylosus*
Lactobacillus reuteri	*Corynebacterium* spp.
Lactobacillus curvatus	*Propionibacterium shermanii*
Lactobacillus johnsonii	*Lactococcus lactis* subsp. *lactis biovar. diacetylactis*
Lactobacillus casei subsp. *rhamnosus*	

(2) express proteolytic enzymes capable of hydrolyzing casein; (3) produce aminopeptidases and other peptidases; (4) metabolize amino acids; (5) not produce defects; and (6) lyse during cheese aging (Briggiler-Marco et al., 2007; Burns et al., 2012). Lysis may be especially relevant, as it promotes release of cytoplasmic peptidases. The latter are then able to hydrolyze so-called bitter peptides that accumulate in cheese as a result of partial casein hydrolysis. Thus, the cheese becomes less bitter, and the released amino acids can be further metabolized to yield desirable end products (Møllera et al., 2013).

It is important to note that flavor formation is not the only function of culture adjuncts. Indeed, there are variety of other functions that adjunct cultures may provide (Table 6.6.1). These include metabolism of residual carbohydrates, formation of exopolysaccharides, and biopreservation.

References

Bockelmann, W. 2010. Secondary cheese starter cultures. In: B.A. Law, A.Y. Tamime, eds. Technology of Cheesemaking. Wiley-Blackwell, Oxford, pp. 193–230.

Burns, P., F. Cuffia, M. Milesi, G. Vinderola, C. Meinardi, N. Sabbag, and E. Hynes. 2012. Technological and probiotic role of adjunct cultures of non-starter lactobacilli in soft cheeses. Food Microbiol. 30:45–50.

Briggiler-Marco´, M., M.L. Capra, A. Quiberoni, G. Vinderola, J.A. Reinheimer, and E. Hynes. 2007. Nonstarter Lactobacillus strains as adjunct cultures for cheese making: *in vitro* characterization and performance in two model cheeses. J. Dairy Sci. 90:4532–4542.

El Soda, M., S.A. Madkor, and P.S. Tong. 2000. Adjunct cultures: recent developments and potential significance to the cheese industry. J. Dairy Sci. 83:609–619.

Fenelon, M.A., T.P. Beresford, and T.P. Guinee. 2002. Comparison of different bacterial culture systems for the production of reduced-fat Cheddar cheese. Int. J. Dairy Technol. 55:194–203.

Gobbetti, M., M. De Angelis, R. Di Cagno, L. Mancini, and P.F. Fox. 2015. Pros and cons for using non-starter lactic acid bacteria (NSLAB) as secondary/adjunct starters for cheese ripening. Trends Food Sci. Technol. 45:167–178.

Law, B. 2010. Cheese adjunct cultures. Aust. J. Dairy Technol. 65:45–49.

Møllera, K.K., F.P. Rattray, and Y. Ardö. 2013. Application of selected lactic acid bacteria and coagulant for improving the quality of low-salt Cheddar cheese: chemical, microbiological and rheological evaluation. Int. Dairy J. 33:163–174.

Reiter, B., T.F. Fryer, A. Pickering, H.R. Chapman, R.C. Lawrence, and M.E. Sharpe. 1967. The effect of microbial flora on the flavor and free fatty acid composition of Cheddar cheese. J. Dairy Res. 34:257–272.

cultures, which typically contain selected strains of *Lactobacillus* and *Lactococcus,* can be added directly as part of the starter culture. In general such strains are selected on the basis of their ability to produce aminopeptidases and degrade bitter peptides. However, because excess acid production is also to be avoided, variants of these strains that are unable to ferment lactose are preferred.

The observation that some strains of lactococci and streptococci were autolytic led to another innovative means for accelerating the ripening process. As described previously, ripening depends largely on the presence of enzymes released by bacteria as they lyse during the aging process. Ordinarily, cell lysis occurs only when the cells are no longer able to maintain cell wall integrity, due to the lack of energy sources and the generally inhospitable conditions (i.e., low pH, high salt, etc.) present within the cheese. In contrast, if lysis and enzyme release were to occur during the early stages of aging, the ripening process could be shortened.

2. Bacteriophages

Bacteriophages are arguably the number one problem in cheese manufacture, at least as far as the fermentation part is concerned. They are certainly the main reason why starter culture activity is sometimes inhibited. Infection of starter cultures by phages may result in slow or sluggish fermentations; occasionally the fermentation may fail completely. Phage infections ultimately can lead to prolonged make times and inferior or poor quality cheese, both of which cause substantial economic hardships on the manufacturer. For many years, phages were thought to be primarily a problem for cheeses made using mesophilic lactococcal cultures. However, as cheeses such as Mozzarella and other Italian cheeses became popular, it became evident that thermophilic species (especially *S. thermophilus*) were also susceptible to attack by phages.

Ever since the phage problem was first recognized (about eighty years ago!), manufacturers and scientists have adopted strategies to prevent or at least minimize the incidence and impact of phage infections. These have been described in detail in Chapter 4, but it is worth emphasizing that phage management is now a common part of most cheese manufacturing operations. Sanitation, plant design, culture rotation, and use of resistant strains are all part of such programs.

3. Microbial defects, preservation, and food safety

Given that cheese is made in a mostly open, non-sterile environment, using non-sterile raw materials, is exposed to or held at non-lethal, non-inhibitory temperatures, it is not surprising that all sorts of microorganisms can gain entry in cheese. Although the combined effects of moderately low pH, moderately high salt concentrations, and low Eh provide a barrier against some microorganisms, there are others, including spoilage organisms and pathogens that can grow in the cheese environment. Thus, microbial defects are not uncommon in cheese and can be responsible for significant economic loss. Of course, chemical and physical defects (e.g., oxidation, crystal formation, discoloration, mechanical openings) can also be responsible for decreased shelf-life or consumer rejection, however, they generally are less serious.

Microorganisms can cause several general types of spoilage in cheese. These include appearance defects, texture defects, and flavor and aroma defects. Appearance defects are those that may cause the consumer to reject a product based strictly on sight. Perhaps

the best example is cheese contaminated with mold growth. Although there are many different fungi capable of growing on cheese, *Penicillium* spp. are the most common. Fungi are obligate aerobes, so growth occurs almost exclusively on the surface, where oxygen is available. However, mold growth on packaged cheese, even vacuum-packaged products, can also occur, provided there is enough oxygen present. Mold growth is more common in block cheese, and even though it can be trimmed prior to cutting and packaging, trimming is time-consuming and results in loss of yield. Since some fungi that grow on cheese belong to species known to produce mycotoxins, there has been concern that mycotoxins could have diffused in the cheese and that trimming visible mold from the surface may not be sufficient to render the product safe. However, numerous studies have revealed that mycotoxins are not produced on cheese, even when the fungi is theoretically capable of synthesizing toxins. Presumably, the cheese environment restricts or inhibits expression of toxin biosynthesis pathways.

Appearance spoilage also occurs due to formation of undesirable pigments. The most common and best-studied example is the pink defect (or pink ring) that occurs around the exterior of several hard cheeses, such as the Parmesan-type cheeses. The defect is worse after prolonged aging, and can occur either inside or near the surface. Several factors contribute to the formation of the pink color, including both chemical and biological reactions (Box 6.7).

Box 6.7 Parmesan, postulates, and pinking

Despite the seemingly simple steps involved in cheese manufacturer, consistent production of defect-free cheese is no easy matter. There is plenty that can go wrong – milk quality, culture performance, curd handling, ripening conditions, all can affect the quality of the final product. When one of these steps go awry, resulting in flavor, texture or appearance defects, there is usually a cause and therefore a correctable action.

One defect for which no cure has been devised is the pink discoloration defect that occurs in many hard cheese. The defect was first reported almost 90 years ago (Morgan, 1933). Affected cheeses include Swiss, Cheddar, and other Cheddar-types (Daly et al., 2012). Parmesan, Romano, and other hard Italian varieties may be particularly susceptible (Shannon et al., 1968). Nonetheless, geography or manufacturing procedures do not seem to matter (Daly et al., 2012). The pink color can form within the interior or near the surface, it can be uniformly distributed or sporadic, and occurs in cheeses made with or without annatto.

If the pink defect were rare, perhaps there would be less concern. Unfortunately, it can affect as much as 5% of retail Cheddar. Frequencies for other cheeses have not been reported, but based on research interest, pinking and other discoloration problems appear to be common (Dairy Pipeline, Wisconsin Center for Dairy Research, 2013). Indeed, researchers have proposed various explanations for why pinking occurs. These include oxidation-reduction reactions, Maillard reactions, nitrite reactions, light-induced reactions, amino acid reactions, starter culture reactions, and annatto reactions (Daly et al., 2012). None of these explanations, however, have evidently resolved the problem.

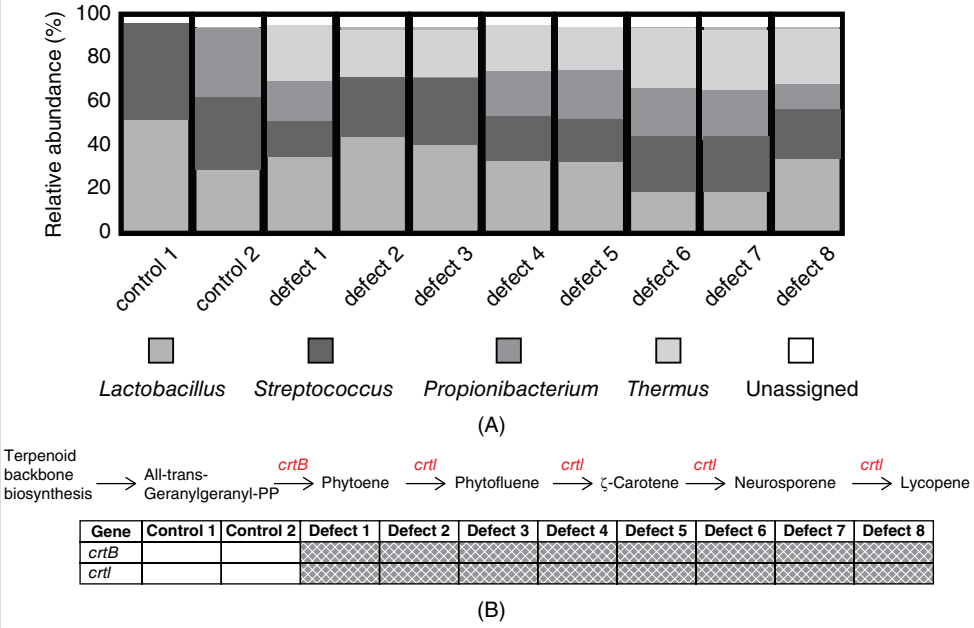


Figure 6.7.1 Relative abundance of genera (A) and presence of carotenoid biosynthesis pathway genes (B) in control and pink defect cheeses. Adapted from Quigley et al., 2016, with permission (colors converted to grey scale). See original text for details.

Recently, researchers in Ireland proposed perhaps the most convincing explanation yet (Quigley et al., 2016). Using 16S rRNA and metagenomics sequencing, they first examined the microbial composition of normal and pink defect cheese. Although the major phyla were similar for both, the pink-defect cheese also harbored one additional taxonomic group that was absent in the normal cheese (Figure 6.7.1A). This group contained organisms belong to the genus, *Thermus*, a group of Gram-negative thermophiles. Importantly, these researchers also performed a metagenome analysis, and discovered that genes encoding for carotenoid metabolism were present only in pink defect-associated cheeses (Figure 6.7.1B). Spectral analysis confirmed that the pink color was due to carotenoid pigments, including those whose syntheses were predicted from the metagenome sequences.

Finally, to establish causality, i.e., that *Thermus* is responsible for the pink defect, the researchers invoked Koch's postulates. Readers may recall from earlier in this text that Robert Koch was among the first microbiologists to study the role of microbes in infectious diseases. The postulates, arguably the most important set of rules in all of biology, form the basis of proving that a particular microorganism causes a specific disease. Simply stated, the four rules require that (i) the organism is always associated with the disease; (ii) it can be isolated from diseased subjects and grown in pure culture; (iii) when introduced into a healthy subject, the organism causes the disease; and (iv) the organism can be recovered in the newly infected subject.

Thus, the experimental plan involved isolation of *Thermus thermophilus* from pink defect cheese and propagating the organism in pure culture. Next, they introduced the organism, along with the varying levels of the starter culture into raw milk, and made cheese. Only the treatments containing *T. thermophilus* became pink, and only the pink–defect cheeses contained this organism during ripening. Thus, the cheese equivalent of Koch's postulates were satisfied.

The researchers were also able to isolate the organism from various locations within the cheese manufacturing environment. They noted that the starter culture used in these experiments may also influence the intensity of *T. thermophilus*-induced pink color formation. That other members of the cheese microbiota might influence formation of the pink color defect was also considered a possibility worthy of additional study. Ultimately, this research provides a rational basis for preventing this problem. Thus, eliminating *T. thermophilus* from the dairy environment, manipulating cheese manufacturing, or adjusting the starter culture may all be effective strategies to prevent expression of the pinking phenomenon.

References

Daly, D.F.M., P.L.H. McSweeney, J.J. Sheehan. 2012. Pink discolouration defect in commercial cheese: a review. Dairy Sci. Technol. 92:439–453.

Morgan, G.F.V. 1933. Discoloration in New Zealand Cheddar cheese. Muddy, pink and bleached defects. I. Bacteriological investigations. J. Dairy Res. 4:226–237.

Quigley, L., D. J. O'Sullivan, D. Daly, O. O'Sullivan, Z. Burdikova, R. Vana, T.P. Beresford, et al. 2016. Thermus and the pink discoloration defect in cheese. mSystems 1:e00023–16.

Shannon, E.L., N.F. Olson, and J.H. von Elbe. 1968. Pink discoloration in Italian varieties of cheese. J. Dairy Sci. 51:613–614.

Finally holes or slits may form as a result of gas production. Several organisms are capable of producing gas (mainly carbon dioxide) in cheese, including yeast, coliforms, propionibacteria, and clostridia. The clostridia, especially *Clostridium tyrobutyricum*, are more common in milk obtained from silage-fed cows and can produce enough CO_2 to cause formation of large holes (>3 cm in diameter). However, the heterofermentative lactic acid bacteria are probably the most common cause of the slit defect. Relevant species include *Lactobacillus fermentum* and *Lactobacillus curvatus.* These bacteria are either naturally present in the milk or are normal contaminants in a cheese plant; thus, they may be particularly difficult to control, especially in raw milk cheese. Residual lactose or galactose in the cheese provides substrate for these anaerobic bacteria, whose growth is enhanced within the interior of large cheese blocks or barrels where the cheese is slow to cool and where low Eh conditions prevail.

The most common texture defects include slime, crystal or sandy mouth feel, and poor body. Polysaccharides that impart a slimy texture to the cheese can be produced by psychrotrophic Gram-negative bacteria such as *Pseudomonas* and *Alcaligenes*, as well as by yeast and lactic acid bacteria. Crystals that form on cheese are usually comprised of calcium lactate and are mainly a physical-chemical problem, caused by low solubility of calcium lactate salts at the cheese surface. However, pediococci, lactobacilli, and other non-starter lactic

acid bacteria may contribute to this problem by converting L(+) lactate to its isomer, D(–) lactate, which is less soluble. Tyrosine-containing crystals can also form in dry cheeses (e.g., Parmesan and Romano).

Flavor defects can be caused by not only by contaminating organisms, but also by the starter culture itself. If the culture is given the opportunity, it may produce too much lactic acid, causing an acid defect. As described earlier, certain peptides generated by starter culture proteinases are bitter-tasting, and unless further metabolized, cause a bitter defect. Heterofermentative, non-starter lactic acid bacteria produce ethanol and acetic acid in addition to CO_2. Acetic acid imparts a highly objectionable vinegar-like sour flavor to the cheese. These bacteria may also hydrolyze triglycerides, releasing free fatty acids that contribute fruity flavors into the cheese. Production of hydrogen sulfide and other sulfur-containing compounds, ammonia, and other volatiles by bacteria and fungi, are also common flavor defects.

Minimizing the entry of spoilage organisms into the cheese milk and subsequently into the cheese is one of the most important steps in preventing spoilage. In most cases, the cheese will only be as good as the milk from which it was made. The use of high quality milk, application of good sanitation practices, and proper cheese manufacture are essential. Pasteurization of milk, even for aged cheese, is now common, not only to inactivate pathogens (see below), but also to kill potential spoilage organisms. Some specific spoilage problems can be addressed by the use of chemical preservatives, either added to the milk or applied to the cheese. For example, mold growth can be inhibited by sorbic acid (usually in the form of potassium sorbate salts) or the antibiotic natamycin (also known as pimaricin). In some European countries, but not in the United States, lysozyme and sodium nitrate can be added to milk to control clostridia.

Although spoilage of cheese by microorganisms is a constant concern, the presence of pathogens in cheese occurs only infrequently, and rarely does foodborne disease result. However, due to the serious public health consequences, as well as potential economic loss due to recalls and liability, attention to food safety is an absolute requirement. Just a single positive test for *L. monocytogenes* or *E. coli* O157:H7 may initiate a product recall. Thus, the goal is not just to prevent the growth of pathogens, but also to reduce or eliminate their very presence in cheese. In the United States, this means more and more cheese is being made from pasteurized milk, and exposure to environmental sources of contamination is minimized (e.g., via the use of enclosed vats). Most cheese manufactures have also adopted Hazard Analysis Critical Control Points (HACCP) or other similar programs that are designed to anticipate and prevent food safety problems.

Another type of foodborne disease that occasionally occurs in cheese is caused by the presence of the biogenic amines histamine and tyramine, but is unrelated to foodborne pathogens. In sensitive individuals, these amines (mainly histamine) can cause headaches, nausea, and cramps and other gastrointestinal symptoms. These compounds are produced from the amino acids histidine and tyrosine, respectively, via specific amino acid decarboxylases. Bacteria that produce these enzymes and form these amines include *Lactobacillus buchneri* and other lactobacilli, lactococci, and enterococci. Aged cheese, particularly aged Cheddar, Gouda, and Swiss, are more likely to contain biogenic amines due to the higher concentration of free amino acid substrates that accumulate during more extensive proteolysis. Because the bacteria responsible for amine production are ordinarily present as raw milk contaminants, pasteurization effectively minimizes this problem. Nonetheless, the presence of biogenic amines is enough of a problem that methods to prevent or reduce their formation are being adopted.

4. Whey utilization

As noted earlier in this chapter, water, in the form of whey, is released when milk is converted into cheese. In fact, whey accounts for 90% of the original milk volume. The dilute nature of whey (about 92% to 94% water) and low protein concentration (<1%) have historically contributed to the perception (at least in the United States) that whey has little economic value. However, in other parts of the world, whey is more widely used, especially in the manufacture of whey-derived cheese. For example, in Norway, the goat whey cheeses Gjetost and Mysost are among the most popular of all cheeses (accounting for about 20% of cheese consumption), and Ricotta, another whey-based cheese, is popular not only in Italy, but throughout Europe and the United States. Whey cheeses are also produced in Greece (Manouri, Anthotyros), Portugal (Requejão), and the Czech Republic (Urda).

Whey cheese manufacture is based on the principle that whey proteins will precipitate when slightly acidified (pH 6) and heated to high temperatures (>70°C). Although whey alone is ordinarily used as the starting material, milk can also be added. Much of the Ricotta produced in the United States, for example, contains a portion of skim or whole milk. The finished cheese, in this case actually consists of a casein-whey co-precipitate. Whey cheeses are not fermented, and since the lactose content of whey may be as high as 75% (on a solids basis), these cheeses will also contain 3% to 4% lactose and are generally sweet. Although Ricotta is a very soft cheese with a white, creamy color, other whey cheeses, such as Gjestot, can be very hard and a light brown color. The tan-to-brown color results from lactose caramelization and Maillard browning reactions that occur during high temperature heating.

During much of the previous century, cheese manufacturers in the US had few options for the large volume of whey that was generated. There was not much of a market for whey cheeses, and instead it was used as animal feed or fertilizer or, more likely, simply discarded altogether. In the past thirty years this practice has changed for several reasons. First, whey is considered to be environmental problem. It has a high biological oxygen demand (BOD), and, given that nearly 25 million kg are generated each year, discharging this much high BOD material down the drain or into the environment exceeds that which sewage treatment plants can handle.

Second, the dairy and food processing industries have developed products and applications in which economic value can be derived from whey and whey components. Many of these applications involve separation technologies (e.g., ultrafiltration and reverse osmosis) in which the more valuable whey protein fractions are obtained.. What remains from these processes is a lactose-rich material (whey permeate) that can be used directly, or further purified, as food- or chemical-grade lactose.

Importantly, it is technologically possible to use whey, whey permeate, or whey-derived lactose as feedstocks for various industrial fermentations, including the production of organic acids, ethanol, and other small molecules. However, given their current market value and the capital and processing costs that would be necessary, it does not make economic sense at the present time to produce these commodity chemicals from whey fermentations.

BIBLIOGRAPHY

Azarnia, S., N. Robert, and B. Lee. 2006. Biotechnological methods to accelerate Cheddar cheese ripening. Crit. Rev. Biotechnol. 26:121–143.

Dalgleish, D.G. and M. Corredig. 2012. The structure of the casein micelle of milk and its changes during processing. Ann. Rev. Food Sci. Technol. 3:449–467.

Donnelly, C.W. 2014. Cheese and Microbes. ASM Press, Washington DC.

Fox, P.F., P.L.H. McSweeney, T.M. Cogan, and T.P. Guinee. 2004. Cheese: Chemistry, Physics and Microbiology, 3rd edn, Volume 1: General Aspects. Elsevier Ltd., London.

Fox, P.F., P.L.H. McSweeney, T.M. Cogan, and T.P. Guinee. 2004. Cheese: Chemistry, Physics and Microbiology, 3rd edn, Volume 2: Major Cheese Groups. Elsevier Ltd., London.

Fox, P.F., T.P. Guinee, T.M. Cogan, and P.L.H. McSweeney. 2017. Fundamentals of Cheese Science, 2nd edn. Springer, New York.

Gobbetti, M., M. De Angelis, R. Di Cagno, L. Mancini, and P.F. Fox. 2015. Pros and cons for using non-starter lactic acid bacteria (NSLAB) as secondary/adjunct starters for cheese ripening. Trends Food Sci. Technol. 45:167–178.

Guinee, T.P. 2016. Protein in cheese products: structure-function relationships. In: P.L.H. McSweeney, J.A. O'Mahony, eds. Advanced Dairy Chemistry, Vol. 1B, 4th edn. Springer, New York, NY, pp. 347–415.

Hickey, C.D., M.A.E. Auty, M.G. Wilkinson, and J.J. Sheehan. 2015. The influence of cheese manufacture parameters on cheese microstructure, microbial localisation and their interactions during ripening: a review. Trends Food Sci. Technol. 41:135–148.

Hou, J., J.A. Hannon, P.L.H. McSweeney, T.P. Beresford, and T.P. Guinee. 2014. Effect of curd washing on cheese proteolysis, texture, volatile compounds, and sensory grading in full fat Cheddar cheese. Int. Dairy J., 34:190–198.

Johnson, M. and B.A. Law. 2010. The origins, development and basic operations of cheesemaking technology. In: Law, B.A. and A.Y. Tamime, eds. Technology of Cheesemaking, 2nd edn, Wiley, Oxford.

Loizzo, M.R., F. Menichini, N. Picci, F. Puoci, U.G. Spizzirri, and D. Restuccia. 2013. Technological aspects and analytical determination of biogenic amines in cheese. Trends Food Sci. Technol., 30:38–55.

Montel, M.C., S. Buchin, A. Mallet, C. Delbes-Paus, D.A. Vuitton, N. Desmasures, and F. Berthier. 2014. Traditional cheeses: rich and diverse microbiota with associated benefits. Int. J. Food Microbiol. 177:136–154.

Steele, J., J. Broadbent, and J. Kok. 2013. Perspectives on the contribution of lactic acid bacteria to cheese flavor development. Curr. Opin. Biotechnol. 24:135–141.

7 Fermented meats

> The smell of rot – the ripe funk you breathe in Italian pork stores and French charcuteries – has always been part of the craft of curing. Traditional dry-cured sausages – the rough-textured, chewy ones like Italian soppressata and French saucisson sec – aren't cooked. Instead, the raw meat is stuffed into natural casings and left exposed to the air, picking up wild yeasts and cultures that start fermentation. Then, like wine and cheese, the sausages are aged in a cool, humid place to develop the rounded, savory taste that comes from slow ripening.
>
> From a description of dry-cured sausages by food writer Julia Moskin, as quoted in the New York Times, 2006.

INTRODUCTION

If the origins of fermentation and the making and eating of fermented foods were based on food preservation, then meat serves as one of the best examples of the application of this technology. When, many thousands of years ago, wild or domesticated animals were slaughtered for food, unless some sort of low temperature storage was available, either the entire animal had to be cooked and consumed within a relatively short amount of time or the meat would spoil. There were no real options. Even if those early consumers were not very discerning, rotten meat must have still had limited appeal. Of course, it would have been far more consequential to eat meat, rotten or not, that caused a serious illness of one form or another. One can imagine that chopped or ground (i.e., comminuted) meat products must have been even more prone to spoilage.

It probably did not take long, therefore, for some aspiring food scientists to develop ways to preserve meat. Drying was undoubtedly among the first of these technologies proven to be successful. Salt combined with drying would have been even more effective. It was probably also observed that smoking preserved fresh meat. If, however, the salt level was not too high and other conditions were just right, then an entirely different type of product could be produced, one that had a tart but appealing flavor and lasted a long time, especially if combined with drying or smoking. Moreover, if the salt had also contained naturally-occurring potassium nitrate (otherwise known as saltpeter), a highly desirable cured meat color and flavor would have been achieved.

Microbiology and Technology of Fermented Foods, Second Edition. Robert W. Hutkins.

HISTORY AND EVOLUTION OF THE FERMENTED MEATS INDUSTRY

Like other fermented foods, recorded references to fermented meats date back thousands of years. The early development of these products likely originated in southern Europe and areas surrounding the Mediterranean Sea during the Roman era, although there were likely Asian counterparts that appeared around the same time. Even though it is not absolutely clear from the historical records whether these early sausage products were actually fermented, it is difficult to imagine, given the circumstances, that there wasn't some sort of natural fermentation occurring.

The manufacture of these early fermented meat products most certainly occurred as a result of accidents, and repeating these successes on a consistent basis must have been very difficult and taken many years. There were probably many failures. Still, naturally-fermented meat products, not too different from those produced centuries ago, are still manufactured and consumed in Europe.

We know now that the key to produce these meat products via a natural fermentation relies on creating conditions that select for the proper organisms (discussed below). It is easy for this type of fermentation to go awry and result in poor quality or unsafe products. However, another technique, called backslopping, was adopted to increase the probability of success. Backslopping relies on taking a portion of a good finished product (i.e., from a "successful fermentation") and adding it back to the starting raw material. Although the early practitioners of this method could not have appreciated the microbiological rationale, backslopping became one of the main ways to produce fermented meat products. This technology is still practiced throughout the world, even though, as we shall see later, there are now far more reliable methods for ensuring a consistent fermentation. In any event, the technology of fermented meats production remained an art for many years. In fact, sausage manufacturing was, like the manufacture of other fermented foods, performed by craftsmen, monks, and other trained specialists.

The actual number and types of fermented meat products that evolved around the world were many, and like other fermented foods, depended largely on geography. Certainly, preservation was the likely driving force for many of the processing practices that were adopted. In warmer areas, such as those in the Middle East and around the Mediterranean, spices were often added, and a drying step was common. Thus, dry, peppery, and spicy sausages, such as Genoa salami and pepperoni, evolved in Italy. In colder, more Northern areas, where sausage technology is more recent, spices were rarely added, and instead products were usually smoked or sometimes cooked following fermentation. Most of the moist and semi-dry German-style sausages, such as Thuringer, Lebanon bologna, and cervelat, are of this variety.

Ultimately, the drying, cooking, and smoking steps, the addition of salt and spices, as well as the incorporation of nitrate salts, not only added flavor and appeal, but also would have given fermented meats a long shelf-life, even at ambient temperature. These products are also enclosed within casings, which contributes to their stability and preservation, as post-processing microbial contaminants would be mostly excluded. In fact, the preservation of fermented meats serves as a perfect example of what food scientists now refer to as the hurdle or barrier concept of food preservation. Preservation is

achieved not by any one specific process, but rather by a combination of processes. Thus, fermentation is combined with drying, salting, smoking, cooking, antimicrobial chemicals, and packaging to ensure safety and to enhance preservation (discussed later in more detail).

CURRENT STATUS

The fermented meats industry, compared to other fermented foods industries, is relatively new. In fact, it was not until well into the twentieth century that manufacturers of fermented meats began to develop and apply modern production technologies. Previously, sausage production was mostly confined to craftsmen and very small-scale producers who made products on a batch-by-batch basis. Product quality, consistency, and especially safety were not always achieved. Equipment for grinding, mixing, and stuffing procedures, as well as fermentation chambers, only became available in the past 60 to 70 years. Moreover, pure curing agents, synthetic casings, and starter cultures have only been available since the 1960s. It is no coincidence that it was during this period that the industry increased in size and that rapid, high throughput manufacturing methods capable of producing products of consistent quality and safety developed.

Today the variety of fermented meat products available around the world is nearly equal to that of cheese. In Spain, for example, there are at least 50 different types of fermented sausages, and in Germany there are more than 350. As one commentator wrote, "Fermented meats are among the most attractive foods from a sensory perspective," adding that "they contribute to cultural and geographical identity" (Vogel, 2012). Not only is this variation due to the meat source (i.e., beef, pork, goat, sheep, etc.), but the cut of meat, the amount and coarseness of the fat, and the casing material used to form the shape all have a profound influence on the finished product. The level of dryness and whether or not smoke is applied or mold growth permitted to occur are especially important factors and form the basis of fermented meats classification (Table 7.1). Within Europe, there are important distinctions with regard to the meaning of "cured". For example, in the Mediterranean, cured usually includes nitrite-treated meats that are fermented, aged, and dried. In contrast, in Northern Europe, cured typically refers to processed meat products containing nitrate curing salts.

To the latter point, it is worth emphasizing, especially for readers less familiar with sausage nomenclature, that many of the sausage products available in the marketplace are simply cured, comminuted products. Thus, they contain curing salts and undergo many of the same processing steps as do fermented sausages, but they are not fermented. Thus, frankfurters, bologna, and breakfast sausages are not fermented and will not be discussed in this chapter. In contrast, not all fermented meat products are made from comminuted meat. There are, for example, fermented meat products that are made from whole, intact meat materials, such as the country hams popular in selected regions in the United States and Europe. These products will be discussed separately.

Although fermented meats are produced and consumed worldwide, they are most popular in Europe. Although consumption figures, when they exist, often combine all processed meats (fermented and not-fermented), available data indicates that European countries are the main consumers of fermented meats (Table 7.2). More than 600×10^6 kg of fermented sausage are consumed in Germany, Spain, France, and Italy. Collectively, about 3% to 5% of all meat consumed in these countries is in the form of fermented sausages.

Table 7.1 Examples of fermented meats and sausages.

Product	Meat	Areas produced	Features
Moist sausages (a_w > 0.94)			
Mettwurst	Beef	Germany	Low pH (<5.0) Smoked Spreadable
Lebanon bologna	Beef	North America	Low pH Smoked
Mortadella	Pork	Italy, France, USA	Low pH Unsmoked Cooked
Semi-dry sausages (a_w = 0.90–0.95)			
Summer sausage	Beef/Pork	USA	Moderate pH (4.9–5.2) Smoked
Thuringer cervelat	Pork	Italy, France, USA	Low pH Unsmoked Cooked
Dry sausages (a_w < 0.90)			
Chorizo	Pork	Spain, Europe, Americas	Low pH
Salchichon	Pork	Spain, Europe, Americas	Spiced Smoked or unsmoked Low pH Unsmoked
Pepperoni	Beef/Pork	Italy, North America	Low pH Unsmoked Spiced
Salami	Beef/Pork	Europe, USA, Mexico	Low pH Unsmoked Raw or cooked
Whole meats			
Country-cured hams	Pork	North America	Heavily salted
Parma hams	Pork	Italy	Heavily salted

Table 7.2 Consumption of fermented meats.

Country	kg/person/year
Canada[1]	0.9
Finland[1]	3.1
France[2]	3.1
Germany[2]	7.1
Italy[2]	4.5
Spain[2]	2.7
United Kingdom[2]	0.1
United States[1]	0.3

[1] Estimated from 2001 WHO statistics.
[2] Adaped from Lucke and Zangeri, 2014.

MEAT COMPOSITION

Fresh meat is a nutrient-rich medium and is an excellent food source for supporting growth of microorganisms. Lean skeletal bovine muscle contains nearly 20% high quality protein, about 2% to 3% lipid, and small amounts of carbohydrate, non-protein nitrogen, and inorganic material. The balance, approximately 75%, is water. Therefore, the water activity (a_w) of fresh meat is nearly 0.99. The pH of the fresh tissue, before rigor, is 6.8 to 7.0, but decreases to about 5.6 to 5.8 following rigor, due to post-mortem glycolysis by endogenous enzymes present within muscle cells.

These values may vary somewhat, depending on the animal and its status before slaughter, but regardless of the source of meat (e.g., pork usually has a slightly higher pH), these conditions are just about perfect for most bacteria, including spoilage organisms and pathogens. Consequently, raw meat is highly perishable. And although the interior of intact meat tissue is sterile, the exterior surface harbors a wide array of aerobic and facultative bacteria. In its comminuted form, i.e., chopped or ground, meat spoilage can be even more rapid, because the members of the surface microbiota become homogeneously distributed throughout the meat. Even though oxygen may also be incorporated into the mixture, enhancing growth of aerobes, anaerobic pockets can also develop within the interior, providing suitable environments for anaerobic bacteria. Among the latter are species of *Clostridium*, including *Clostridium botulinum* and other related species capable of causing serious, sometimes fatal food poisoning disease. Since fermented sausages are typically made using raw, comminuted meat, controlling the resident microbiota, especially pathogens, is of utmost importance. If control is not maintained, the results can be disastrous.

FERMENTATION PRINCIPLES

In contrast to the lactic acid fermentation that occurs in milk, the meat fermentation has been, until recently, considerably less well studied and understood. In fact, the use of pure, defined starter cultures in the fermented meats industry is a relatively recent development, having began only in the 1950s and 1960s. Before the use of meat starter cultures, the most common way to start the fermentation practice was, as noted above, backslopping.

Backslopping works for several reasons. First, backslopping ordinarily selects for those bacteria that are well suited for growth in the sausage environment. Strains that are slow to scavenge for carbohydrates, are inhibited by fermentation acids, or are sensitive to salt or nitrite will not be maintained. Instead, they will be displaced by more competitive bacteria that have particular metabolic and physiological traits that confer selective advantages in that environment. Thus, even a prolific acid-forming strain does not have much of a chance if it is not also tolerant of salt and nitrite and is able to grow in a low oxygen environment.

Second, the bacterial population that becomes established during repeated transfers and fermentations will likely be heterogeneous in nature, consisting of multiple species and strains. If, for example, one such strain were to suddenly die or otherwise be lost due to the presence of bacteriophages or an inhibitory agent, then the remaining strains, acting as back-up, would still be able to complete the fermentation.

Finally, backslopping is effective simply due to the size of the inoculum, which is usually around 5%, but which can be as high as 20% of the total mass. Such a large inoculum provides reasonable assurance that the desired organisms will overwhelm the background microbiota and that undesirable interlopers will have little chance of competing.

Despite backslopping's long history and wide use, there are several drawbacks associated with it. Fermentation times may be subject to considerable variation and can be unreliable and difficult to control. Products made by backslopping may have inconsistent quality, including flavor and color. In large production facilities in Europe, and especially in the United States, short and consistent fermentation times, standardized production schedules, and consistent product quality have become essential. A manufacturer producing a single, small batch of product can perhaps tolerate delays, and may even come to appreciate modest differences in product quality. However, inconsistent product quality would be unacceptable for consumers, and production delays due to sluggish fermentations would be intolerable for large manufacturers who have substantial employee payrolls and tight production schedules. Above all, the entire backslopping process can be considered microbiologically risky, since any deviation from the norm (i.e., slow or delayed fermentation) may permit growth of *Staphylococcus aureus*, *Listeria monocytogenes, C. botulinum*, or a range of other pathogens of public health significance. This would also be problematic if a recall were to occur, as no separation of lots would exist.

Although obvious, the following point cannot be over-emphasized: sausage is made from raw meat that may well contain pathogenic organisms. If a cooking step is not included, fermentation represents the primary means of preservation and the main barrier against pathogens. Since the actual fermentation can take many hours or even days, a slow or failed fermentation may not be discovered right away, permitting growth of pathogenic bacteria. If the bacteria are able to reach high levels, even subsequent acid production (or even a heating step) may not be sufficient to inactivate these pathogens.

It is important to mention, as noted above, that it is entirely possible to produce fermented sausages without any type of culture at all. This practice is not uncommon, and is still used in many parts of the world. One can simply prepare the sausage mixture and wait for the natural lactic flora to take over. This method is successful because the formulation and fermentation conditions (i.e., salt, nitrate, low temperature, and an anaerobic environment) provide sufficient selection of desirable lactic acid bacteria. However, for obvious food safety reasons, none of the large, modern manufacturers in the United States or elsewhere rely on natural fermentations. Finally, there is one other way to produce sausages that have a tangy or acid-like flavor without a starter culture and without relying on the indigenous microbiota. The meat mixture can be directly acidified using a food-grade acidulant (e.g., citric or lactic acid or glucono-δ-lactone), which results in a product with sensory properties that mimic (somewhat) those of fermented sausage.

Meat starter cultures

Microbiologists did not really begin to study the microbial ecology of fermented sausage until the 1940s. It soon became clear that lactic acid bacteria were the primary organisms responsible for the fermentation. This conclusion was based on the fact that the predominant organisms isolated from naturally fermented sausages were species of *Lactobacillus*. When the isolates were propagated and re-inoculated into fresh meat, a well-fermented sausage could be produced with all the expected characteristics.

Patents, based on using these bacteria as meat starter cultures, were assigned in Europe and the United States. However, applications of this technology were initially unsuccessful. This was because the *Lactobacillus* strains that had been identified and used successfully in trial situations were difficult to mass produce in a form convenient for sausage manufacturers. For any organism to function well as a starter culture, it must not only satisfy the performance criteria, but it must also be present in high numbers and be viable at the time of

use. In the case of the *Lactobacillus* cultures, cell viability following lyophilization (or freeze drying, the main form of starter culture preservation) was poor, leading to slow and unacceptable fermentation rates.

Demand for a culture that could be used for fast, consistent, large-scale production of fermented sausage eventually led to the discovery of other lactic acid bacteria that not only had the relevant performance characteristics, but also the durability required for commercial applications. One organism, classified as *Pediococcus cerevisae*, was found to have these properties. Even though pediococci are not normally found in fermented sausage (they are, however, involved in vegetable fermentations), this organism was introduced in the United States in the late 1950s as the first meat starter culture. Strains of this species, later re-classified as either *Pediococcus acidilactici* or *Pediococcus pentosaceus,* are still widely used today.

In addition, improvements in starter culture technology and the development of frozen concentrated cultures (see Chapter 4) enhanced culture viability such that *Lactobacillus* starter cultures are now widely used. Initially, the *Lactobacillus* strains that were isolated from fermented sausage and used as starter cultures were classified as *Lactobacillus plantarum*. Other strains of *Lactobacillus* that had different physiological properties and that performed well in sausage manufacture were subsequently isolated. Thus, the closely related species, *Lactobacillus sakei* and *Lactobacillus curvatus,* are also used in starter culture preparations.

Although the different *Pediococcus* and *Lactobacillus* strains used as starter cultures perform the same basic role – they ferment sugars and produce organic acid, they vary with respect to several important physiological and biochemical properties (Table 7.3). These differences influence how they are used as starter cultures. First, different species have different temperature optima and different thermal tolerances. For example, *L. sakei* and *L. curvatus* are considered psychrotrophic, meaning they are capable of growth, albeit slowly, at temperatures as low as 4°C. Thus, they are suitable for fermentations conducted at cool ambient temperatures.

Many of the European sausages are fermented at low temperatures (20°C) to control the fermentation rate and to provide sufficient time for nitrate-reduction and subsequent color and flavor development (Box 7.1). In contrast, *P. acidilactici* has a growth optimum near

Table 7.3 Properties of bacteria used in meat starter cultures.[1]

Organism	Minimum temperature	Temperature optimum	Acid from glucose[2]	Nitrate reductase	Primary function
Lactobacillus sakei	4°C	42°C	+	–	acid
Lactobacillus curvatus	4°C	42°C	+	–	acid
Lactobacillus plantarum	10°C	42°C	+	–	acid
Pediococcus pentosaceus	15°C	28–32°C	+	–	acid
Pediococcus acidilactici	15°C	40°C	+	–	acid
Kocuria varians	10°C	25–37°C	–	+	flavor, aroma
Staphylococcus carnosus	10°C	30–40°C	–	+	flavor, aroma
Staphylococcus xylosus	10°C	25–35°C	–	+	flavor, aroma

[1] Adapted from Bergey's Manual of Systematic Bacteriology, Volume 3, 8th edn.
[2] Under anaerobic conditions.

Box 7.1 *Micrococcaceae*, nitrate, and "old world" sausage

In the United States, most fermented sausage starter cultures contain only lactic acid bacteria, usually species of lactobacilli and pediococci. These organisms perform one primary function - they produce lactic acid, decreasing the pH of the meat to levels that inhibit growth of pathogenic and spoilage organisms. The fermentation temperatures are usually at the optimum for the specific strain, and fermentation times are short.

In contrast, for many European products (and a few in the United States), the cultures contain lactic acid bacteria as well as coagulase negative species of *Staphylococcus* and *Kocuria* from the family Micrococcaceae (Leroy et al., 2006). Fermentation temperatures are lower for European-style products, such that growth of lactic acid bacteria occurs at a slower rate. The cocci are therefore able to grow at these low fermentation temperatures and over longer fermentation times.

For fermented sausages manufactured in the United States, nitrite is, by far, the most common curing agent. In contrast, nitrate salts are generally used in the manufacture of European-style sausages. These differences in culture composition and choice of curing agent reflect the desired quality attributes relative to so-called "new world" and "old world" sausage-making technologies and styles (Aquilanti et al., 2016).

Note that all cured meats (whether fermented or not) contain either nitrate or nitrite salts as the curing agent. However, it is the nitrite form (i.e., NO_2^-), and not the nitrate (NO_3^-) that actually reacts with the meat pigments and provides the curing effect. If nitrate is used as the curing agent, it must first be converted to nitrite (Figure 7.1.1). This conversion is simply a reduction reaction catalyzed by the enzyme nitrate reductase. However, there are additional independent enzymatic and chemical reactions that occur that are responsible for color and flavor formation. These are influenced by pH, moisture and other environmental factors (Mainar et al., 2017).

In general, bacteria that produce nitrate reductase are respiring aerobes that use nitrate as an electron acceptor during growth under anaerobic or low oxygen conditions. Among the organisms having high nitrate reductase activity are species of *Staphylococcus* and *Kocuria*. Thus, if the sausage formulation contains nitrate, then

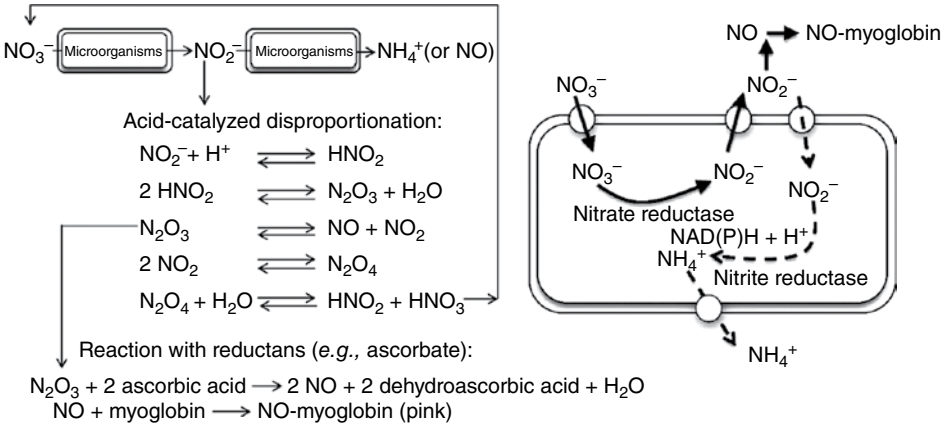


Figure 7.1.1 Conversion of nitrate into nitrite and formation of fermented meat color by coagulase-negative cocci. Adapted from Mainar et al., 2017 and Hammes, 2012, with permission.

its conversion to nitrite will depend on the presence of nitrate reductase-producing strains of these bacteria, either those naturally present in the raw meat or added in the form of a starter culture. In the latter case, it might seem rather odd to add staphylococci to food, since some coagulase-positive staphylococci (e.g., *Staphylococcus aureus*) are pathogenic and produce exotoxins. However, the strains of *Staphylococcus carnosus* and *Staphylococcus xylosus* used for sausage fermentations are coagulase-negative and non-toxigenic. This emphasizes why it is important to accurately identify strains of *Staphylococcus* that might end up in a starter culture (Morot-Bizot et al., 2004).

If nitrate must be converted to nitrite to produce cured sausage, then the obvious question to ask is why add nitrate in the first place? Why depend on the nitrate-reducing bacteria to perform this important function? In other words, why wouldn't all sausage manufacturers simply add nitrite as the curing agent, as is widely done in the United States? To answer these questions, one must consider what other functions the micrococci and other nitrate-reducing bacteria might perform in fermented sausage, other than to reduce nitrate. It turns out there are several.

The actual nitrate-to-nitrite reaction occurs rather slowly during the sausage fermentation because the prevailing conditions (low temperature and low pH) are less than optimal for nitrate reductase activity. It can take several weeks for the conversion of nitrate to nitrite. This affords the relevant *Kocuria* and *Staphylococcus* spp. the opportunity to secrete lipases and other enzymes that ultimately generate flavor precursors in the meat. If, instead of ripening the sausage at 20°C, it was fermented at 38°C to 40°C, the lactic acid bacteria would produce acid too quickly, and the non-lactic acid bacteria would be inhibited and unable to reduce nitrate or otherwise influence the properties of the finished product. It is also argued that slow conversion of nitrate to nitrite enhances color development (although it's not clear why this should be the case).

Among the flavor compounds produced by staphylococci and kocuriae are metabolic end products resulting from protein and fatty acid metabolism. Most strains are lipolytic and many are also proteoyltic. In addition, amino acid metabolism may also generate flavor and aroma compounds. For example, it was reported that metabolism of leucine by *Staphylococcus xylosus* resulted in formation of various metabolites, such as 3-methylbutanol and 2-methylpropanol, that contribute to the flavor properties of fermented meats (Beck et al., 2004). Protein hydrolysis by these organisms may also enhance growth of the lactobacilli that are necessary for lactic acid formation (Tremonte et al., 2010). Finally, it should be noted that although endogenous lipases (i.e., in the muscle tissue) contribute to flavor formation, coagulase-negative cocci also play an important role, via hydrolysis of triglycerides as well as by subsequent oxidation and metabolism of free fatty acids (Mainar et al., 2017).

References

Aquilanti, L., C. Garofalo, A. Osimani, and F. Clementi. 2016. Ecology of lactic acid bacteria and coagulase negative cocci in fermented dry sausages manufactured in Italy and other Mediterranean countries: an overview. Int. Food Res. J., 23:429–445.

Beck, H.C., A.M. Hansen, and F.R. Lauritsen. 2004. Catabolism of leucine to branched-chain fatty acids in *Staphylococcus xylosus*. J. Appl. Microbiol. 96:1185–1193.
Hammes, W.P. 2012. Metabolism of nitrate in fermented meats: The characteristic feature of a specific group of fermented foods. Food Microbiol. 29:151–156.
Leroy, F. J. Verluyten, and L. De Vuyst. 2006. Functional meat starter cultures for improved sausage fermentation. Int. J. Food Microbiol. 106:270–285.
Mainar, M.S., D.A. Stavropoulou, and F. Leroy. 2017. Exploring the metabolic heterogeneity of coagulase-negative staphylococci to improve the quality and safety of fermented meats: a review. Int. J. Food Microbiol. 247:24–37.
Morot-Bizot, S.C., R. Talon, and S. Leroy. 2004. Development of a multiplex PCR for the identification of *Staphylococcus* genus and four staphylococcal species isolated from food. J. Appl. Microbiol. 97:1087–1094.
Tremonte, P., A. Reale, T. Di Renzo, L. Tipaldi, A. Di Luccia, R. Coppola, E. Sorrentino, and M. Succi. 2010. Interactions between *Lactobacillus sakei* and CNC (*Staphylococcus xylosus* and *Kocuria varians*) and their influence on proteolytic activity. Lett. Appl. Microbiol. 51:586–594.

40°C and is preferred by manufacturers interested in fast, high temperature fermentations. Metabolic differences also exist between these organisms. Whereas all pediococci and lactobacilli ferment glucose, other sugars are fermented only by specific strains. Pediococci, for example, do not ferment lactose. Sausage formulations must, therefore, account for the metabolic capacity of the culture.

There are occasions when starter culture metabolism can lead to problems and defects in the finished product. All pediococci are considered to be homofermentative, producing only lactic acid from glucose. However, under some circumstances, such as during sugar limitation, heterofermentative products such as ethanol, carbon dioxide, and acetic acid can be formed. Similarly, small amounts of these end products can also be produced by homofermentative *L. plantarum* during sugar limitation or aerobic growth. Of the heterofermentative end products that are formed, acetic acid is especially undesirable in fermented sausage because it imparts a sour, vinegar-like flavor rather than the tart, tangy flavor contributed by lactic acid.

The ability of some strains to produce peroxides is also a serious problem. Depending on the starter culture strain and the level of oxygen in the environment, hydrogen peroxide can be formed directly in the fermenting sausage. Hydrogen peroxide can react with heme proteins in the muscle tissue to form undesirable, green pigments. In addition, hydrogen peroxide and peroxide radicals promote lipid oxidation, a serious flavor defect. Although some strains may also produce catalase or catalase-like enzymes that degrade hydrogen peroxide, the production of these enzymes cannot always be counted on to inactivate accumulated peroxides.

Protective properties of cultures

It has long been suggested that lactic acid starter cultures were responsible for preservation effects beyond their acidification and pH-lowering effects. During growth in meat, they scavenge sugars and other nutrients faster than competitors and lower the oxidation-reduction potential (Eh) of the environment such that growth of aerobic organisms is inhibited. In the 1980s it was discovered that some of the strains used as meat starter cultures may provide additional preservation effects via production of antagonistic agents that inhibited other bacteria. Although some strains could produce inhibitory levels of hydrogen peroxide,

experimental evidence indicated that a substance other than hydrogen peroxide was responsible for the inhibitory effects. It was subsequently discovered that these bacteria were capable of producing a class of substances known as bacteriocins.

Bacteriocins are proteinaceous substances with bactericidal activity, usually against bacteria that are closely related to the producer organism. Among the bacteria used as starter cultures for fermented sausage, several species have been shown to produce bacteriocins, including strains of *P. acidilactici, L. plantarum,* and *L. sakei*. Moreover, several of these bacteriocins, in particular the plantaricins and sakacins, have a somewhat broad spectrum of activity, inhibiting pathogens such as *L. monocytogenes*, *S. aureus*, and *C. botulinum*, as well as Gram-positive spoilage organisms. Greater application of these so-called bioactive or protective cultures is likely to increase (Box 7.2), perhaps leading to their use in traditionally non-fermented sausages. For example, various research laboratories have shown that *P. acidilactici* can be incorporated into frankfurter mixtures, and even in the absence of fermentation, can reduce *L. monocytogenes* populations.

Box 7.2 Fermented meats and bioprotective cultures

Protecting meat with bacteriocins

It has long been known that lactic acid bacteria have inhibitory activity against other bacteria, above and beyond that due to the lactic acid they produce. Some lactic acid bacteria, for example, also produce acetic acid, hydrogen peroxide, and diacetyl, all of which can inhibit potential spoilage or pathogenic organisms. However, the antimicrobial compounds that have attracted the most attention are the bacteriocins. These agents are now being used, in a variety of delivery formats, in a wide range of foods, including fermented and other processed meats. Although fermented meats, by virtue of their intrinsic properties (organic acids, low pH, low a_w, smoke, spices) are not prone to microbial spoilage and infrequently serve as vehicles for foodborne pathogens, the addition of a bacteriocin to these products provides manufacturers with one more barrier and an extra margin of safety.

Bacteriocins defined and classified

Bacteriocins are defined as proteinaceous compounds produced by bacteria that inhibit other closely related bacteria. They are distinguished from antibiotics in several respects (Perez et al., 2014). Specifically, bacteriocins are ribosomally-synthesized, they usually have a narrow spectrum of activity, and they are stable to heat and pH. Although the bacteriocins described in this discussion are produced by lactic acid bacteria, the ability to produce bacteriocins is widespread among all major groups of bacteria. In fact, a 2017 PubMed search on the term "bacteriocin" led to more than 7000 published article hits since 1980, with more than 2300 published since 2010 (about one per day!).

Classification of lactic acid bacteria-produced bacteriocins has been based on genetic, structural, and physiological properties, and numerous different classification schemes have been proposed (Klaenhammer, 1993; Cotter et al., 2013; Perez et al., 2014; Cavera et al., 2015). In general, class I bacteriocins are small peptides that undergo post-translational modification. These bateriocins are often referred to as

lantibiotics, because they contain the unusual amino acid, lanthionine. Nisin, the most well-studied bacteriocin, belongs to this class. Class II bacteriocins consist of several small non-modified peptides that are heat stable, making them good antimicrobials for heat-treated products, like cooked sausages. Class III contains the high molecular weight (>10 kDa), heat-sensitive bacteriocins. Most of the bacteriocins used in food applications belong to Class I or II.

Bacteriocin applications in sausage

On a practical basis, there are two main reasons why bacteriocins are used in meat products: (1) to prevent growth of spoilage organisms and the production of spoilage end products; and (2) to inhibit or kill organisms of food safety concern. Lactic acid bacteria, enterococci, *Brochothrix*, and clostridia are among the spoilage organisms inhibited by specific bacteriocins. However, control of pathogens and *Listeria monocytogenes*, in particular, is one of the main reasons for why bacteriocins are being used in fermented meat products. The United States has a zero-tolerance policy for *Listeria* in fermented meat products, so any technology or product that would reduce the frequency of a positive test result for *Listeria* would be valuable.

It should also be noted that these same concerns about *Listeria* and other pathogens have led manufacturers of non-fermented, ready-to-eat meat products (e.g., hot dogs, luncheon meats) to consider the use live bacteriocin-producing cultures, but under conditions in which fermentation does not occur or is minimized (Woraprayote et al., 2016).

Among the bacteriocins inhibitory or bacteriocidal to *L. monocytogenes* are those produced by *Pediococcus acidilactici*, *Lacobacillus sakei*, and *Lactobacillus plantarum*. Because these organisms (and other related species) are ordinarily used as starter cultures for fermented meats, if the appropriate bacteriocin-producing strains were included in the culture blend, bacteriocins could be produced *in situ* during the fermentation. This approach was first described in 1992 (Foegeding et al.), and many more examples have since been described (Table 7.2.1). Likewise, adjunct cultures, added only for their bacteriocin-producing ability, can also be added.

Although including bacteriocin-producing strains in a starter culture is a convenient way to introduce bacteriocins into a product, there is no assurance that the bacteriocin will always be produced on a consistent basis and at levels necessary to achieve the desired effect. Thus, other approaches have been considered to ensure that sufficient amounts are actually delivered. For example, a pure bacteriocin can either be added directly to the sausage batter or applied to the surface in the form of a dip or spray. It is also possible to incorporate bacteriocins into packaging films. However, nisin is the only bacteriocin that has been purified and granted "generally recognized as safe" (GRAS) status. Purification processes and clearing regulatory hurdles are expensive activities, which explains why so few "pure" bacteriocins have been commercialized.

An alternate strategy is to use bacteriocins in a non-purified form. This can be achieved by growing the producer strain in food-grade medium under conditions optimized for bacteriocin expression. The entire contents of the fermentation can be pasteurized to inactivate the bacteria and then concentrated by drying or ultrafiltration.

Table 7.2.1 Application of protective cultures in fermented sausages.

Product	Producer(s)	Target(s)	Reference
Dry sausage	*Carnobacterium piscicola CS526*	*E. coli* *Listeria*	Azuma et al., 2007
Fermented sausage (Alheira)	*Pediococcus acidilactici* HA-6111-2	*Listeria*	Albano et al., 2008
Belgian-type sausage and Italian salami	*Lactobacillus sakei* CTC *494*	*Listeria*	Ravyts et al., 2008
Spanish dry-fermented sausage	*Pediococcus acidilactici* MCH14	*Clostridium* *Listeria*	Nieto-Lozano et al., 2010
Chorizo sausage	*Lactobacillus sakei* CTC494	*Listeria*	Ortiz et al., 2014
Fermented sausage	*Lactobacillus sakei* C2	*Listeria*	Gao et al., 2014
Salami	*Leuconsotoc carnosum B4010* *Pediococcus acidilactici DSM10313*	*Listeria*	Raimondi et al., 2014
Salami	*Lactobacillus curvatus MBSa2*	*Listeria*	De Souza Barbosa., 2015
Dry fermented sausage	*Lactobacillus curvatus* 54M16	*Listeria* *Bacillus*	Casaburi et al., 2016
Fermented pork	*Lactobacillus plantarum* PCS20	*Clostridium*	Di Gioia et al., 2016

These products have several advantages. First, they not only contain the active bacteriocin, but also the antimicrobial organic acids that were produced during fermentation. These so-called "fermentate" antimicrobial products do not require regulatory approval as food additives, as would purified bacteriocins. Finally, with many food companies concerned about "clean" labels, these products can be labeled in a more consumer-friendly as "cultured dextrose" or "cultured whey".

References

Albano, H., C. Pinho, D. Leite, J. Barbosa, J. Silva, L. Carneiro, R. Magalhães, T. Hogg, and P. Teixeira. 2009. Evaluation of a bacteriocin-producing strain of *Pediococcus acidilactici* as a biopreservative for "Alheira", a fermented meat sausage. Food Control. 20:764–770.

Azuma, T., D.K. Bagenda, T. Yamamoto, Y. Kawai, and K. Yamazaki, K. 2007. Inhibition of *Listeria monocytogenes* by freeze-dried piscicocin CS526 fermentate in food. Lett. Appl. Microbiol. 44:138–144.

Casaburi, A., V. Di Martino, P. Ferranti, L. Picariello, and F. Villani. 2016. Technological properties and bacteriocins production by *Lactobacillus curvatus* 54M16 and its use as starter culture for fermented sausage manufacture. Food Control. 59:31–45.

Cavera, V.L., T.D. Arthur, D. Kashtanov, and M.L. Chikindas. 2015. Bacteriocins and their position in the next wave of conventional antibiotics. Int. J. Antimicrob. *Agents.* 46:494–501.

Cotter, P.D., R.P. Ross, and C. Hill. 2013. Bacteriocins – a viable alternative to antibiotics? Nat. Rev. Microbiol. 11:95–105.

Di Gioia, D., G. Mazzola, I. Nikodinoska, I. Aloisio, T. Langerholc, M. Rossi, S. Raimondi, B. Melero, and J. Rovira. 2016. Lactic acid bacteria as protective cultures in fermented pork meat to prevent *Clostridium* spp. growth. Int. J. Food Microbiol. 235: 53–59.

de Souza Barbosa, M., S.D. Todorov, I. Ivanova, J.-M. Chobert, T. Haertlé, and B.D.G. de Melo Franco. 2015. Improving safety of salami by application of bacteriocins produced by an autochthonous *Lactobacillus curvatus* isolate. Food Microbiol. 46:254–262.

Foegeding, P.M., A.B. Thomas, D.H. Pilkington, and T.R. Klaenhammer. 1992. Enhanced control of *Listeria monocytogenes* by in situ-produced pediocin during dry fermented sausage production. Appl. Environ. Microbiol. 58:884–890.

Gao, Y., D. Li, and X. Liu. 2014. Bacteriocin-producing *Lactobacillus sakei* C2 as starter culture in fermented sausages. Food Control 35:1–6.

Klaenhammer, T.R. 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. 12:39–85.

Nieto-Lozano, J.C., J.I. Reguera-Useros, M.d.C. Peláez-Martínez, G. Sacristán-Pérez-Minayo, A.J. Gutiérrez-Fernández, and A.H.d. la Torre. 2010. The effect of the pediocin PA-1 produced by *Pediococcus acidilactici* against *Listeria monocytogenes* and *Clostridium perfringens* in Spanish dry-fermented sausages and frankfurters. Food Control 21:679–685.

Perez, R.H., T. Zendo and K. Sonomoto. 2014. Novel bacteriocins from lactic acid bacteria (LAB): various structures and applications. Microb. Cell Fact.13(Suppl 1):S3.

Ortiz, S., V. López, M. Garriga, and J.V. Martínez-Suárez. 2014. Antilisterial effect of two bioprotective cultures in a model system of Iberian chorizo fermentation. Int. J. Food Sci. Technol. 49:753–758.

Raimondi, S., M. Popovic, A. Amaretti, D. Di Gioia, and M. Rossi. 2014. Anti-*Listeria* starters: *in vitro* selection and production plant evaluation. J. Food Prot. 77:837–842.

Ravyts, F., S. Barbuti, M.A. Frustoli, G. Parolari, G. Saccani, L. De Vuyst, and F. Leroy. 2008. Competitiveness and antibacterial potential of bacteriocin-producing starter cultures in different types of fermented sausages. J. Food Prot. 71:1817–1827.

Woraprayote, W., Y. Malila, S. Sorapukdee, A. Swetwiwathana, S. Benjakul, and W. Visessanguan. 2016. Bacteriocins from lactic acid bacteria and their applications in meat and meat products. Meat Sci.120:118–132.

Non-lactic acid bacteria cultures

As described above, most meat starter cultures available in the United States contain species belonging to one of two genera of lactic acid bacteria, *Lactobacillus* and *Pediococcus*. In Europe, a quite different type of starter culture has historically been used. Specifically, cultures used for European or European-style fermented sausages contain not only lactic acid bacteria, but also organisms belonging to the family *Micrococcaceae*. These include species of Gram-positive, coagulase-negative *Staphylococcus, Micrococcus,* and *Kocuria*. In fact, when lactic acid bacteria starter cultures were first introduced in the United States nearly 50 years ago for sausage manufacture, the first European cultures contained only *Micrococcus*. These micrococci cultures are still available.

Whereas the main function of the lactic starter culture is essential – to produce lactic acid and lower the pH, the inclusion of *Micrococcaceae* in meat starter cultures, while important, is strictly optional. These bacteria are not fermentative and they produce no acid end products. Moreover, although they are metabolically active, they hardly even grow in the sausage. Rather, these bacteria are included in starter cultures for other functional purposes. First, they convert nitrate to nitrite via expression of the enzyme nitrate reductase. Nitrite, after all, is the active form that participates in color and flavor reactions. It's also nitrite that has antimicrobial properties. Along the way, the *Micrococcaceae* also contributes directly to flavor development via metabolism of lipids and proteins. Is it important to note that because they

are mesophilic, the fermentation temperature must fall within the range of 18°C to 25°C. Thus, the lactic acid bacteria present in the culture must also be capable of growth within this range. If fermentation were to occur at higher temperatures (e.g., 32°C to 40°C), the rapid acid development would inhibit growth of the micrococci and the benefits they provide would not be achieved.

In summary, there are at least five functions performed by meat starter cultures (Table 7.4). The culture must: (1) produce lactic acid and lower the pH; (2) produce desirable flavors; (3) out-compete spoilage and pathogenic microorganisms for substrates and nutrients; (4) lower the Eh, since *Salmonella*, *S. aureus*, and other pathogens grow better aerobically; and (5) in the case of the *Micrococcaceae* cultures, enhance flavor and color development via reduction of nitrate. Although a number of factors account for the excellent safety record of fermented meat products (Box 7.3), the role of the culture in producing cannot be overemphasized (see below).

Table 7.4 Desirable properties of meat starter cultures.[1]

Bacteria	Fungi
• Non-pathogenic	• Non-pathogenic
• No toxins produced	• No toxins produced
• Grows well in meat	• Competitive at the surface
• Stable	• Firm surface mycelium
• Produces good flavor	• Proteolytic and lipolytic
• Nitrate/nitrite resistant	• Moldy aroma
• Salt-tolerant	
• Bioprotective	
• Easy to identify	

[1] Adapted from Ojha et al., 2015 and Cocconcelli and Fontana, 2010.

Box 7.3 Safety record of fermented sausage

All fermented sausages are made from raw meat. Many versions, including dry fermented and other traditionally-produced sausages are never cooked and are consumed without any heat processing. Thus, it is not unreasonable to for public health authorities to be concerned about the microbiological safety of these products. The emergence of several serious foodborne pathogens in the 1980s, such as enterohemorrhagic *Escherichia coli* (EHEC) O157:H7, other so-called Shiga toxin-producing *E. coli*, and *Listeria monocytogenes*, has raised additional concerns, since these organisms are frequently present in raw meat. Both of these organisms have high mortality rates. In addition, *Listeria* can tolerate hostile environments better than other foodborne pathogens. Of course, if not properly manufactured, other foodborne pathogens, such as *Staphylococcus aureus*, *Clostridium botulinum*, and *Salmonella*, can present significant food safety risks in fermented sausages.

Despite these concerns, manufacturers, as well as consumers of fermented sausages have long considered these products to be safe. Indeed, fermented sausages, and dry fermented sausages, in particular, have generally withstood the test of time (Holck et al., 2017). Their safety is undoubtedly due to the presence of multiple antimicrobial

barriers or hurdles that exist in fermented sausages (Roccato et al., 2017; Table 7.3.1). After all, for a given organism to survive and grow in these products, it would have to overcome each of these individual barriers. That is, at minimum, it would have to be nitrite-resistant, acid-resistant, salt-resistant, and osmotolerant. Moreover, when barriers are combined, there is a synergistic effect, such that the net antimicrobial effect of multiple barriers is greater than would be predicted based on their singular effects. For example, a strain of *Salmonella* may be fully capable of surviving a pH of 5.2, provided that all other conditions are optimal for growth. But if another hurdle is added (e.g., the water activity is reduced from 0.99 to 0.97), then pH 5.2 may be sufficient to inhibit this organism.

The problem, however, is now more complicated, because it has been recognized that some pathogens are tolerant even to multiple barriers. For example, *L. monocytogenes* can be resistant to low pH, low water activity (a_w), high salt, and nitrite, and can even grow at refrigeration temperatures (Meloni, 2015). Nonetheless, since 2007, there have been few food poisoning outbreaks caused by *E. coli*, *Salmonella*, or other pathogenic organisms in fermented sausage (Table 7.3.2). In general, short ripening

Table 7.3.1 Antimicrobial barriers in fermented meats.

Property	Level, range, or function
pH	4.5–5.5
Acids	1–2%
Water activity	0.7–0.9
Salt	2–4%
Culture activity	Competition
Bacteriocins	Inhibition
Oxidation-reduction (Eh)	Anaerobic (reduced)
Nitrite	100 ppm
Smoke	Inhibition
Casing	Exclusion

Table 7.3.2 Recent food safety outbreaks involving fermented meats (since 2007).

Year	Country	Product	Organism	Cases	Reference
2007	Italy	Dry fermented salami	*Escherichia coli* 0157:H7	3	Conedera et al.
2007	Italy	Pork salami	*Salmonella* Typhimurium DT 104A	63	Luzzi et al.
2008	Norway	Mutton sausage	*Escherichia coli* 0103:H25	17	Schimmer et al.
2008	Sweden	Cold-smoked sausage	*Escherichia coli* 0157:H7	39	Sartz et al.
2009	Denmark	Beef sausage	*Escherichia coli* 026:H11	21	Ethelberg et al.
2010	Denmark	Salami	*Salmonella* Typhimurium	20	Kuhn et al.
2010	France	Dry pork sausage	*Salmonella enteric* 4,12:i	54	Bone et al.
2011	France	Dry pork sausage	*Salmonella enterica* 4, [5],12:i:	337	Gossner et al.

times, sluggish culture activity, and incomplete fermentations were responsible for the growth of the pathogens. However, even the mere presence of these organisms in ready-to-eat meat products (like dry fermented sausages) would likely initiate a product recall. Indeed, the US Department of Agriculture (USDA) has established rules that now require a minimum five-log reduction of pathogenic organisms (*Salmonella* and EHEC) during the manufacture of uncooked, ready-to-eat fermented sausage. Manufacturers are also required to develop and implement HACCP plans that describe the relevant intervention steps necessary to produce safe products.

Of course, the simplest solution and the most effective way to ensure a greater than five-log reduction would be to include a heating step somewhere during the process. In the US, many manufacturers have adopted this practice. However, throughout Europe, and even in the US, manufacturers of traditional dry fermented sausage are unwilling to accept the changes in the sensory properties caused by heating, and instead rely on ensuring that natural barriers are sufficient. It is also possible to include additional intervention measures into the product or process. For example, meat starter cultures capable of producing bacteriocins and other antagonistic agents that are inhibitory to pathogens are now available.

In the early 2000s, the USDA Food Safety and Inspection Service (FSIS) began a testing program for *E. coli* O157:H7 in dry and semi-dry fermented sausage. Of the 10,000 samples that were subsequently collected, none tested positive. In 2011, the FSIS suspended the testing program and diverted resources to focus on "raw products that pose a more immediate public health impact." Thus, this announcement reinforces what producers of traditional dry and semi-dry fermented sausage products have long stated - when produced under appropriate conditions and with relevant controls in place, these products are safe.

References

Bone, A., H. Noel, S. Le Hello, N. Pihier, C. Danan, M.E. Raguenaud, S. Salah, H. Bellali, V. Vaillant, F.X. Weill, and N. Jourdan-da Silva. 2010. Nationwide outbreak of *Salmonella enterica* serotype 4,12:i:- infections in France, linked to dried pork sausage, March–May 2010. *Euro. Surveill*. 15:pii=19592.

Conedera, G., E. Mattiazzi, F. Russo, E. Chiesa, I. Scorzato, S. Grandesso, A. Bessegato, A. Fioravanti, and A. Caprioli. 2007. A family outbreak of *Escherichia coli* O157 haemorrhagic colitis caused by pork meat salami. *Epidemiol. Infect.* 135:311–314.

Ethelberg, S., B. Smith, M. Torpdahl, M. Lisby, J. Boel, T. Jensen, E.M. Nielsen, and K. Mølbak. 2009. Outbreak of non-O157 shiga toxin-producing *Escherichia coli* infection from consumption of beef sausage. *Clin. Infect. Dis.* 48:78–81.

Gossner, C.M., D. van Cauteren, S. Le Hello, F.X. Weill, E. Terrien, C. Janin, A. Brisabois, V. Dusch, V. Vaillant, and N. Jourdan-da Silva. 2012. Nationwide outbreak of *Salmonella enterica* serotype 4,[5],12:i:- infection associated with consumption of dried pork sausage, France, November to December 2011. *Euro. Surveill*. 17:pii=20071.

Holck, A., L. Axelsson, A. McLeod, T.M. Rode, and E. Heir. 2017. Health and safety considerations of fermented sausages. *J. Food Qual*.

Kuhn, K., M. Torpdahl, C. Frank, K. Sigsgaard, and S. Ethelberg. 2011. An outbreak of *Salmonella Typhimurium* traced back to salami, Denmark, April to June 2010. *Euro. Surveill.* 16.

Luzzi, I., P. Galetta, M. Massari, C. Rizzo, A.M. Dionisi, E. Filetici, A. Cawthorne, A. Tozzi, M. Argentieri, S. Bilei, L. Busani, C. Gnesivo, A. Pendenza, A. Piccoli, P. Napoli, L. Loffredo, M.O.

Trinito, E. Santarelli, and M.L. Ciofi degli Atti. 2007. An Easter outbreak of *Salmonella Typhimurium* DT 104A associated with traditional pork salami in Italy. *Euro Surveill.* 12:E11–E12.

Meloni, D. 2015. Presence of *Listeria monocytogenes* in Mediterranean-style dry fermented sausages. *Foods* 4:34–50.

Roccato, A., M. Uyttendaele, F. Barrucci, V. Cibin, M. Favretti, et al. 2017. Artisanal Italian salami and soppresse: Identification of control strategies to manage microbiological hazards. *Food Microbiol.* 61:5–13.

Sartz, L, B. De Jong, M. Hjertqvist, L. Plym-Forshell, R. Alsterlund, S. Lofdahl, B. Osterman, A. Stahl, E. Eriksson, H.B. Hansson, and D. Karpman. 2008. An outbreak of *Escherichia coli* O157:H7 infection in southern Sweden associated with consumption of fermented sausage; aspects of sausage production that increase the risk of contamination. *Epidemiol. Infect.* 136:370–380.

Schimmer, B., K. Nygard, H.-M. Eriksen, J. Lassen, B.-A. Lindstedt, L.T. Brandal, G. Kapperud, and P. Aavitsland. 2008. Outbreak of haemolytic uraemic syndrome in Norway caused by stx_2-positive *Escherichia coli* O103:H25 traced to cured mutton sausages. *BMC Infect. Dis.* 8:41.

PRINCIPLES OF FERMENTED MEAT PRODUCTION

There are actually only a few general steps involved in fermented sausage manufacture. First, the ingredients are selected, weighed, mixed, and stuffed into casings. Second, the stuffed sausages are held under conditions necessary to promote fermentation. Third, the sausage is subjected to one or more post-fermentation steps whose purpose is to affect flavor, texture, and preservation properties. These latter steps can range in duration from as little as one week in the case of moist or semi-dry sausages to more than two months for very dry, strongly flavored sausages such as Italian salamis.

Ingredients

It is entirely possible to manufacture a fermented sausage with just a handful of ingredients. In fact, only five ingredients are essential: meat, sugar, salt, culture, and a curing agent. As discussed above, fermented meats can indeed be made without adding a culture, but most large-scale manufacturers would not consider making product without a culture. Likewise, fermented sausages also can be manufactured in the absence of the curing agent. However, these agents, either in the form of nitrite or nitrate, perform such important microbiological and organoleptic functions that they are nearly universally used. That being said, there is a small (but growing) market among organic foods proponents for reduced or even nitrite-free meat products. Even if the organoleptic properties provided by nitrite could be provided by other agents (a big if), removing nitrite from cured meat products would expose these products to a potentially serious food safety threat. Still, provided that other barriers are in place, especially low pH and low temperature, theoretically safe products can be produced.

1. Meat

Of the ingredients listed above, the main ingredient is obviously the meat, which contributes not only the protein and the bulk of the product matrix, but also the fat. The latter provides much of the flavor and also imparts juiciness and texture. The fat-containing cuts usually are chopped or ground separately from the leaner portions to impart a desired appearance and

flavor. The grind also affects texture and accordingly determines the type of product. For example, some sausages (e.g., Plockwurst) have large visible fat particles, whereas others (e.g., cervelat) are ground to a fineness such that the fat particles are so small as to be indistinguishable from the sausage matrix.

The fat and lean portions may even be derived from different animals. Pork fat contains more unsaturated lipids than beef fat and imparts flavors generally preferred by consumers. Thus, many sausage products, such as the popular U.S. product summer sausage, are typically made with mixtures of beef and pork. Cuts are also important – shanks, chucks, and bull meat have binding properties that are especially important in sausage manufacture. Obviously, many sausages have a reputation for using less expensive cuts, but high quality meats are often still used.

2. Sugar

The next essential ingredient is the sugar or carbohydrate. Although glucose, in the form of the polymer glycogen, is initially present in muscle tissue of slaughtered animals, glycogen stores are quickly depleted during the post-mortem period. Thus, fresh meat contains little fermentable sugar, and addition of sugar is generally necessary to obtain adequate production of lactic acid and reach a suitably low pH. Glucose is most commonly used in the United States, and is added to about 0.5% to 2% of the total batter weight. Since the amount of acid produced by the lactic culture is directly related to the amount of available glucose, the sugar concentration in the batter can be adjusted, in general, to give a particular final pH. Also, higher sugar levels promote faster fermentations, which are preferred in the United States. In contrast, many European fermented sausage manufacturers prefer less tanginess and more diverse flavor development. Achieving these characteristics require slower fermentation rates, thus less rapidly fermentable sugar is added (as little as 0.1% to 0.2%).

3. Salt

Salt is an essential ingredient in all types of sausage products (whether fermented or not). Salt, usually added in concentrations of 2% to 3%, performs several critical functions. First, it is responsible for extracting and solubilizing the muscle proteins, which are ordinarily in an insoluble form. Once extracted and solubilized, the proteins form a "sticky" film around the meat particles, creating an emulsion-type structure. Second, salt provides flavor. Finally, salt is the primary means, at least initially, for controlling the microflora. Although salt at a concentration of even 3% might not appear to be sufficiently high enough to inhibit many organisms, the actual concentration within the aqueous phase (about 75% of the total mass) is considerably higher.

4. Culture

Most commercial cultures for sausage are supplied in either a frozen or lyophilized form. Frozen cultures are supplied as thick slurries in peel-back or flip-top containers ranging in size from 20 ml to 250 ml. Cell densities typically range from 10^8 to 10^9 cells per ml. A typical 70-ml containers is sufficient for about 150 kg of sausage batter. These cultures are shipped frozen under dry ice and users are instructed to store containers at –40°C or below. They should be thawed in cold water prior to use.

Proper handling of frozen cultures is absolutely necessary to maintain culture viability and to ensure that culture performance (i.e., rapid fermentation) is not impaired. Lyophilized cultures, which have gained in popularity, have the advantage of not requiring low-temperature storage. They are stable at refrigeration or even ambient temperatures. As free-flowing powders, they are easily measured and distributed into the batter. These cultures, which can contain up to 10^{10} cells per gram, are usually more expensive, however, than frozen cultures.

5. Curing agents

Finally, with few exceptions, fermented meat products include nitrite or nitrate as curing agents. These are added as either the sodium or potassium salt. Although nitrite salts are now used far more frequently, until the 1970s, nitrate salts were more common. For reasons discussed previously, some sausage manufacturers still prefer nitrate. In any case, nitrite is added at a maximum of 156 ppm for dry and semi-dry sausages. Since a single ppm translates to 1 gram per 1000 kg, only 156 grams (about one-third of a pound) are all that can be added to a 1000-kg batch of sausage (more than 2200 pounds). Despite this relatively small amount, nitrite performs a number of important microbiological and organoleptic functions. In fact, without nitrite (or nitrate), fermented meat products would be far less popular and considerably less safe to eat.

Nitrite is mainly added to sausages (and not just those that are fermented) because of its effectiveness as an antimicrobial agent. In particular, nitrite inhibits the out-growth of *C. botulinum* spores, making it one of the most powerful anti-botulinum agents available to the processed meats industry. Although fermented meats contain combinations of organic acids and salt, both effective antimicrobials, neither provide a sufficient degree of inhibition against this organism. It should be emphasized, however, that nitrite alone, at the levels currently used, does not entirely inhibit *C. botulinum*. Rather, it is the combined effects of nitrite along with organic acids, low pH, and low a_w that effectively control the growth of this organism during the manufacture and storage of fermented sausage.

Other reasons for adding nitrite to fermented meats are related to the organoleptic properties this agent imparts. Nitrite fixes color, acts as an antioxidant and prevents a warmed-over flavor, and imparts a desirable cured meat flavor.

6. Spices, flavoring and other ingredients

A wide variety of spices, seasonings, and other flavoring agents are often added to fermented sausages. These include pepper (black and red), paprika, garlic, mustard, mace, and cardamom. Flavorings can be added in their natural form or as extracts. Levels vary, depending on the nature of the product and consumer preferences. In general, moist, smoked sausages that are popular in Germany and northern Europe are only slightly spiced, whereas the dried, non-smoked products consumed in southern Europe (e.g., Italy and Spain) and other Mediterranean regions are more heavily spiced. Among the optional ingredients commonly added to fermented meats, ascorbate and erythorbate are perhaps the most important. Both provide similar functional roles as nitrite in that they inhibit autoxidation and increase color and flavor intensity. Finally, some manufacturers of "naturally cured" fermented sausage, presumably in effort to have a friendlier ingredient label, omit the nitrite altogether. Instead, they add celery powder or juice which provides nitrate in a natural form. These concentrated celery products can contain as much as 3% nitrate, and when combined with a suitable

nitrate-reducing culture, make it possible to obtain the benefits of nitrite without a nitrite label declaration.

Manufacture of fermented sausage

1. Cutting and mixing

Once the ingredients have been collected and weighed, the manufacturing process is remarkably simple (Figure 7.1). The meat portions (lean and fat) are usually ground separately to produce varying degrees of coarseness. A silent cutter – a rotating, bowl-shaped device that chops and mixes the sausage batter or a similar grinding device are used for this purpose. Then the ground meat, along with all of the remaining ingredients, are combined and mixed. Mixing should be done to minimize or exclude oxygen, which not only can interfere with color and flavor development, but also is less conducive to growth of the starter culture. Above all, the mixing step, as well as all the grinding and weighing steps, must be performed at low temperatures for both quality and safety. In general, the temperature in processing rooms are held at 45°C or below. In fact, in the United States, all meat processors must have a Hazard Analysis Critical Control Points (HAACP) plan, with the temperature during processing listed as a control point (Box 7.4). In Canada, Latin America, Europe, and much of Asia, HACCP or equivalent plans are also required (with exceptions) for most processed meat products.

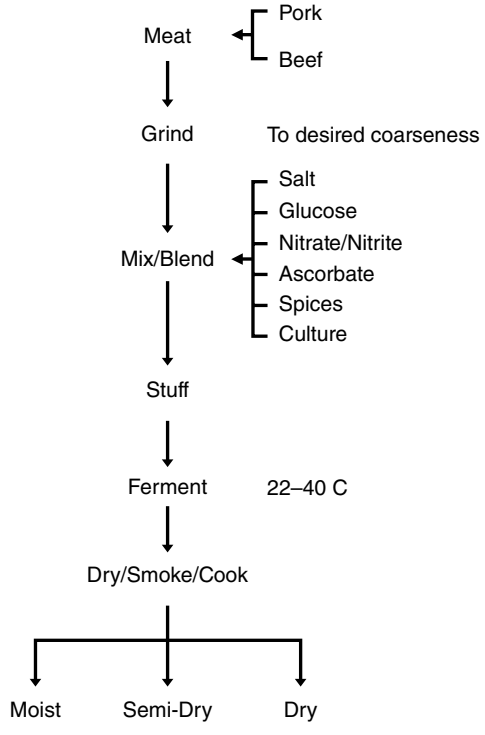


Figure 7.1 General processing steps for manufacture of fermented sausage.

Box 7.4 Ensuring safety of fermented meats

More than 20 years ago (July, 1996), the Pathogen Reduction/Hazard Analysis and Critical Control Point (HACCP) Systems became US regulatory law for all USDA-regulated facilities. The rationale for this rule, was in large part, because the Food Safety and Inspection Service (FSIS), an agency within the USDA, considered HACCP as "the optimal framework for building science-based process control to prevent food safety hazards into food production systems". The regulation applied broadly to the processed meats industry, including fermented sausages. In addition, after a phase-in period, large as well as small producers were required to comply. More recently, the US Food Safety Modernization Act (FSMA) spelled out measures called "preventive controls" that are consistent with HACCP. Collectively, these principles are now widely applied throughout the world. This is the case even in regions

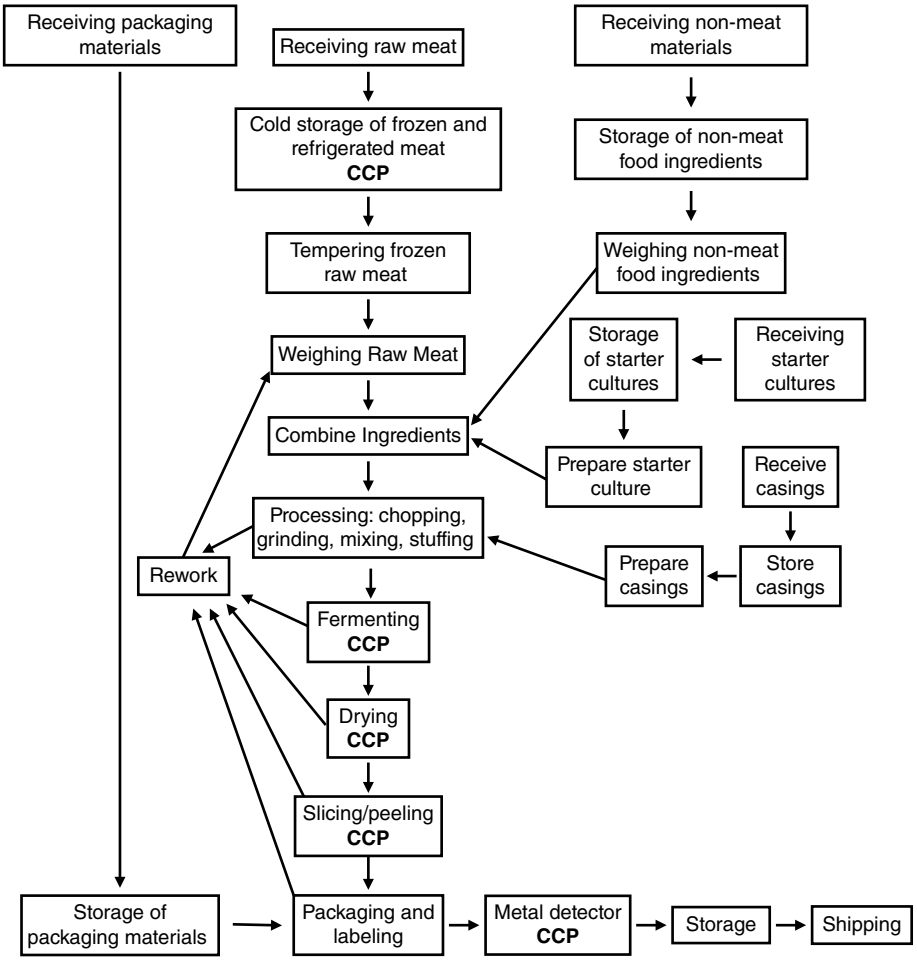


Figure 7.4.1 Generic process flow diagram for dry fermented dry sausage. The main critical control points (CCP) are shown.

where fermented sausages are still produced on a small scale by traditional manufacturing practices.

The general principles and steps for developing a HACCP plan are well-known (Fraqueza and Barreto, 2014) and will not be described here. Rather, only those processing steps specific to fermented sausages will be discussed. Accordingly, a representative processing flow chart and the critical control points designed to prevent potential hazards relative to an uncooked dry fermented sausage are shown in Figure 7.4.1.

It is important to emphasize that for most of the fermented sausages produced in the U.S., a thermal process or "kill" step is included. Having a cooking step as part a critical control point provides manufacturers with reasonable assurance that potential hazards are controlled. However, for traditional dry fermented or smoked sausages, including those widely produced and consumed in Europe, no such step occurs. Thus, for these products, other controls must be in place to produce safe products. For example, achieving a particular pH (e.g., 5.3) within a particular time could be considered a critical control point. Similarly, drying conditions and final moisture and water activity levels must be reached to ensure safety.

Corrective actions are also developed for each critical control point and documented in the HACCP plan. These corrective actions are applied whenever deviation from a critical limit occurs; specific and exacting records are kept. A separate HACCP program is designed and documented for each manufacturing process used in an establishment. Finally, the HACCP plan must be accompanied by proper prerequisite programs such as Good Manufacturing Practices and Sanitation Standard Operating Procedures.

Finally, the reader is reminded that regulations and inspection requirements to ensure meat safety depend on the country. Still, the USDA, FSMA, as well as comparable agencies in Canada, Australia, and other counties generally rely on HACCP or similar programs.

Reference

Fraqueza, M.J. and A.S. Barreto. 2014. HACCP: Hazard Analysis and Critical Control Points. In: F. Toldra, Y.H. Hui, I. Astiasaran, J. Sebranek, and R. Talon, eds. *Handbook of Fermented Meat and Poultry*, 2nd edn. Wiley-Blackwell, Oxford.

2. Stuffing

After the batter has been sufficiently mixed, it is moved to a stuffer, a device that pumps the mix into casings. The casings are essentially long flexible tubes that give the product its characteristic shape. The diameter of the casings can vary from less than 1.5 cm to more than 9 cm. As the tubes are filled, they are tied off or cut to give desired section lengths, again depending on the product being made. Lengths can vary from 5 cm to 100 cm. Shape and diameter size are important, not only because they are specific for a given product, but more importantly, because they influence the rate of drying, cooking, smoking, and ultimately the flavor and texture of the finished product. Casings must be permeable to both moisture and smoke.

Two general types of casing materials are used, natural and synthetic. These terms are somewhat nebulous, since the latter are often made from natural sources. Traditionally, casings were made of animal intestines. Although there is still a significant market for products made using natural casings, especially in Europe, synthetic casings have several advantages and are now widely used. They are usually made using cellulose or collagen.

3. Fermentation

Once the sausage batter is packed into casings, the material is moved into specially designed ripening chambers where the fermentation occurs. These facilities, often referred to as the green room or smoke house or simply the "house", have controls for maintaining temperature, humidity, and air movement. Moreover, control systems in modern facilities are fully programmable so conditions can be ramped or adjusted depending on how the fermentation is proceeding or the particular specifications of the manufacturers. Thermocouples and pH probes inserted directly into product samples can feed the appropriate information into a computer to provide constant monitoring, record-keeping, and feed-back control. Thus, the entire fermentation could proceed in the absence of a full-time operator.

Fermentation parameters vary depending on the culture and the desired product qualities. In general, lower incubation temperatures require longer fermentation times. For example, at the low temperature range of 21°C to 24°C (70°F to 75°F), which is very near ambient, fermentation can take several days or even longer. In contrast, at 29°C to 32°C (85°F to 90°F), 12 to 16 hours of fermentation will be required. In the United States, where faster overall production times are preferred, the incubation temperature can be as high as 37°C to 40°C (98°F to 102°F) for as little as 12 to 18 hours.

Since individual culture strains may have different temperature optima and tolerances, selection of cultures that perform under the selected conditions is critical. It is also important, for some applications, that the fermentation rate is controlled such that it does not proceed too fast. This is particularly relevant when flavor- or color-producing organisms (e.g., *Micrococcus* or *Staphylococcus*) are used, because they may be inhibited by fast lactic acid producers. However, if the fermentation is too slow, there may be sufficient opportunity for pathogens or spoilage organisms to grow. Ultimately, the pH at the end of fermentation should be less than 5.3, which, by itself, will not make the product shelf-stable, but which does provide a reasonable protective barrier against most foodborne pathogens. In addition, fermented sausages at pH 5.0 to 5.1 will have only a slight tart flavor that may actually be indistinguishable from non-fermented products.

Alternatively, depending on the culture, the incubation conditions, and the amount of substrate (i.e., fermentable sugar) provided, much higher acidities can be achieved. Typically, summer sausage has a pH around 4.7 to 4.9 but can be as low as 4.5. When the pH reaches 4.8 or less, a definite "tangy" flavor becomes apparent.

Fermentation rooms are also equipped with air movement devices and humidistats to maintain the relative humidity (RH) at desired levels. Since the RH in the atmosphere surrounding a food directly influences the water activity of that food (where $a_w \times 100 = RH$), as RH is decreased, the more moisture is lost to the atmosphere and the lower will be the a_w in the sausage. Typically, the RH should be about 10% less than the $a_w \times 100$ of the finished product.

4. Cooking, drying, and smoking

Several different treatments and combinations of treatments can be applied at end of the fermentation. These include cooking, drying, and smoking. In the United States, fermented sausages are often cooked after fermentation, whereas, in Europe and elsewhere, raw sausages are the norm, and post-fermentation heating steps are rarely applied. Moreover, the manner in which drying or smoking are performed usually is dictated by the particular style, quality traits, or geographical regions in which the products were produced.

In general, properly made dry, fermented, uncooked sausages, like salami and pepperoni, are still considered to be shelf-stable and ready-to-eat even in the absence of a cooking or heat treatment step. Cooking, however, does provide important advantages, and in the US pepperoni is normally heat-processed. Cooking not only inactivates the culture and stops the fermentation, but it also kills pathogenic microorganisms that may have been present in the raw meat. Thus, the cooking step, at least in the United States, may be an important component of HACCP programs used by sausage manufacturers, since it serves as a terminal process step providing manufacturers (and consumers) with reasonable assurance of product safety.

Another reason for including a cooking step is to inactivate *Trichinella*, a nematode that infects swine and that can cause the disease trichinellosis in humans. In the United States, any pork-containing sausage must, by federal regulations, be cooked to destroy *Trichinella*. The USDA has developed various time-temperature regimens that are effective (e.g., 2 minutes at 59°C), and which can easily be implemented as part of the cooking process. The USDA does provide for alternative methods to control *trichinella*. It is possible, for example, to inactivate *Trichinella* in pork by freezing, and the USDA has established guidelines for freeze-inactivation of the cysts. Exceptions to the cooking requirement are also allowed if the pork is certified as *Trichinella*-free.

If a cooking step is included, the product can be moved to a separate chamber or, as is more common, the fermentation chamber itself is equipped with heating capability. The temperature is slowly raised until the desired internal temperature (usually 60°C to 62°C) is achieved. During the cooking step, the product can be smoked. If the product is not cooked, it can be dried after fermentation. In general, Mediterranean sausages (e.g., Genoa and Milano) are dried, but not smoked, and northern sausages (e.g., German) are smoked, but not dried. In contrast, some semi-dry products, such as summer sausage, are slightly smoked.

If the product is to be dried, the chamber environment is set at about 7°C to 13°C (45°F to 55°F) and 70% to 72% RH. Good air movement is necessary to rapidly remove water vapor and any condensate that collects at the surface. The rate of drying is also critical. If the RH is too low and the temperature too high, drying will initially be rapid. However, at these high drying rates, the surface will become dehydrated and form a hard, water-impermeable skin. This phenomenon, called case hardening, results in slower drying and poor product quality, since water molecules are unable to diffuse through the hardened surface and are trapped within the sausage interior.

Drying times depend on product specifications and desired quality characteristics. Obviously, large diameter products require longer drying times than small diameter products. The longer the drying, the more water is lost, and the lower will be the final water activity. Semi-dry products are typically dried to remove about 20% to 30% of the original water and to have a final moisture of about 45% to 50%. The a_w of semi-dry products ranges from 0.90 to 0.94. Such products may be stable at ambient temperature, with a shelf-life of

several months. Examples include Thuringer, cervelat, Lebanon bologna, summer sausage, and semi-dry salami. Dry fermented sausages will lose about 35% water and have a final moisture of 35% and an a_w between 0.85 and 0.91. Dry products can be shelf-stable for several months or longer. Pepperoni and salami, especially the Italian, Mediterranean, and eastern European varieties, are the most common examples.

As noted above, most modern facilities are equipped with programmable systems that allow operators to set fermentation and cooking parameters, as well as air movement, smoking, and drying conditions. Recording devices provide a record of these conditions during the manufacturing process. Thus, once the sausages are loaded into the ripening chamber, they can be left on their own, more or less, until the process is completely finished.

Mold-ripening

Many of the European-style sausages are ripened by mold. These products are particularly popular in Hungary and Romania, as well as throughout the Mediterranean region. For many of these products, fungal growth can be extensive, with the mycelia covering the entire surface. Mold-fermented sausages are not nearly as common in the United States. However, there are some whole meat products, in particular, country-cured hams, that are fermented by yeasts and fungi and that are very popular in various regions of the United States. Although technically not fermented, aged meat owes its improved flavor and texture properties, in part, to growth of surface fungi, in particular, *Thamnidium elegans*.

The role of fungi in fermented whole meats and sausages is similar to that of other mold-fermented products. That is, the fungi are not involved in the primary lactic acid fermentation, but rather their function is to enhance flavor and texture properties. This is accomplished by production and secretion of proteinases and lipases and their diffusion into the meat, generating flavor and aroma products or their precursors. Ammonia, released from protein and amino acid metabolism, and ketones and other rancid and oxidized flavor notes, derived from fatty acid metabolism, are among the end products that accumulate in mold-ripened sausages. In this respect, the fungi produce organoleptic properties not unlike that found in mold-ripened cheese.

The manufacture of mold-ripened sausages is similar to that of normal fermented sausage, in that ingredients are mixed and stuffed into casings. Inoculation of sausages or whole meats (like hams) with fungi can occur either naturally, via the fungal spores present in the manufacturing environment, or more commonly, by dipping or spraying the product with a defined spore suspension. Yeast and fungal starter cultures, which are becoming more widely available for fermented sausage and ham manufacture, are generally comprised of strains of *Debaryomyces hansenii*, *Penicillium nalgiovense*, *Penicillium camemberti*, and *Penicillium chrysogenum*. For natural fermentations of country-cured hams, Parma hams, and other whole meats, salt can be applied to the surface to reduce the water activity and provide a selective environment for resident yeasts and fungal organisms. The main problems, however, with naturally-occurring fungi are (1) they may yield products with inconsistent quality; and (2) perhaps more importantly, they may also produce mycotoxins. Thus, the use of pure fungal starter cultures, selected on the basis of their functional properties as well as their inability to produce mycotoxins, have obvious advantages, since greater quality control and product safety can be achieved (Table 7.4).

FLAVOR OF FERMENTED MEATS

Like other fermented foods made with a lactic acid starter culture, the main flavor compounds are acids, principally lactic and acetic, derived via metabolism of sugars. In many of the US-produced products that are cured with nitrite, fermented at high temperatures for a short time, and cooked following fermentation, there is only a brief opportunity for development of other flavors. In contrast, a much more complex array of flavor compounds is produced in sausages in which nitrate is used as the curing agent, that are fermented slowly, and are uncooked. Flavor development may be especially enhanced if adjunct microorganisms, such as micrococci and staphylococci, are included in the starter culture. The ripening process for these sausage products is not unlike that for aged cheese, in that microbial as well as endogenous enzymes act on proteins and fats in the raw material, generating hydrolysis products that contribute to the flavor and texture of the finished product. Importantly, the source of the microbial enzymes may be the lactic starter culture or adjunct organisms, or naturally-occurring bacteria, yeasts, and molds present in the raw meat material.

DEFECTS AND SPOILAGE OF FERMENTED MEATS

Defects of fermented meats can occur before, during, or after manufacturing. Like all fermented foods, the production of high quality products depends largely on the microbiological quality of the raw ingredients. Perhaps this is even more so for fermented sausage. This is because the starting material, meat, is raw and cannot be heat-processed to inactivate spoilage or other undesirable microorganisms prior to fermentation. Thus, any organisms present in the raw meat will be present in the sausage batter and may even survive fermentation.

Of course, fermentation acids kill or inhibit many organisms, and, if the sausage is cooked after fermentation, most of the remaining organisms will be killed. However, spoilage products may have already been produced. For example, psychrotrophic bacteria, such as *Brochothrix thermosphacta* and various *Pseudomonas* spp., may produce ammonia and other volatile off-odors and flavors in the meat during storage even before fermentation. Lipases and other enzymes may also be produced by these organisms prior to fermentation, resulting in rancid, "cheesy," or bitter end-products.

The other main group of spoilage organisms is lactic acid bacteria. These bacteria are part of the natural meat microflora, and are quite tolerant of the barriers that ordinarily control microbial activity in fermented sausage (i.e., low pH, low a_w, low Eh, nitrite, salt, etc.). Under appropriate conditions, lactic acid bacteria can cause flavor, color, and tactile defects, and some strains are also responsible for a particular type of foodborne disease, due to their production of biogenic amines (Box 7.5). Of course, the causative organisms are not necessarily endogenous to the product, because the starter culture itself is comprised of lactic acid bacteria, including *L. sakei*, *L. curvatus*, *L. plantarum, P. acidilactici,* and other species capable, in theory, of producing these defects. Therefore, it is important that screening and selection of strains for starter cultures be based, in part, on their ability (or inability) to produce undesirable end-products.

Among the spoilage products formed by lactic acid bacteria, hydrogen peroxide is probably the most problematic. It is produced primarily by lactobacilli, but only by specific strains, and only under specific conditions. Oxygen is required for production of hydrogen

Box 7.5 Safety of fermented sausage: Bacteria are not the only challenge

Bacterial pathogens are not the only food safety issue of concern to the fermented meats industry. Parasites and viruses can also be present in raw meat, and toxigenic fungi can contaminate sausage during fermentation and ripening. The parasite most commonly associated with meat, and pork in particular, is the nematode, *Trichinella spiralis*, the causative agent of trichinosis. Although certified *Trichinella*-free pork is available in the United States (and in Europe), it is also possible to inactivate this nematode by a freezing treatment, as per USDA guidelines. Otherwise, a cooking step is required to destroy the cysts.

In contrast to parasites, viruses have not been considered as a serious food safety problem in fermented meats, and this is still largely true. Viruses found in meat are not usually pathogenic to humans, they do not replicate, and they are mostly sensitive to the acidification and drying steps used in sausage manufacture.

Several different genera of fungi, including species of *Penicillium* and *Aspergillus*, are frequent contaminants of fermented meats. For many products, including both whole fermented meats (i.e., hams), as well as fermented sausages, their growth is encouraged, due to their ability to produce flavor-generating enzymes (as discussed previously). However, some strains isolated from mold-fermented products are capable of producing mycotoxins. Moreover, in laboratory conditions, inoculation of ham and sausage with toxigenic fungi and incubation under optimized conditions results in toxin formation (Ferrara et al., 2016; Peromingo et al., 2016). Despite these findings, the presence of mycotoxins in mold-fermented meat products appears to occur rarely, a situation not unlike that for fungal-ripened cheese and other mold-fermented products.

Still, the potential for mycotoxin production in fermented meats has led researchers to use defined, non-toxigenic strains rather than the wild or house strains that have commonly been used. For example, *Penicillium nalgiovense,* a fungal meat starter culture, has many desirable properties, but since some strains produce mycotoxins, only strains demonstrated to be non-toxin producers are in commercial use. Undesirable mold growth on sausage can also be controlled by antimycotic agents such as sorbic acid and, if permitted, the antibioic pimaricin. Smoke, which is usually applied to high moisture, but not dry products, also contributes antimycotic constituents.

Finally, another biologically-active group of microbial end products found in a wide variety of fermented foods, including fermented meats, are referred to as biogenic amines (Lorenzo et al., 2016). These compounds are formed via decarboxylation of amino acids by various bacteria that are commonly found in fermented meats, including lactobacilli and other lactic acid bacteria, enterococci, *Enterobacteriaceae*, and *Micrococcaceae*. In some individuals (see below), ingestion of biogenic amines results in a particular food poisoning syndrome marked by headache, nausea, and dilation of blood vessels.

In fermented meats, the most common biogenic amine capable of causing food poisoning symptoms is tyramine derived from the amino acid, tyrosine. Others also include histidine, putrescine and cadaverine (from histidine, ornithine and lysine, respectively). In general, dry fermented sausages contain about 50 to 300 mg tyramine per kg. Other amines are usually present at lesser concentration (Table 7.5.1).

Table 7.5.1 Biogenic amines in fermented meats (mg/kg)

Product	Tyramine	Histamine	Putrescine	Cadaverine	Reference
Chorizo	79	n.d	28	1	De May et al.
Salami (North European)	27	n.d	67	2	De May et al.
Garlic salami	60	n.d	20	1	De May et al.
Paio (Portuguese)	7	108	181	74	Alves et al.
Kobasica (Serbian)	12	10	20	5	Alves et al.
Sichuan-style sausages	165	196	59	142	Sun et al.
Soppressata (Italian)	178	22	99	61	Suzzi et al.
Salsiccia (Italian)	77	n.d.	20	7	Suzzi et al.
Fuet (Spanish)	191	2	72	19	Suzzi et al.
Chorizo	90–130	1–10	30	5–20	Miguelez-Arrizado et al.
Fuet	20	1	30–40	5	Miguelez-Arrizado et al.
Salchichon	130–170	2–20	80–115	5–10	Miguelez-Arrizado et al.

n.d.: Not determined.

However, only when concentrations are very high (>1000 mg per kg of dry weight) do these products pose a health risk in most normal individuals. In one recent survey of 20 retail dry fermented sausages from Portugal and Serbia, the levels of biogenic amines were well under this threshold (Alves et al., 2017).

Other biogenic amines may also be formed in meat at appreciable levels, including putrescine and cadaverine, but these compounds do not generally elicit symptoms described above. Since formation of biogenic amines requires the presence of free amino acids, the amount produced depends on the extent of protein hydrolysis that had occurred in the food. Thus, the longer the meat is aged or fermented, the higher will be the concentration of amino acid substrates and the more likely it is that the product will contain biogenic amines (assuming the relevant decarboxylating enzymes are also present).

In general, biogenic amines pose a public health risk when: (1) concentrations in foods are high or (2) if consumed by sensitive individuals (Latorre-Moratalla et al., 2017). The latter include those individuals who cannot metabolize or detoxify the amines due to concomitant consumption of antidepressant monoamine oxidase inhibitors.

Because some lactic acid bacteria have the ability to produce amino acid decarboxylases, starter cultures should be screened to eliminate such strains from use (Holck et al., 2017; Moracanin et al., 2014). Moreover, it may be possible to use starter cultures that have inhibitory activity against potential bioamine producing bacteria, either by virtue of their ability to produce acids rapidly, produce bacteriocins, or outcompete them for nutrients. Indeed, one recommended strategy is based on identifying products with low biogenic amine levels, isolating strains from those products that do not

make biogenic amines, and then to use those organisms as starter cultures (Lorenzo et al., 2017).

References

Alves, S.P., C.M. Alfaia, B.D. Škrbić, J.R. Živančev, M.J. Fernandes, R.J.B. Bessa, and M.J. Fraqueza. 2017. Screening chemical hazards of dry fermented sausages from distinct origins: Biogenic amines, polycyclic aromatic hydrocarbons and heavy elements. *J. Food Comp. Anal.* 59:124–131.

De Mey, E., K. De Klerck, H. De Maere, L. Dewulf, G. Derdelinckx, M.-C. Peeters, I. Fraeye, Y.V. Heyden, and H. Paelinck. 2014. The occurrence of N-nitrosamines, residual nitrite and biogenic amines in commercial dry fermented sausages and evaluation of their occasional relation. *Meat Sci.* 96:821–828.

Ferrara, M., D. Magistà, V. Lippolis, S. Cervellieri, A. Susca, and G. Perrone. 2016. Effect of *Penicillium nordicum* contamination rates on ochratoxin A accumulation in dry-cured salami. *Food Control* 67:235–239.

Holck, A., L. Axelsson, A. McLeod, T.M. Rode, and E. Heir. 2017. Health and safety considerations of fermented sausages. *J. Food Qual*.

Latorre-Moratalla, M.L., O. Comas-Baste, S. Bover-Cid, M.C. Vidal-Carou. 2017. Tyramine and histamine risk assessment related to consumption of dry fermented sausages by the Spanish population. *Food Chem. Toxicol.* 99:78–85.

Lorenzo, J.M., D. Franco, and J. Carballo. 2016. Biogenic amines in fermented meat products. In: N. Zdolec, ed. *Fermented Meat Products: Health Aspects*. CRC Press, Cambridge.

Lorenzo, J.M., P.E.S. Munekata, and R. Domínguez. 2017. Role of autochthonous starter cultures in the reduction of biogenic amines in traditional meat products. *Curr. Opin. Food Sci.* 14:61–65.

Miguelez-Arrizado, J., S. Bover-Cid, M.L. Latorre-Moratalla, and M.C. Vidal-Carou. 2006. Biogenic amines in Spanish fermented sausages as a function of diameter and artisanal or industrial origin. *J. Sci. Food Agric.* 86:549–557.

Moracanin, S.V., S. Stefanovic, T. Radicevic, and D. Djukic. 2014. Screening procedure for biogenic amine production by lactic acid bacteria isolated from traditionally fermented sausages. *J. Hyg. Eng. Design* 6: 202–207.

Peromingo, B., A. Rodríguez, V. Bernáldez, J. Delgado, and M. Rodríguez. 2016. Effect of temperature and water activity on growth and aflatoxin production by *Aspergillus flavus* and *Aspergillus parasiticus* on cured meat model systems. *Meat Sci.* 122:76–83.

Sun, X., K. Zhou, Y. Gong, N. Zhang, M. Yang et al., 2016. Determination of biogenic amines in Sichuan-style spontaneously fermented sausages. *Food Anal. Methods* 9:2299–2307.

peroxide by lactic acid bacteria. Not only does oxygen serve as a reactant in the hydrogen peroxide-generating reactions, it may also induce expression of the enzymes involved in these reactions. Thus, minimizing exposure to air during mixing and subsequent steps is critical.

Once formed, hydrogen peroxide participates in several undesirable reactions. First, it reacts with heme-containing pigments, especially nitrosyl myochromogen, formed as a result of curing. When the heme iron is oxidized by peroxide, the desirable pink color is lost and an undesirable green pigment is formed. Second, hydrogen peroxide can form hydroxyl radicals (e.g., O_2^-) that serve as initiators of lipid oxidation reactions.

One way to limit the formation of hydrogen peroxide in fermented meats is to include catalase-producing strains in the starter culture. Although some lactic acid bacteria produce a small amount of catalase or a pseudo-catalase, micrococci (present in some cultures) produce much greater levels of this enzyme.

Another less common defect caused by lactic acid bacteria is slime formation. In particular, strains of *L. sakei* have been shown to produce exopolysaccharides (i.e., slime) in vacuum-packaged meat products, and have occasionally been implicated in this form of spoilage in fermented sausages.

Ultimately, microbial spoilage can best be prevented first by using meat that has been kept cold and that has been minimally exposed to psychrotrophic bacteria. Second, other potential spoilage organisms should be excluded from the finished fermented product via a comprehensive sanitation program. Few organisms should be present in cooked fermented sausages, provided post-processing contamination does not occur. While vacuum or modified atmosphere packaging with carbon dioxide is effective against aerobic psychrotrophs, facultative and anaerobic spoilage organisms, such as *B. thermosphacta* and lactic acid bacteria will not be inhibited. Antimicrobial agents, including organic acids and bacteriocins, however, may be effective against these bacteria, as described previously.

BIBLIOGRAPHY

Cocconcelli, P.S., and C. Fontana. 2010. Starter cultures for meat fermentation. In: F. Toldra, ed. Handbook of Meat Processing, Blackwell Publishing, Oxford.

Hammes, W.P. 2012. Metabolism of nitrate in fermented meats: the characteristic feature of a specific group of fermented foods. Food Microbiol. 29:151–156.

Holck, A., L. Axelsson, A. McLeod, T.M. Rode, and E. Heir. 2017. Health and safety considerations of fermented sausages. J. Food Qual. 3:1–25.

Iacumin, L. G. Comi, C. Cantoni, and L. Cocolin. 2006. Ecology and dynamics of coagulase-negative cocci isolated from naturally fermented Italian sausages. *S*yst. Appl. Microbiol. 29:480–486.

Leroy, F., A. Geyzen, M. Janssens, L. De Vuyst, and P. Scholliers. 2013. Meat fermentation at the crossroads of innovation and tradition: A historical outlook. Trends Food Sci. Technol. 31:130–137.

Lücke F-K, and P. Zangerl. 2014. Food safety challenges associated with traditional foods in German-speaking regions. Food Control 43:217–230.

Maddock, R. 2015. US products – dry sausage. In: F. Toldrá, ed. *Handbook of Fermented Meat and Poultry*, 2nd edn. Wiley, Oxford.

Ojha, K.S., J.P. Kerry, G. Duffy, T. Beresford, and B.K. Tiwari. 2015. Technological advances for enhancing quality and safety of functional meat products. Trends Food Sci. Technol. 44:105–116.

Toldrá, F., Y. Hui, I. Astiasarán, J. Sebranek, and R. Talon. 2015. *Handbook of Fermented Meat and Poultry*, 2nd edn. John Wiley & Sons, Oxford.

Vignolo, G., C. Fontana, S. Fadda. 2010. Semidry and dry fermented sausages. In: F. Toldra, ed. *Handbook of Meat Processing*. Blackwell Publishing, Oxford.

Vogel, R.F. 2012. Preface. Food Microbiology 29:149–150.

Zdolec, N. 2016. *Fermented Meat Products: Health Aspects*. CRC Press, Boca Raton, FL, USA.

8 Fermented vegetables

> Sour Kraut of which we had a large quantity, is not only a wholesome vegetable food, but in my judgement, highly antiscorbutic; and it spoils not by keeping. A pound of this was served to each man, when at sea, twice a week, or oftener, as was thought necessary.
>
> From the journal of Captain James Cook, recipient of the Royal Society of London, Copley Medal in 1776.

INTRODUCTION

Wherever vegetables are grown and consumed, it is almost certain that fermented versions exist. Moreover, despite the wide diversity of vegetables produced around the world, the general principles involved in the manufacture of fermented vegetables are very near the same. However, like other fermented foods, readily observed variations certainly exist. Often, these differences are based on the types of raw materials available in particular regions, as well as aesthetic or cultural preferences in those regions. While fermented vegetables are seemingly among the easiest fermented foods to make, their manufacture is influenced by many of the same microbiological and production variables that contribute to the diversity of other fermented food products.

A good example of the cultural differences that exist for fermented vegetables is the cabbage fermentation. Cabbage is widely used as a fermentation substrate, but the actual cultivar and ingredients that are used varies depending on culture and geography. Thus, in Germany, a mild-tasting, white European cabbage is used to make sauerkraut, with no other ingredients (other than salt) added. In contrast, in South Korea, several cultivars of cabbage are used to make kimchi, depending on the region and season. In addition, the cabbage used for kimchi is mixed with a variety of other ingredients, including peppers, radishes, garlic, spices, and even seafood, to give a much spicier and flavorful product.

The manufacture of fermented vegetables most likely evolved from simply dry-salting or brining vegetables. Salting vegetables was a common means of food preservation and was practiced for thousands of years in Europe, the Middle East, and Asia, and for several centuries in the Americas. In general, salt or brine was added to the fresh raw material as a preservation aid, and then the mixtures were packed into suitable containers and stored at a cool ambient temperature. Ordinarily, high salt concentrations (much above 15%) would

Microbiology and Technology of Fermented Foods, Second Edition. Robert W. Hutkins.

have precluded fermentation. However, if the salt concentration was not too high, this practice would have established ideal conditions for growth of naturally-occurring lactic acid bacteria. The ensuing fermentation would have not only enhanced preservation, but it would have also created highly desirable flavor and aroma characteristics.

Researchers have suggested that fermented vegetable technology actually began more than 2000 years ago. In Asia and the Far East, fermented products were made from cabbage, turnips, radishes, carrots, and other vegetables endemic to the local areas. This technology was exported to Europe sometime in the 1500s, with regional vegetables, such as European round cabbages, serving as the starting materials. Eventually, European settlers to the New World brought with them cabbages and procedures for the manufacture of sauerkraut. It is also likely that other fermented vegetables, pickles and olives in particular, were produced and consumed in the Middle East, at least since biblical times.

Fermented vegetables have long been a staple of Middle-East, Western, Indian, and Far East diets, not only because of their enhanced preservation and desirable flavor and texture properties, but also because these products had important nutritional properties. In particular, sauerkraut has long been known to have anti-scurvy properties, due to the high vitamin C content of cabbage. Thus, as noted by Captain Cook (see above), sauerkraut was an essential food for naval personnel and other seafarers because, in the absence of fresh fruits and vegetable, it served as a stable source of this vitamin. Cabbage also contains high concentrations of thiocyanates and other sulfur-containing compounds that may have antimicrobial activity. In the Far East, and Korea in particular, kimchi, has become the most popular of all fermented vegetables. Not only does kimchi have unique and desirable flavor and sensory attributes, its popularity is also due to suggested nutritional properties (see below).

PRODUCTS AND CONSUMPTION

In the United States, there are only three main fermented vegetable products that are produced and consumed on a large scale basis. These include sauerkraut, pickles, and olives. The raw materials used for these products – cabbage, cucumbers, and olives, are high moisture foods, with little protein or fat (except for olives), and just enough fermentable carbohydrates to support a fermentation (Table 8.1).

Other fermented vegetables, such as peppers, cauliflower, and green tomatoes, are also produced, but they are not as common, at least in the West. There are also many acidified or pickled vegetable products that are made by adding mixtures of vinegar, salt, and flavoring materials to fresh vegetables. In fact, most of the pickle products consumed in the United States, including the most popular brands, are not fermented, but rather are simply "pickled" by packing fresh cucumbers in vinegar or salt brines (discussed later). Likewise, most of the olives consumed in the United States are similarly produced.

Table 8.1 Composition of food substrates used in vegetable fermentations (%).

Vegetable	H_2O	Carbohydrate	Protein	Fat
Cabbage	92 – 94	5–6	1	–
Cucumbers	95	2–3	1	–
Olives	78–80	2–4	1–2	12–14

Although data for total production of fermented vegetables in the US is not readily available, on a per capita basis, consumption of fermented vegetables is rather modest. US consumers in 2016 ate about 0.6 kg of sauerkraut (based on USDA data) and about the same amount of olives (although not all of the latter were fermented). Per capita US consumption of pickles is somewhat higher, at about 4 kg. However, less than half of those pickles are of the fermented variety. In contrast, Germans eat about 1.8 kg of sauerkraut per person per year, and Albanians eat nearly 11 kg of fermented olives. Although kimchi consumption in Korea has decreased by more than 20% since 2005, in 2015, Koreans were still consuming 35 kg per year (nearly 100 g per day!).

PRODUCTION PRINCIPLES

As noted above, fermented vegetable technology is based on the same principles as other lactic acid fermentations, in that sugars are converted to acids, and the finished product takes on new and different characteristics. However, there is one major difference between the vegetable fermentation and the cheese, cultured milk, and fermented sausage fermentations described previously. Whereas these latter products (with just a few exceptions) are now almost always produced using starter cultures, the modern manufacture of pickles, olives, sauerkraut, and kimchi still relies on the natural microbiota to carry out the fermentation. Although starter cultures for these products have been developed (see below), they have not been widely adopted by the industry.

The microbial ecology of vegetable fermentations has long been the subject of considerable study. In particular, researchers at Cornell University and later at North Carolina State University and the USDA described the overall dynamics of the vegetable fermentation and developed technologies to improve the quality and consistency of these fermentations. More recently, molecular methods have been used to identify organisms that may have escaped detection in previous studies that relied on cultural means. Recent studies have shown, for example, that compared to the relatively few strains used for dairy fermentations, a diverse assortment of lactic acid bacteria are involved in vegetable fermentations. These genera include *Lactobacillus*, *Pediococcus*, *Leuconsotoc*, and *Weissella*, a genus that has not received much attention so far in this book. Moreover, while homofermentative lactic acid bacteria are important in fermented vegetables, heterofermentative species also make crucial contributions, especially in the early stages. Yeast may also be relevant in certain olive fermentations, especially when high salt concentrations are used.

It is important to note that although the plant-based substrates (i.e., cabbage, cucumbers, and olives) ordinarily contain the relevant lactic acid bacteria necessary to perform a lactic fermentation, they also harbor a complex microbiota consisting of other less desirable organisms. In fact, the resident lactic acid bacteria population represents only a small fraction of the total microflora present in the starting material. Unlike dairy fermentations, where pasteurization can substantially reduce the indigenous microflora present in raw milk, no such heating step can be used to produce fermented vegetables (lest one end up with cabbage soup!). Chemical pasteurization procedures have been developed and can effectively reduce the resident population. However, such methods are not common. Therefore, the essential requirement for a successful fermentation is to create environmental conditions that are conducive for the lactic acid bacteria, but that inhibit or otherwise restrict the non-lactic microbiota.

THE MICROBIOTA OF FRESH VEGETABLES

Plant material, including edible vegetables, serves as the natural habitat for a wide variety of microorganisms (Table 8.2). The endogenous or epiphytic microbiota consists of yeast, fungi, and both Gram-positive and Gram-negative bacteria. The plant environment is exposed to the air and the surfaces of plant tissue have a high Eh. Thus, aerobic bacteria, such as *Pseudomonas*, *Flavobacterium*, *Bacillus*, and various aerobic fungi, would be expected to dominate freshly harvested material, as is indeed the case.

Facultative anaerobes, including *Enterobacter*, *Escherichia coli*, *Klebsiella*, and other enteric bacteria, as well as spore-forming clostridia, are also part of the resident microbiota. Various yeasts, including *Candida*, *Saccharomyces*, *Hansenula*, *Pichia*, and *Rhodotorula,* may also be present. Lactic acid bacteria, mainly species belonging to the genera *Lactobacillus*, *Pediococcus*, *Streptococcus*, *Weissella*, and *Enterococcus*, are ordinarily present, but at surprisingly low numbers. In fact, whereas the total population of *Pseudomonas*, *Flavobacterium*, *Escherichia*, and *Bacillus* may well reach levels as high as 10^7 cells per gram, lactic acid bacteria are normally present at only about 10^3 cells per gram. Thus, the lactic acid bacteria are outnumbered by non-lactic competitors by a thousand times or more, putting them at a serious disadvantage.

Given the diversity of microorganisms initially present in the raw material and the numerical disparity between the lactic and non-lactic bacteria, it would seem that rather severe

Table 8.2 Representative microbiota of fresh vegetables.

Organisms	Log CFU/g	Abundance (%)
Aerobic bacteria	4–6	1–6
Pseudomonas		
Flavobacterium		
Micrococcus		
Bacillus		
Lactic acid bacteria	0.7–4	1–7
Lactobacillus		
Pediococcus		
Streptococcus		
Tetragenococcus		
Leuconostoc		
Enterobacteriaceae	3–3.5	20–60
Enterococcus		
Enterobacter		
Klebsiella		
Escherichia		
Yeasts and mold	0.3–4.6	Not determined
Fusarium		
Ascochyta		
Aspergillus		
Penicillium		
Rhodotorula		

Enumerative data adapted from Nout and Rombouts, 1992 and abundance data from Leff and Fierer, 2013.

measures must be adopted to establish the selective environment necessary for a successful lactic acid fermentation. In reality, selection is based on only a few simple factors – salt, temperature, and anaerobiosis. Thus, under appropriate conditions, most non-lactic acid bacteria will grow slowly, if at all. In contrast, lactic acid bacteria will generally be unaffected (but not totally) by these restrictions, and will instead grow and produce acidic end products. The acids, along with CO_2 that may also be produced, creates an even more stringent environment for would-be competitors. Within 6–12 hours, growth of lactic acid bacteria will be evident, and as the lactic acid fermentation proceeds, the number of competing organisms will decline.

The lactic acid fermentation that occurs during most vegetable fermentations does not depend on any single organism, but rather on a consortium of bacteria representing several different genera and species (Table 8.3). Moreover, there is a predictable order to the fermentation, with the various organisms having temporal functions. Thus, a given organism (or group of organisms) initiates growth and becomes established for a particular period of time. Then, due to accumulation of acidic end products or other inhibitory factors, growth of those organisms will begin to slow down or cease. Eventually, the initial microbial population gives way to other species that are less sensitive to those inhibitory factors. Microbial ecologists refer to this ecological process as a succession. This is one reason why vegetable fermentations are ordinarily conducted without starter cultures, since duplicating a natural succession of organisms is generally not achievable on a consistent basis with cultures.

MANUFACTURE OF SAUERKRAUT

Few fermented foods are produced in such a seemingly simple process as is sauerkraut (Figure 8.1). Only two ingredients, cabbage and salt, are necessary, and once these ingredients are properly mixed and placed into suitable fermentation vessels, there is little that the manufacturer needs to do until the fermentation is completed. The simplicity of the process is reflected by the US Standards that states that sauerkraut is the "product of characteristic acid flavor, obtained by the full fermentation, chiefly lactic, of properly prepared and shredded cabbage in the presence of not less than 2 percent nor more than 3 percent of salt." When properly fermented, sauerkraut should contain not less than 1.5% acid (expressed as lactic acid) and have a pleasant tart flavor.

Table 8.3 Main lactic acid bacteria involved in vegetable fermentations.

Sauerkraut	Kimchi	Pickles	Olives
Leuconostoc mesenteroides	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides*
Leuconostoc fallax	*Leuconostoc kimchii*	*Lactobacillus plantarum*	*Lactobacillus plantarum*
Lactobacillus plantarum	*Leuconostoc gelidum*	*Lactobacillus brevis*	*Lactobacillus brevis*
Lactobacillus brevis	*Leuconostoc inhae*	*Pediococcus pentosaceus*	*Lactococcus* spp.
Pediococcus pentosaceus	*Leuconostoc citreum*		
	Lactobacillus plantarum		
	Lactobacillus brevis		
	Lactococcus lactis		
	Weissella kimchii		

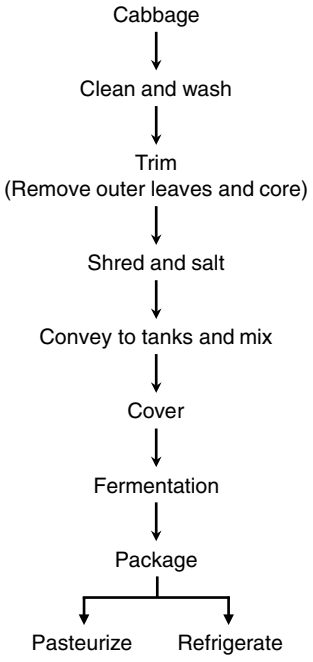


Figure 8.1 Manufacture of sauerkraut. Adapted from Holzapfel et al., 2008.

The manufacture of sauerkraut starts with the selection of the raw substrate material. Although various cabbage cultivars exist, white cabbage is typically used because it has a mild, slightly sweet flavor and contains 5% or more fermentable sugars. The latter are comprised mainly of nearly equimolar amounts of glucose and fructose, with a very small amount of sucrose. Cabbage used to make sauerkraut should be fully mature, and should contain few outer leaves. Some manufacturers allow the cabbage heads to wilt for a day or two.

1. Shredding and salting

Once the outer leaves and any spoiled leaves are removed from the cabbage heads (with or without washing), the core is removed. The cabbage is shredded according to the manufacturer's specifications to make a slaw. The shredded leaves are then weighed and conveyed directly to tanks or are deposited first into tubs or carts and then transferred into tanks. Salt can be added as the slaw is conveyed or it can be added to the slaw when it arrives in the tanks. In either case, both the amount of salt added and the means by which mixing and distribution occur are critical.

Usually, between 2% and 3% salt is added (by weight). This is a fairly narrow range, with about 2.25% generally considered to be the optimum. Because salt performs several essential functions during the sauerkraut fermentation, problems are almost certain to occur if too much or not enough salt is added or if the salt is not uniformly distributed (see below). Very soon after the salt is mixed with the shredded cabbage, water begins to diffuse out from the interior of the plant tissue to the exterior medium, due to simple osmosis. The brine that forms also contains sugars and other dissolved nutrients that diffuse out with the water. Thus, it is this water phase that ultimately serves as the location for most of the microbial activity.

The salt dissolved in the brine also provides the selective conditions that discourage growth of most of the non-lactic microorganisms that would otherwise compete with the lactic microbiota. Although salt at concentrations ordinarily used, is by itself, not sufficient to inhibit all of the indigenous, non-lactic bacteria, it is enough to provide the lactic acid bacteria with a substantial growth advantage. Furthermore, combined with other environmental factors, the selective effects of this relatively moderate salt concentration can be increased appreciably. Thus, once the pH has been decreased by fermentation and the ensuing production of organic acids, the combination of salt plus acid contributes significantly to the long preservation properties of the finished product. Finally, salt imparts a desirable flavor to the product and helps to maintain a crisp texture by inhibiting pectinolytic enzymes responsible for softening of the tissues.

2. Mixing

The shredded and salted cabbage is then placed into tanks and mixed well to distribute the salt. As noted above, mixing is an important step, because localized regions within the rather heterogeneous material may contain more or less than the 2–3% salt that was added to the bulk mixture. Within those pockets, therefore, it is entirely possible that the salt concentration may vary by as much as 0.5–1%. This may result in either too little or too much inhibitory control over the organisms that reside in that microenvironment. If spoilage organisms were able to grow, their products (e.g., slime, pigments, off-flavors) could accumulate in those pockets. Then, when the sauerkraut is mixed prior to packaging, the entire batch of product would be contaminated with those products.

It is worth noting that, despite the challenges, it is possible to produce sauerkraut using lower levels of salt (around 1%). Low salt sauerkraut is of interest, in part, for nutritional purposes, but also because of environmental reasons (i.e., disposal of brines). Finally, high salt levels can promote spoilage just as readily as low salt levels. For example, the "pink" defect (discussed below) is caused by growth of salt-tolerant yeasts that ordinarily would be suppressed by lactic acid bacteria whose growth is impaired at high salt levels.

The sauerkraut fermentation was traditionally performed in wooden barrels. Wood-stave tanks are still used; however, concrete vats are now common. The latter are lined with fiberglass or plastic, and can hold as much as 80,000 kg. The cabbage is covered with a plastic, tarp-like material, large enough to drape over the sides of the tank. Water (or brine) is then placed on top to weigh down the cabbage and to drive out and exclude air. This also reduces exposure to air-borne organisms, foreign matter, and insects. The weight further enhances formation of a brine, which soon completely covers the shredded cabbage. Importantly, this step also enhances the anaerobic atmosphere in the cabbage-brine mixture.

3. Fermentation

Sauerkraut fermentation has long been a subject of interest among food microbiologists as well as microbial ecologists. In fact, many of the biochemical and microbiological details of the sauerkraut fermentation were described as long ago as the 1930s. This interest has undoubtedly been due, in large part, to the very nature of the fermentation process, in that it involves several different naturally-occurring microorganisms acting as part of a complex ecosystem. Studies suggest that bacteriophages may also play an important role in the microbial ecology of the sauerkraut fermentation (Box 8.1).

Box 8.1 Bacteriophages get into the mix

For microbial ecologists with a food orientation, the vegetable fermentation is a near-perfect model system. Consider what transpires in the course of a typical sausage or kimchi or other vegetable fermentation. There are, for example, major shifts in the environment, from aerobic to anaerobic, and from a neutral to acidic pH. The presence of salt also provides selection. The availability of nutrients, in particular, fermentable carbohydrates, decreases during the fermentation. At the same time, there is a succession of microorganisms, such that some genera and species, present at the outset, are displaced by other microbial communities, and cannot even be detected at later stages. Moreover, these fermentations are remarkably repeatable. Finally, modern molecular methods now provide the means to study these microbial interactions with high resolution (Perez-Díaz et al., 2017).

While bacteriophages are common in dairy fermentations, their role in vegetable fermentations was not recognized until relatively recently (Lu et al., 2003 and Yoon et al., 2002). These initial studies showed that phages are widespread in commercial sauerkraut fermentations. Interestingly, appearance of specific members of the natural microbiota correlated with the appearance of their homologous phages. That is, phages capable of infecting *Leuconostoc* and *Weissella* were only isolated during the first few days of the fermentation, when these were the dominant organisms. Phages that infected *Lactobacillus*, in contrast, were not found until later in the fermentation, when *Lactobacillus plantarum* and other species had become established. Thus, these phage infection events coincided with the main population shift that occurs during the sauerkraut fermentation, i.e., from mainly heterofermentative species to mainly homofermentative species (Lu et al., 2003). These researchers suggested that the well-studied phenomenon of succession that occurs during the sauerkraut fermentation may be mediated not just by the changing environment, but also by the emergence of bacteriophages.

More recently, the role of phage in cucumber fermentations was described (Lu et al., 2012; Jung et al., 2011). For cucumber fermentations (i.e., pickles), as much as 10% of the representative population of lactic acid bacteria was sensitive to phage (Lu et al., 2012). Species from all relevant genera, including *Leuconostoc*, *Weissella*, *Lactobacillus*, and *Pediococcus* were identified as suitable hosts. Moreover, some of these phages had surprisingly broad host range, infecting multiple species. Fermented vegetable brines may therefore be considered a major reservoir for phages that infect *Leuconostoc* and *Weissella* (Kot et al., 2014).

On a practical basis, the presence of phage in wild vegetable fermentations would not be expected to be problematic, given the diversity of species and strains able to carry out the fermentation. However, there has long been interest in developing pure culture fermentations, in part because of the large volume of high salt brines that must be managed as waste. Low salt fermentations are possible (for pickles and olives, in particular), but require starter cultures to ensure a prompt and proper fermentation. Such pure culture fermentations could be adversely affected by phage, were they to be present. Because the raw materials used in fermented vegetable manufacture cannot be heated, nor are these fermentations conducted under aseptic conditions, it is not possible to exclude phages from the production environment. As noted by these researchers,

phage-resistant strains would therefore be necessary to ensure the success of low salt pickle fermentations.

Lactic bacteriophage have also been isolated from fermented olives (Lanza et al., 2012; Zago et al., 2013). One of the motivations for this research was to identify strains of *Lactobacillus plantarum* that degrade oleuropein, a bitter compound associated with olives. Such strains could then be used as an olive starter culture. However, these researchers observed that more than half of the strains they screened were sensitive to phage (Zago et al., 2013).

Finally, only a few studies have reported the presence of phage during kimchi fermentations. Nonetheless, it would certainly not be surprising if they played a similar role as in other fermented vegetables. A lytic phage that infects the kimchi isolate, *Weissella cibaria*, was recently described (Kleppen et al., 2012). DNA encoding for putative phage capsid proteins were also detected in a kimchi fermentation metagenome analysis, and had sequences similar to a *Leuconostoc* phage (Jung et al, 2011). The abundance of phage sequences also increased during the fermentation, suggesting that phage infections and lysis occur during the kimchi fermentation and that phage may have a major influence on the composition of the microbial community. As the authors concluded, the interactions between different microbial members in the kimchi community, as well as phage, may ultimately affect production of metabolic products, including those responsible for flavor (Jung et al., 2011).

References

Jung, J.Y., S.H. Lee, J.M. Kim, M.S. Park, J.-W. Bae, Y. Hahn., E.L. Madsen, and C.O. Jeon. 2011. Metagenomic analysis of kimchi, a traditional Korean fermented food. Appl. Environ. Microbiol. 77:2264–2274.

Kleppen, H.P., H. Holo, S.-R. Jeon, I.F. Nes, and S.-S. Yoon. 2012. Novel Podoviridae family bacteriophage infecting Weissella cibaria isolated from kimchi. Appl. Environ. Microbiol. 78:7299–7308.

Kot, W., H. Neve, K.J. Heller, and F.K. Vogensen. 2014. Bacteriophages of *Leuconostoc*, *Oenococcus*, and *Weissella*. Front. Microbiol. 28:186.

Lanza, B., M. Zago, D. Carminati, L. Rossetti, A. Meucci, P. Marfisi, F. Russi, E. Iannucci, M.G. Di Serio, and G. Giraffa. 2012. Isolation and preliminary characterization of *Lactobacillus plantarum* bacteriophages from table olive fermentation. Ann. Microbiol. 62:1467–1472.

Lu, Z., F. Breidt, V. Plengvidhya, and H.P. Fleming. 2003. Bacteriophage ecology in commercial sauerkraut fermentations. Appl. Environ. Microbiol. 69:3192–3202.

Lu, Z., I.M. Perez-Diaz, J.S. Hayes, and F. Breidt. 2012. Bacteriophage ecology in a commercial cucumber fermentation. Appl. Environ. Microbiol. 78:8571–8578.

Perez-Díaz, I.M., J. Hayes, E. Medina, K. Anekella, K. Daughtry, S. Dieck, M. Levi, R. Price, N. Butz, Z. Lu, and M.A. Azcarate-Peril. 2017. Reassessment of the succession of lactic acid bacteria in commercial cucumber fermentations and physiological and genomic features associated with their dominance. Food Microbiol. 63:217–227.

Yoon, S.S., R. Barrangou-Poueys, F. Breidt, Jr., T.R. Klaenhammer, and H.P. Fleming. 2002. Isolation and characterization of bacteriophages from fermenting sauerkraut. Appl. Environ. Microbiol. 68:973–976.

Zago, M., B. Lanza, L. Rossetti, I. Muzzalupo, D. Carminati, and G. Giraffa. 2013. Selection of *Lactobacillus plantarum* strains to use as starters in fermented table olives: oleuropeinase activity and phage sensitivity. Food Microbiol. 34:81–87.

The manufacture of sauerkraut and other fermented vegetables depends on a succession of organisms that are naturally present in the raw material. Some appear early on in the fermentation, perform a particular function, and then, for all practical purposes, disappear from the product. Other organisms, in contrast, emerge later in the fermentation and then remain at moderate to high levels throughout the duration of the fermentation and post-fermentation process. However, growth of those organisms that occur late in the process depends on the organisms that had grown earlier and that had established the correct environmental conditions.

Microbial activity begins almost immediately after the cabbage is shredded, salt is added, and the brine has formed. Initially, the atmosphere is aerobic, with redox potentials (or Eh values) of over 200 mV. However, the combined effects of physical exclusion of air as well as residual respiration and oxygen consumption by plant cells quickly reduces the Eh and makes the environment anaerobic. Thus, pseudomonads, fungi, and other obligate aerobic microorganisms that are initially present at high levels, have little opportunity to grow. Some of these organisms are also salt-sensitive, further reducing their ability to compete in this environment. Still, at the temperatures used during the sauerkraut fermentation (16°C to 20°C), many other indigenous salt-tolerant, mesophilic, facultative organisms might be expected to grow, including *Enterobacter*, *Klebsiella*, *E. coli*, and *Erwinia*. Instead, these organisms persist for only a short time, perhaps as little as a few hours, due to competition by lactic acid bacteria and the inhibitory effects of the acids produced by these bacteria.

The lactic fermentation in sauerkraut occurs in a series of overlapping stages or sequences. These stages and the succession of microorganisms associated with each stage have been very well studied. Remarkably, the fermentation almost always follows the same pattern (Figure 8.2).

The first stage, variously referred to as the initiation, heterofermentative, or gaseous phase, is marked by growth of *Leuconostoc mesenteroides* subsp. *mesenteroides*. This

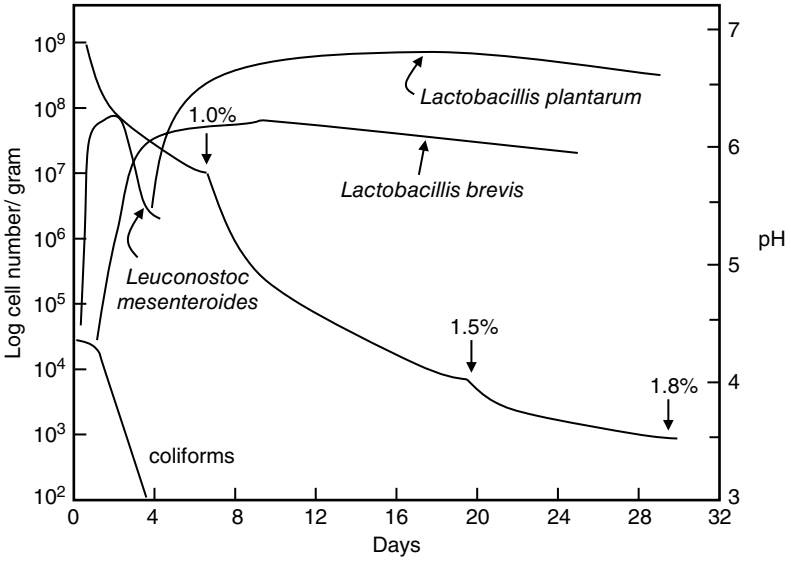


Figure 8.2 Fermentation succession. Idealized model for successive growth of lactic acid bacteria during the sauerkraut fermentation. The approximate acidities (as lactic) at varying pHs are indicated. Adapted from Di Cagno et al., 2013, Lu et al., 2003 and other sources.

organism is salt-tolerant and has a relatively short lag phase and high growth rate at low temperatures (15°C to 18°C). Importantly, it metabolizes sugars via the heterofermentative pathway, yielding lactic and acetic acids, CO_2, and ethanol. As noted above, *Weissella cibaria* and other species of this genus may also appear at the beginning of the fermentation. Ultimately, the acidic environment (0.6% to 0.8%, as lactic acid) created by growth of these heterofermentative bacteria not only inhibits non-lactic competitors, but it also favors other lactic acid bacteria. The production of CO_2 also contributes to making the environment even more anaerobic (as low as –200 mV), which again favors the more anaerobic lactic acid bacteria. Eventually, however, as the acid concentration approaches 1.0%, *L. mesenteroides* is, itself, inhibited, and within four to six days, this organism is barely detectable.

In the next stage – the so-called primary or homofermentative phase – the decrease in the *Leuconostoc* population coincides with the succession of several other lactic acid bacteria. Included among the latter are *Lactobacillus plantarum* and *Lactobacillus brevis.* Although *L. plantarum* is a facultative heterofermentor (meaning it has the metabolic capacity to ferment different sugars via homo- or heterofermentative pathways) and *L. brevis* is an obligate heterofermentor, both organisms are strong acid producers, nearly doubling the acid content to about 1.4% to 1.6%. They are also quite stable in this acidic environment and dominate the fermentation during this period (especially *L. plantarum*). It is not unusual, however, for other lactic acid bacteria, including *Pediococcus pentosaceus*, *Pediococcus acidilactici*, *Lactobacillus curvatus*, and *Enterococcus sp.*, to be present during the primary fermentation. Finally, as the acidity approaches 1.6% and the pH decreases below 4.0, the acid-tolerant *L. plantarum* dominates the finished product. The entire process can take up to one to two months, and the fermentation is generally considered complete when the acidity is at about 1.7%, with a pH of 3.4 to 3.6.

End products

Although lactic acid is the major compound produced during the fermentation, other metabolic end products are also formed. Importantly, many of these products contribute to the overall flavor of sauerkraut. In particular, end products produced by *Leuconostoc* sp. and other heterofermentative lactic acid bacteria are essential for good-tasting sauerkraut. As much as 0.3% acetic acid and 0.5% ethanol can be present in the finished sauerkraut. In addition, these bacteria may also synthesize small amounts of diacetyl, acetaldehyde, and other volatile flavor compounds. Finally, the CO_2 that accumulated during the initiation stage of the fermentation provides carbonation and enhances mouthfeel.

Mannitol is another end product that accumulates during the sauerkraut fermentation. It is formed directly from fructose by heterofermentative leuconostocs and lactobacilli via the NADH-dependent enzyme, mannitol dehydrogenase. As shown in Figure 8.3, regeneration of NAD in the heterofermentative pathway ordinarily occurs twice, first when acetyl CoA is reduced to acetaldehyde and then when acetaldehyde is reduced to ethanol. However, fructose, when available in excess, can serve as an alternative electron acceptor. This allows the cell to use acetyl phosphate as a phosphoryl group donor in the acetate-generating reaction catalyzed by acetate kinase. As a consequence, one additional molecule of ATP is synthesized by substrate level phosphorylation.

More than 100 mM mannitol can be formed in sauerkraut by this pathway (with a commensurate amount of ATP made for the cell). Eventually, some of this mannitol may be consumed by lactobacilli that emerge later in the fermentation. Although most of the mannitol

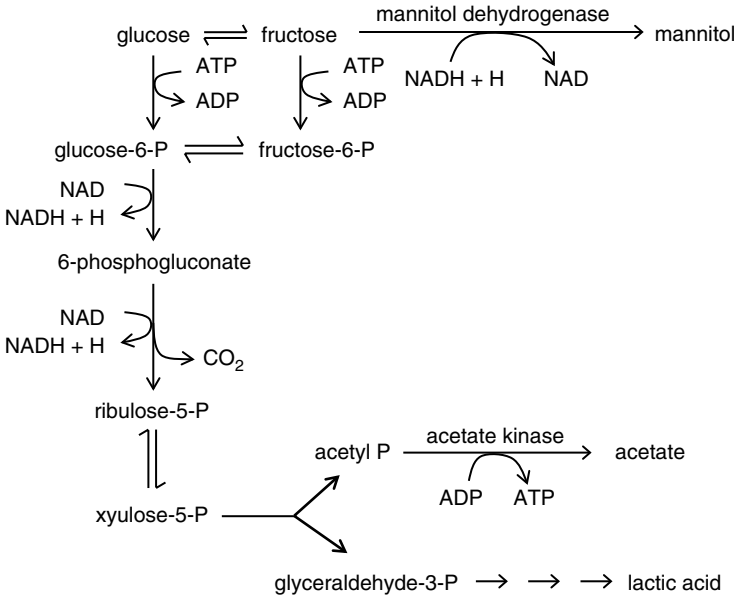


Figure 8.3 Mannitol formation in heterofermentative lactic acid bacteria. Phosphorylated glucose and fructose are metabolized by the phosphoketolase pathway. The reactions leading to lactic acid (from glyceraldehyde-3-P) are not shown. Adapted from Saha and Racine, 2010.

is produced directly from fructose, the appearance of mannitol even when fructose has been depleted suggests that some might be formed indirectly from glucose (following its conversion to fructose via glucose isomerase).

Packaging and processing

In the United States, commercial products are usually thermally processed, much like other high-acid foods (about 75°C), prior to packaging in cans or jars. Such products are essentially commercially sterile such that they are stable at room temperature. There is also an emerging market for non-pasteurized, refrigerated sauerkraut that is packaged in glass jars or sealed plastic bags. The latter products may still evolve CO_2, which may build up pressure inside the package. Recently, plastic pouches have been developed that contain venting systems that release the accumulated CO_2.

Spoilage and defects

Although chemical or physical reactions are occasionally responsible for sauerkraut spoilage, most defects are caused by microorganisms (Table 8.4). Defects are more likely to occur if the production conditions are not properly controlled, leading to deviations in the fermentation pattern.

Two of the most common factors that influence the fermentation and that may lead to quality defects are temperature and salt. If, for example, the temperature is too high (>30°C) or if too much salt is added (>3%), then *L. mesenteroides* may be inhibited. This could result

Table 8.4 Microbial defects in fermented vegetables.

Product	Defect	Causative organisms
Sauerkraut	Pinking	*Rhodotorula*
	Ropy/slimy	*Leuconostoc, Lactobacillus*
	Softening	*Enterobacter, Flavobacterium, Pseudomonas*
Pickles	Bloating/floating	*Bacillus, Aeromonas, Achromobacter, Aerobacter, Fusarium, Penicillium*
	Softening	*Fusarium, Penicillium, Ascochyta*
Olives	Gassy/floater/fish eyes	*Enterobacter, Citrobacter, Klebsiella, Escherichia, Aeromonas, Hansenula, Saccharomyces*
	Softening	*Bacillus, Aeromonas, Achromobacter, Aerobacter, Fusarium, Penicillium*
	Zapatera/malodorous	*Clostridium, Propionibacterium*

in the absence of heterofermentative end-products, and higher levels of lactic acid and a harsher more acidic flavor. Worse yet, when prompt acid formation is delayed by high salt conditions (or pockets of high salt, due to uneven mixing), growth of salt-tolerant yeasts and other organisms may occur. Growth of *Rhodotorula* sp. is a particular problem, due to the undesirable pink pigment this yeast produces.

If, in contrast, the temperature is too low (<10°C) or too little salt is added (<2%), various Gram-negative bacteria, including *Enterobacter*, *Flavobacterium*, and *Pseudomonas*, may grow. Some of these bacteria are capable of producing pectinolytic enzymes that cause a "soft kraut" defect, resulting from pectin hydrolysis.

Finally, some strains of *L. mesenteroides* produce dextrans and other polysaccharides that give a slimy or ropy texture to the product. Other lactic acid bacteria, including *L. plantarum*, may produce capsular materials that cause similar texture defects.

KIMCHI

As noted previously, the Korean version of sauerkraut is called kimchi. Actually, given the emerging global popularity of kimchi perhaps sauerkraut should be referred to as the European version of kimchi! Although kimchi is a staple food in Korea and is popular in Japan and throughout the Far East, it has become one of the trendiest "new" foods in the US in the past decade. Kimchi-serving restaurants, sushi bars, and even food trucks are now located in cities throughout the US

Although the manufacture of kimchi and sauerkraut are very similar, the characteristics of the finished products are quite different. The main difference between sauerkraut and kimchi is that the latter product contains ingredients other than simply cabbage and salt. Kimchi, for example, not only can be made from one of several different cultivars of cabbage, but other vegetables, including radishes and cucumbers, can also be used, alone or mixed with cabbage. Indeed, more than 200 different types of kimchi are produced and consumed in Korea. A variety of other vegetables, spices, and flavoring agents are also commonly added to kimchi, depending on the particular type of kimchi being produced. Garlic, green

onion, ginger, and red peppers are among the typical ingredients, but fish, shrimp, fish sauce, fruits, and nuts can also be added.

Many kimchi products contain hot peppers, which when combined with lactic and acetic acids, can provide substantial heat and tanginess. Kimchi, therefore, has a much more complex flavor and texture profile as compared to other fermented vegetables. However, another reason why kimchi has become popular is because it has been suggested to have unique nutritional properties. These benefits are conferred in part by the raw materials, but also by the fermentative microorganisms and their end products (Box 8.2).

Box 8.2 Health properties of kimchi

In Korea, kimchi is arguably the most popular of all fermented foods. As many as 100 different types are produced using various raw materials and processes. On an annual basis, about 360 million kg are produced commercially, with an economic value over 2 billion dollars (Jung et al., 2014). A large amount is also made directly in homes. Per capita consumption in 2012 was more than 120 grams per day, which is still nearly 20% less than in the previous 15 years (Kim et al., 2016). In Korea, it is common to eat kimchi at every meal, all year round (Surh et al., 2008).

The manufacturing steps involved in kimchi production are, in principle, similar to those used for other fermented vegetables (Figure 8.2.1). The main difference between kimchi and sauerkraut, its European counterpart, is that kimchi contains several additional ingredients that impart considerably more flavor and texture properties. While the popularity of kimchi can certainly be ascribed to the combination of these desirable sensory characteristics, there are also nutritional reasons that contribute to its widespread consumption. For example, kimchi can contain appreciable amounts of ascorbic acid (vitamin C), B vitamins, calcium, iron, potassium, dietary fiber, and

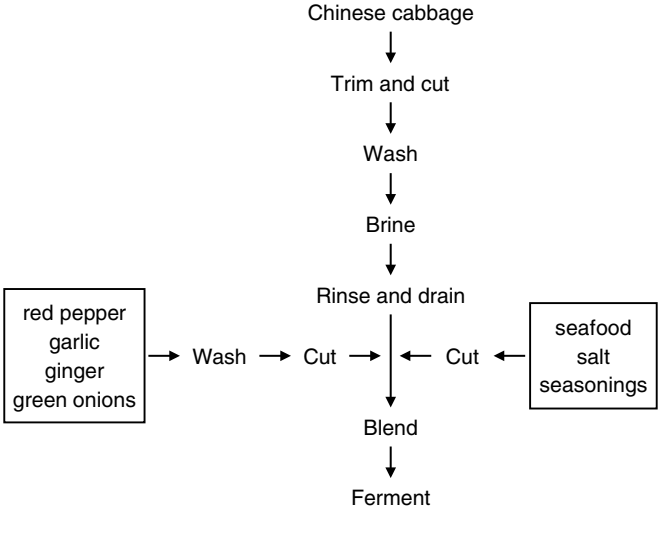


Figure 8.2.1 Kimchi flow chart. Adapted from Rhee et al., 2011.

naturally occurring antioxidants. As discussed below, kimchi is most often eaten in its uncooked or raw state, such that live microorganisms are ordinarily present.

The putative nutritional and health-promoting properties of kimchi are now the subject of considerable research. Several reports suggest that kimchi may reduce the risks of various cancers, ameliorate inflammation due to colitis, alleviate allergic reactions, and improve serum lipids (Choi et al., 2013; Jeong et al., 2015; Kim et al., 2013; Kim et al., 2014b; Kim et al., 2015; Park et al., 2017). However, these studies have been mostly performed *in vitro* or using animals models.

Epidemiological studies have also suggested that consumption of kimchi may be associated with reduced incidence of cancer, metabolic syndrome, asthma, and other diseases among the Korean population (Kim et al., 2014a; Lee and Cho, 2014; Park and Bae, 2016; Wie et al., 2017). For example, in the latter study, 8000 individuals were followed for 9 years. The researchers observed a significant inverse relationship between consumption of kimchi and rice and non-gastrointestinal cancers, and concluded that such diets might reduce the risk of cancer. Other studies, however, have showed that consumption of kimchi and other high salt foods may increase the risk of some cancers, especially gastric cancer (Shin and Park, 2011; Woo et al., 2014).

If, indeed, kimchi has health-promoting properties beyond those present in the raw material, then those effects must be due either to the kimchi microbiota directly, to products produced by the microbiota during the fermentation, or to transformation of kimchi constituents by the microbiota. Regular consumption of kimchi could serve as a significant dietary source of live lactic acid bacteria. There are numerous reports in the literature that describe lactic acid bacteria isolated from kimchi and their putative health benefits. As noted above, these were mostly done in animal models. For example, the kimchi isolate, *Lactobacillus sakei* probio 65, was found to inhibit atopic dermatitis-like skin lesions in mice, an effect mediated in part by reduced serum levels of IgE and skin-associated chemokines (Kim et al., 2013). Another kimchi strain, *Weissella cibaria* WIKIM28 had similar effects in a mouse model, suppressing allergic Th2 responses while inducing a Treg response (Lim et al., 2017).

In another recent study, *Lactobacillus plantarum* HAC01 and *L. sakei* HAC10, isolated from kimchi and fed to obese mice, reduced weight gain compared to control-fed mice (Park et al., 2016). Finally, the ability of kimchi bacteria to degrade potentially harmful metabolites was recently described (Kim et al., 2017). Cabbage, as well as other fermented vegetables, can contain high levels of naturally-occurring nitrates that can be enzymatically reduced to nitrite, which is subsequently formed, *in vivo*, into nitrosamines. As shown in this study, the formation of these toxic compounds was inhibited by kimchi-derived *L. sakei*, *Lactobacillus curvatus*, and *Lactobacillus brevis*. These organisms also had the capacity to degrade nitrosamines and its precursors.

Kimchi is made from a wide variety of raw materials, including several that may have biological activity and enhance human health (e.g., cabbage, garlic, radishes, peppers, ginger, and onion). The lactic acid microbiota is also complex and variable. Thus, identifying the actual components or processes that may be responsible for the suggested health benefits of kimchi, and then validating those benefits *in vivo*, remains a significant challenge.

References

Choi, I.H., J.S. Noh, J.-S. Han, H.J. Kim, E.-S. Han, and Y.O. Song. 2013. Kimchi, a fermented vegetable, improves serum lipid profiles in healthy young adults: randomized clinical trial. J. Med. Food 16:223–229.

Jeong, M., J.-M. Park, Y.-M. Han, K.Y. Park, D.H. Lee, J.-H. Yoo, J.Y. Cho, and K.-B. Hahm. 2015. Dietary prevention of *Helicobacter pylori*-associated gastric cancer with kimchi. Oncotarget 6:29513–29526.

Jung, J.Y., S.H. Lee, and C.O. Jeon. 2014. Kimchi microflora: history, current status, and perspectives for industrial kimchi production. Appl. Microbiol. Biotechnol. 98:2385–2393.

Kim, B. J.-L. Song, J.-H. Ju, S.-A. Kang, and K.-Y. Park. 2015. Anticancer effects of kimchi fermented for different times and with added ingredients in human HT-29 colon cancer cells. Food Sci. Biotechnol. 24:629–633.

Kim, E.-K. A.-W. Ha, E.-O. Choi, and S.-Y. Ju. 2016. Analysis of kimchi, vegetable and fruit consumption trends among Korean adults: data from the Korea National Health and Nutrition Examination Survey (1998–2012). Nutr. Res. Pract. 10:188–197.

Kim, H., S.-Y. Oh, M.-H. Kang, K.-N. Kim, Y. Kim, and N. Chang. 2014a. Association between kimchi intake and asthma in Korean adults: the fourth and fifth Korea National Health and Nutrition Examination Survey (2007–2011). J. Med. Food 17:172–178.

Kim, H.-Y., J.-L. Song, H.-K. Chang, S.-A. Kang, and K.-Y. Park. 2014b. Kimchi protects against azoxymethane/dextran sulfate sodium-induced colorectal carcinogenesis in mice. J. Med. Food. 17:833–841.

Kim, J.-Y., B.-K. Park, H.-J. Park, Y.-H. Park, B.-O. Kim, and S. Pyo. 2013. Atopic dermatitis-mitigating effects of new *Lactobacillus* strain, *Lactobacillus sakei* probio 65 isolated from kimchi. J. Appl. Microbiol. 115:517–526.

Kim, S.-H., K.H. Kang, S.H. Kim, S. Lee, S.-H. Lee, E.-S. Ha, N.-J. Sung, J.G. Kim, and M.J. Chung. 2017. Lactic acid bacteria directly degrade N-nitrosodimethylamine and increase the nitrite-scavenging ability in kimchi. Food Control 71:101–109.

Lee, K.W. and M.S. Cho. 2014. The traditional Korean dietary pattern is associated with decreased risk of metabolic syndrome: findings from the Korean National Health and Nutrition Examination Survey, 1998–2009. J. Med. Food 17:43–56.

Lim, S.K., M.-S. Kwon, J. Lee, Y.J. Oh, J.-Y. Jang, J.-H. Lee, H.W. Park, Y.-D. Nam, M.J. Seo, S.W. Roh, and H.-J. Choi. 2017. *Weissella cibaria* WIKIM28 ameliorates atopic dermatitis-like skin lesions by inducing tolerogenic dendritic cells and regulatory T cells in BALB/c mice. Sci. Rep. 7:40040.

Park, J.-S., I. Joe, P.D. Rhee, C.-S. Jeong, and G. Jeong. 2017. A lactic acid bacterium isolated from kimchi ameliorates intestinal inflammation in DSS-induced colitis. J. Microbiol. 55:304–310.

Park, S., and J.-H. Bae. 2016. Fermented food intake is associated with a reduced likelihood of atopic dermatitis in an adult population (Korean National Health and Nutrition Examination Survey 2012–2013). Nutr. Res. 36:125–133.

Park, S., Y. Ji, H. Park, K. Lee, H. Park, B.R. Beck, H. Shin, and W.H. Holzapfel. 2016. Evaluation of functional properties of lactobacilli isolated from Korean white kimchi. Food Control 69:5–12.

Rhee, S.J., J.-E Lee, and C.-H. Lee. 2011. Importance of lactic acid bacteria in Asian fermented foods. Microb. Cell Fact. 10(Suppl. 1):S5.

Shin, A., J. Kim, and S. Park. 2011. Gastric cancer epidemiology in Korea. J. Gastric Cancer 11:135–140.

Surh, J., Y.H.L. Kim, and H. Kwon. 2008. Korean fermented foods, Kimchi and Doenjang. In: Handbook of Fermented Functional Foods. E.R. Farnworth, ed. Boca Raton, FL, pp. 333–351.

Wie, G.-A., Y.-A. Cho, H.-Hh. Kang, K.A. Ryu, M.-K. Yoo, J. Kim, S. Shin, O.K. Chun, and H. Joung. 2017. Identification of major dietary patterns in Korean adults and their association with cancer risk in the Cancer Screening Examination Cohort. Eur. J. Clin. Nutr. 71:1223–1229.

Woo, H.D., S. Park, K. Oh, H.J. Kim, H.R. Shin, H.K. Moon, and J. Kim. 2014. Diet and cancer risk in the Korean population: a meta-analysis. Asian Pac. J. Cancer Prev. 15:8509–8519.

PICKLE PRODUCTION PRINCIPLES

In a very general sense, pickles refer to any vegetable (or fruit) that is preserved by salt or acid. Certainly, the vegetable most often associated with pickles, at least in the US, is the cucumber. The cucumber, however, is not native to the US, rather it originated in southern Asia. However, it is now cultivated throughout the world, with China, the leading producer (according to FAO United Nations statistics). In 2015, the US grew nearly 1 billion kg of cucumbers; most are consumed as fresh, with about 40% being used for pickles.

As previously noted, not all pickles are fermented. In fact, only a third of the pickles produced in the United States undergo a lactic acid fermentation. Rather, acetic acid can be added directly as the pickling acid, omitting the fermentation step. The pickle slice on the top of a fast-food hamburger is not the fermented type. Indeed, fermented pickles can be difficult to find in US supermarkets.

Pickles and pickling technology have a long and rich history. The cucumber was brought from India to the Middle East about 4000 years ago, and cured versions were eaten at least 3000 years ago. Cleopatra reportedly endorsed pickle consumption, claiming they were responsible (in part) for her beauty. The ancient Roman historian and natural scientist Pliny the Elder and his contemporaries, Roman emperors Julius Caesar and Tiberius, were all fond of pickles. Columbus introduced pickles to the Americas, eventually leading to the origins of a pickle industry on the Lower East Side of New York City. American founding fathers George Washington, John Adams, and Thomas Jefferson reportedly derived inspiration from the pickle.

In the United States, pickles are generally divided into three different groups, based on their means of manufacture. Fresh-packed pickles are simply cucumbers that are packed in jars, covered with vinegar and other flavorings, and pasteurized by heat. They have a long shelf-life, even at room temperature. Fresh packed pickles are crisp, mildly acidic, and are the most popular. Refrigerated pickles are also made by packing cucumbers into jars with vinegar and various flavorings, but they are not heated. Instead these pickles are maintained at refrigeration temperatures, giving them a crisp, crunchy texture and bright green color. Because refrigerated pickles are not heat treated, they have a shorter shelf-life than fresh-packed pickles. Some products contain sodium benzoate as an antimicrobial preservative. One of the main advantages of both fresh-packed and refrigerated-style pickles is that their manufacture is relatively fast and easy and requires few steps (Figure 8.4). Consumers have probably also grown accustomed to these products.

The only pickles that are fermented are those referred to as salt-stock or genuine pickles. They may also be referred to as processed, although this may be somewhat confusing since non-fermented pickles can be made into relishes and other processed pickle products. While fermented pickles are not as popular as non-fermented versions in the US, they still have about one-third of the pickle market. Fermented pickles have a distinctly different flavor and texture compared to fresh-packed or refrigerated pickles, which may account for different consumer preferences. Although they take much longer to make, fermented or processed pickles also have a very long shelf-life, about two years. Details regarding their manufacture and the fermentation process are described below.

It should also be noted that within the three groups of pickles described above, pickles can be further distinguished based on the types of spices, herbs and flavoring agents used, the size or type of cucumber (gherkins and midgets), and the form or shape of the pickle (i.e., whole, spear, slice, etc.). For example, dill pickles, the most popular, refer to any type of

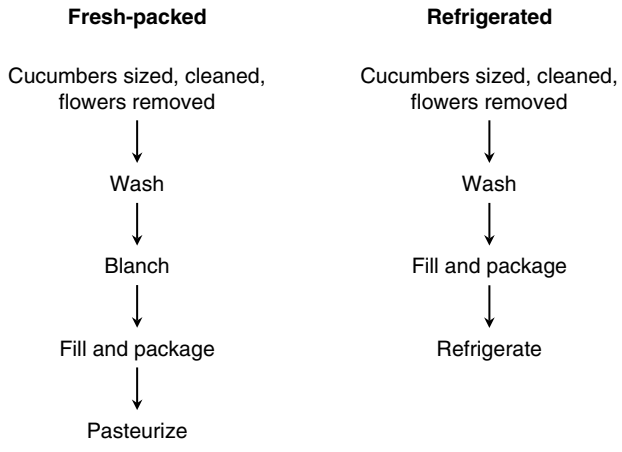


Figure 8.4 Non-fermented pickles and their manufacture. Adapted from Harris, 1998.

pickle to which dill weed (either the seed or oil) is added. If the dill pickles are labeled as genuine dill, it means the pickles are of the processed type (i.e., fermented) and are dill-flavored. Otherwise, dill pickles are usually made from fresh-packed or refrigerated pickles. Other common flavored pickle types include sweet, bread-and-butter, kosher (style), and garlic.

Manufacture of fermented pickles

The actual process steps used for the manufacture of fermented pickles are similar to those used for making sauerkraut. Both rely on salt, oxygen exclusion, temperature, and anaerobiosis to provide the appropriate environmental conditions necessary to select for growth of naturally-occurring lactic acid bacteria. There are, however, several differences between pickle and sauerkraut fermentations. First, a brine, rather than dry salt, is used for pickle fermentations. In addition, the salt concentration is higher than that used for sauerkraut, in part because the water in the cucumbers dilutes the salt level in the brine. Still, the final salt concentration will be higher resulting in development of a less diverse microbiota. Finally, the pickle fermentation process, unlike sauerkraut, is amenable to the use of pure starter cultures and a more controlled fermentation. Indeed, such cultures are now available and some pickle manufacturers have adopted controlled fermentation processes (Box 8.3).

The manufacture of fermented pickles starts with selection and sorting of cucumbers (Figure 8.5). Cultivars for pickles are different from those intended for the fresh market. Furthermore, only small or immature cucumbers, harvested when they are green and firm, are used for pickles. They are then washed, sorted, and transferred to tanks, and a brine solution is added.

The brine typically contains at least 5% salt (or about 20 salometer, where 100 salometer = 26% salt). Because the cucumbers-to-brine ratio is nearly 1:1, the actual salt concentration is actually less. For so-called salt stock pickles, which may be held in bulk for long periods, the initial brine may contain 7% to 8% salt, which is followed by the addition of more salt to raise the total salt concentration to above 12%. For genuine dill-type pickles, the brine concentration is usually between 7.5% and 8.5%. Dill weed is also added, usually in the seed or oil form.

Box 8.3 Starter cultures and fermented vegetables

The modern manufacture of most fermented vegetables, in contrast to cheese, sausage, and other fermented food products, still relies on a natural fermentation. In large part, this is because vegetable fermentations occur as a succession, and duplicating this process with "controlled fermentations" using starter cultures has not been a viable option. Also, vegetable fermentations are often conducted in less than aseptic conditions, so adding a culture to a raw material comprised of a complex, well-populated background microbiota is unlikely to be very effective. Finally, paying for cultures to perform a step that ordinarily costs the manufacturer nothing makes little economic sense.

Yet, for all of the same reasons that eventually drove other industries to adopt pure starter culture technology (i.e., consistency, improved flavor, control, safety, and convenience), the fermented vegetable industries have indeed developed manufacturing procedures that depend on starter cultures, rather than the natural microbes, to perform the fermentation (Lee et al., 2015). Research on pure culture technology for fermented vegetables actually began nearly 60 years ago, and cultures were eventually developed in the 1960s (Daeschel and Fleming, 1987). Despite the availability of these cultures, however, they have not been widely used.

In the past two decades, other factors have provided additional motivation for the use of starter cultures. As mentioned previously, one particularly relevant issue relates to the large volumes of high salt brines that are generated by pickle and olive fermentations. These salt solutions create significant environmental problems. The most obvious way to reduce the discharge of this material into the environment is to use less salt. However, since salt provides the major means for controlling the microbiota, conducting a natural fermentation at low-salt concentrations is a risky proposition. In contrast, less salt could be used if the background microbiota was controlled by other means (see below), and a starter culture was used instead to dominate the environment and to carry out the fermentation.

The first requirement for performing a controlled fermentation is to remove and/or inactivate the endogenous microbiota. This can be done with chemical agents that kill organisms at the surface. Specifically, the raw product is washed first with dilute chlorine solutions, then with acetic acid. In reality, chemical pasteurization is possible only for cucumbers and olives and not for shredded cabbage due to surface area considerations. Although olives can tolerate a modest heat treatment, cabbage and cucumbers suffer severe texture defects if heated. Finally, a nitrogen purge drives out air and creates anaerobic conditions, and an acetate buffered brine is then added, followed by the starter culture.

The organisms that are currently available or are being considered for use as starter cultures include many of the same species ordinarily isolated from vegetable fermentations, e.g., *Lactobacillus plantarum*, *Leuconostoc mesenteroides, Pediococcus acidilactici*, *Lactobacillus pentosus*, and *Lactobacillus brevis.* Strain selection, however, is necessary and must be based on the specific application and desired characteristics of the particular fermented food (Table 8.3.1). For example, strains to be used as starter cultures for fermented olives must resist the antimicrobial phenolic compounds ordinarily present in olives.

Table 8.3.1 Desirable properties of starter cultures for fermented vegetables.

- Minimum nutritional requirements
- Able to grow at low temperatures
- Able to ferment diverse carbohydrate substrates
- Able to compete against wide array of organisms
- Able to produce desirable flavor
- Rapid growth and acid production
- Tolerant to acids and low pH
- Tolerant to salt
- Tolerant to antimicrobial phenolics
- Resistant to bacteriophage
- Non-pectinolytic
- Unable to produce dextrans or other polysaccharides
- Unable to produce biogenic amines
- Minimum loss of viability during storage

As noted above, another inherent problem in controlled fermentation technology is the difficulty in establishing a microbial succession, such that a heterofermentative phase always precedes the homofermentative phase. That is, how could the initiating organism, *L. mesenteroide*s, and one of the late-fermenting organisms, *L. plantarum,* both be added at the outset of a vegetable fermentation, yet have conditions controlled such that growth of *L. plantarum* is delayed until the later stages of the fermentation? Indeed, the absence of methods for reducing initial microbial levels in the raw materials is one of several challenges recently noted by Lee et al. (2015). They also noted that making kimchi on an industrial scale is limited by (a) lack of commercial starter cultures; (b) increases in the price due to starters; (c) the absence of safety assessment guidelines.

Ultimately, interest in the use of starter cultures for the manufacture of fermented vegetables is likely to increase as the size of the production facilities and the demand for speed, efficiency, and throughput both increase. In addition, the starter culture industry is now able to develop strains that have specific physiological properties, satisfy specific performance characteristics, are stable during storage, and are easy to use. Recent reports provide examples of the successful application of this technology (Campus et al., 2017; Xionga et al., 2014).

References

Campus, M., E. Cauli, E. Scano, F. Piras, R. Comunian, A. Paba, E. Daga, R. Di Salvo, P. Sedda, A. Angioni, and R. Zurru. 2017. Towards controlled fermentation of table olives: LAB starter driven process in an automatic pilot processing plant. Food Bioprocess. Technol. 10:1063–1073.

Daeschel, M.A. and H.P. Fleming. 1987. Achieving pure culture cucumber fermentations: a review. In: G. Pierce, ed. Developments in industrial microbiology. Society for Industrial Microbiology, Arlington, VA, pp. 141–148.

Lee, M.-E., J.-Y. Jang, J.-H. Lee, H.-W. Park, H.-J. Choi, and T.-W. Kim. 2015. Starter cultures for kimchi fermentation. J. Microbiol. Biotechnol. 25:559–568.

Xiong, T., F. Peng, Y. Liu, Y. Deng, X. Wang, and M. Xie. 2014. Fermentation of Chinese sauerkraut in pure culture and binary co-culture with *Leuconostoc mesenteroides* and *Lactobacillus plantarum.* LWT – Food Sci. Tech 59:713–717.

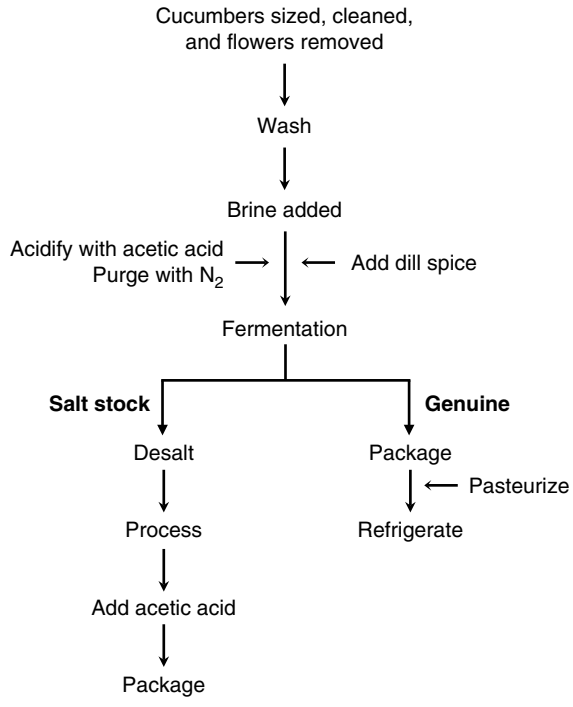


Figure 8.5 Manufacture of fermented pickles. Adapted from Harris, 1998.

Care must be taken when weighing down the pickles, because the buoyancy of the cucumbers may cause those at the top to become damaged. The large tanks used by large pickle manufacturers are usually located outdoors, and temperatures may vary between 15°C to 30°C. The lower the temperature, the longer it takes to complete the fermentation. Thus, in Michigan (a large Northern producer of pickles), fermentation may require up to two months, whereas in North Carolina (the main southern producer), fermentations may be complete in three weeks. Some producers perform the fermentation in the absence of covers, exploiting the sun's ultraviolet radiation as a means of controlling surface yeasts. At the end of the fermentation, the pH will be about 3.5, with acidities between 0.6% and 1.2% (as lactic).

Pickle fermentation

As noted above, the high salt concentrations used in pickle manufacturing cause the fermentation to proceed quite differently from that in sauerkraut. Only those pickles made using brines at less than 5% salt will allow for growth of *L. mesenteroides*. Although heterofermentative fermentations may promote more diverse flavor development, the formation of CO_2 is undesirable, because it may lead to bloater or floater defects (see below). Moreover, low salt brines may also permit growth of unwanted members of the natural microbiota, including coliforms, *Bacillus*, *Pseudomonas,* and *Flavobacterium*. At salt concentrations between 5% and 8%, growth of *Leuconostoc* is inhibited and instead

the fermentation is initiated and dominated by lactobacilli and pediococci, and *L. plantarum*, *L. pentosus*, and *P. pentosaceus*, in particular.

Pickle fermentation brines typically contain high concentrations of salt and organic acids and have a pH less than 4.0. These conditions are especially inhibitory to coliforms, pseudomonads, bacilli, clostridia, and other non-lactic acid bacteria that would otherwise cause flavor and texture problems. This environment, in fact, is hard even on lactic acid bacteria. However, the latter have evolved sophisticated physiological systems that enable them to survive in under very uncomfortable circumstances (Box 8.4).

Box 8.4 Life in a pickle: How lactic acid bacteria deal with acids, salts, and other stresses

The successful initiation of vegetable fermentations depends on modification of the environment such that most of the resident microorganisms are unable to grow. This is accomplished by adding salt, excluding oxygen, and maintaining a somewhat cool temperature. These conditions select for heterofermentative lactic acid bacteria that produce lactic and acetic acids and CO_2. The formation of these end-products causes the pH and Eh to decrease, providing additional hurdles against most remaining neutrophilic, aerobic competitors.

The net effect of these environmental changes is the rather broad inhibition of resident pseudomonads, clostridia, enteric bacteria, fungi, and other undesirable organisms. However, these same conditions can also impose significant challenges for the lactobacilli and other lactic acid bacteria that ultimately remain and whose growth and fermentative activity are to be encouraged (Papadimitriou et al., 2016). Indeed, low pH, high salt, high osmolality, and the accumulation of organic acids can have serious detrimental effects on cell metabolism, growth, and viability. Thus, how the lactic acid bacteria cope with these so-called "self-imposed" stresses is of practical importance (Papadimitriou et al., 2016).

Lactic acid bacteria are prolific producers of lactic acid (no surprise there) and can tolerate high lactic acid concentrations (>2% or 0.22 M) and low pH (<3.5), more so than most of their competitors. At least several physiological strategies have been described that enable these bacteria to tolerate high acid, low pH conditions.

First, recall that one of the most important functions of the bacterial cell envelope (including both the wall and membrane) is to serve as a barrier separating the intracellular environment from the extracellular medium. Thus, even as acids accumulate in the medium (or brine) and the pH decreases, the bacteria are able to maintain a much higher intracellular pH, at least for a time. Indeed, even when the medium pH is as low as 3.0, the cytoplasmic pH (the relevant pH for the cell's metabolic and reproductive machinery) can be one or more pH units higher. For example, when held in medium acidified to pH 3.0 with HCl, *Lactobacillus plantarum* and *Leuconostoc mesenteroides* maintained pH gradients of 1.3–1.5 (McDonald et al., 1990). However, in the presence of lactate or acetate, much lower pH gradients were maintained. This is because organic acids diffuse across the cytoplasmic membrane at low pH (based on their pKa), release protons, and ultimately cause acidification of the intracellular medium. The collapse of the pH gradient spells real trouble for the cells, as enzymes, nucleic acid replication, ATP generation, and other essential functions would be inhibited.

If maintenance of a pH gradient is important for acid tolerance, then the next question to ask is how such a gradient can be maintained. That is, how does the cell extrude the pH lowering protons that accumulate inside the cell?

Several mechanisms exist to maintain pH homeostasis. Perhaps the most important is the proton-translocating F_0F_1-ATPase (or H^+-ATPase). This multi-subunit, integral membrane-associated enzyme pumps protons from the inside to the outside using ATP hydrolysis as the energy source (Figure 8.4.1A). This enzyme is widely conserved in bacteria (in fact, throughout nature), but the specific properties of enzymes from different species show considerable variation. Thus, the H^+-ATPases from lactobacilli have a low pH optima, accounting, in large part, for the ability of these bacteria to tolerate low pH relative to less tolerant lactic acid bacteria.

Although the H^+-ATPase system is the primary means by which lactic acid bacteria maintain pH homeostasis, other systems also exist (Figure 8.4.1A). For example, deamination of the amino acid arginine releases ammonia, which raises the pH. Decarboxylation of malic acid, commonly present in fermented vegetables, also increases the pH by conversion of a dicarboxylic acid to a monocarboxylic acid. Accumulation of histidine was reported to increase acid resistance in *Lactobacillus casei* (Broadbent et al., 2010). One specific enzyme induced by low pH in lactic acid bacteria is glutamate decarboxylase. This enzyme, encoded by *gadAB* genes in *Lactobacillus brevis*, converts glutamic acid into gamma-aminobutyric acid (Wu et al., 2017). The reaction also yields carbon dioxide, and importantly consumes an intracellular proton, raising the intracellular pH.

When lactobacilli and other lactic acid bacteria are exposed to low pH, a wide array of genes are induced (Van de Guchte et al., 2002). Collectively, this adaptation to low pH is referred to as the acid tolerance response. This response can be exploited to enhance tolerance of strains used in kimchi and other fermented vegetables such that survival at low pH is increased by several orders of magnitude (Kim et al., 2014).

The molecular basis for acid tolerance has also been investigated based on proteomics approaches (Huang et al., 2011; Hamon et al., 2013). In general, induced proteins encode for repair systems, stress proteins, and chaperones. The latter are important for folding and unfolding of proteins previously affected by low pH, osmotic pressure, or temperature (Sugimotoa et al., 2008). Not surprisingly, many of these proteins are also involved in the machinery used by the cell to deal with other physical or chemical stresses. Thus, the acid tolerance response may not only protect the cell against low pH, but also heat and oxidative stress.

In vegetable fermentations, the other important stresses encountered by lactic acid bacteria are high salt concentrations and high osmotic pressures. Salt concentrations in sauerkraut brines are around 0.4 M, giving an osmolality of about 0.8 Osm. Pickle and olive brines may contain more than 1.0 M salt, or osmolalities above 2 Osm. Salt is an extremely effective antimicrobial agent due to its ability to draw water from the cytoplasm, thereby causing the cell to become dehydrated, lose turgor pressure, and eventually plasmolyze (and die!). The ability of lactic acid bacteria to tolerate high salt conditions varies (just as it did for acid tolerance), depending on the organism. Given that *L. plantarum* usually dominates high salt fermentations, it should be no surprise

that this organism has also evolved physiological and genetic mechanisms that make it salt- and osmotolerant.

Like acid tolerance, the salt tolerance system depends on the activity of membrane pumps. However, in the latter case, the pumps are actually transport systems that take up a special class of molecules called compatible solutes. By accumulating these non-toxic solutes inside the cytoplasm to high concentrations, cell water is retained and osmotic homeostasis is maintained.

Compatible solutes are also referred to as osmoprotectants because they not only maintain osmotic balance, they also protect enzymes, proteins, and other macromolecules from dehydration and misfolding. Among the osmoprotectants accumulated by

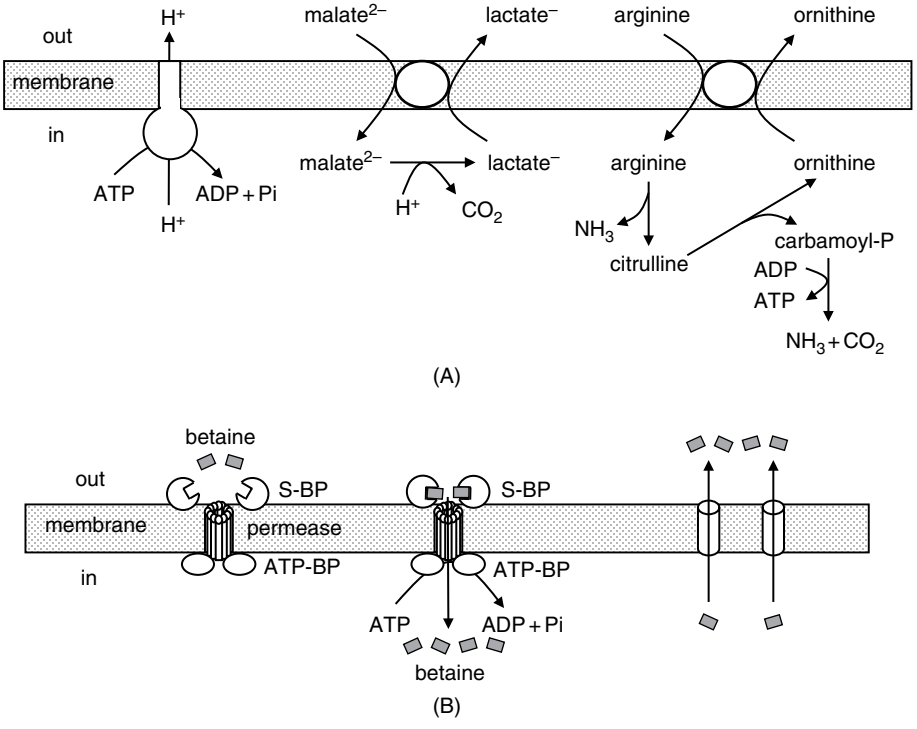


Figure 8.4.1 pH and osmotic homeostastis in *Lactobacillus plantarum*. Shown in Panel A are three main systems whose function is to maintain pH homeostasis in *L. plantarum*. The F_0F_1-ATPase (left) is a primary proton pump that extrudes protons from the inside to the outside, using ATP as the energy source. In contrast, the malate and arginine systems rely on product efflux to drive uptake (no energy is required). In the malolactate system (center), proton consumption de-acidifies the medium and raises the pH. In the arginine diiminase system (right), medium pH is raised by virtue of the two molecules of NH_3 that are released per mole of arginine.

Panel B shows the QacT system responsible for osmotic homeostasis in *L. plantarum*. The components of this putative opuABCD-encoded system (left) include membrane-associated substrate-binding proteins (S-BP), a betaine permease, and ATP-binding proteins (ATP-BP). When the osmotic pressure is high, betaine is bound by the S-BP and taken up by the permease (center). Transport is driven by an ATPase following ATP-binding. If the osmotic pressure is reduced, accumulated betaine is effluxed via membrane channels (right).

L. plantarum, the quaternary amine, glycine betaine (or simply betaine) is the most effective. Potassium ion, glutamate, and proline are also accumulated, but to lower concentrations, at least in lactobacilli. In *L. plantarum*, betaine is preferentially transported by the quaternary ammonium compound or QacT transport system (Glaasker et al., 1998). This transporter is a high affinity, ATP-dependent system whose activity is stimulated by high osmotic pressure (Figure 8.4.1B).

In contrast, at low osmotic pressure, the efflux reaction is activated, and pre-accumulated betaine is released back into the medium. In *L. plantarum*, the expression of several genes involved in betaine transport increased as the salt concentration was raised from 0 to 9% (Wu et al., 2016). The natural source of betaine, it is worth noting, is plant material, so perhaps it is no coincidence that *L. plantarum* is unable to synthesize betaine *de novo* and instead relies on a transport system to acquire it from the environment.

References

Broadbent, J.R., R.L. Larsen, V. Deibel, and J.L. Steele. 2010. Physiological and transcriptional response of *Lactobacillus casei* ATCC 334 to acid stress. J. Bacteriol. 192:2445–2458.

Glaasker, E., E.H. Heuberger, W.N. Konings, and B. Poolman. 1998. Mechanism of osmotic activation of the quaternary ammonium compound transporter (QacT) of *Lactobacillus plantarum*. J. Bacteriol. 180:5540–5546.

Hamon, E., P. Horvatovich, E. Marchioni, D. Aoude-Werner, and S. Ennahar. 2013. Investigation of potential markers of acid resistance in *Lactobacillus plantarum* by comparative proteomics. J. Appl. Microbiol. 116:134–144.

Huang, G., C. Li, and Y. Cao. 2011. Proteomic analysis of differentially expressed proteins in *Lactobacillus brevis* NCL912 under acid stress. FEMS Microbiol. Lett. 318:177–182.

Kim, J.E., H.-J. Eom, L. Li, K.-S. Yoo, and N.S. Han. 2014. Induction of the acid tolerance response in *Leuconostoc mesenteroides* ATCC 8293 by pre-adaptation in acidic condition. Food Sci. Biotechnol. 23:221–224.

McDonald, L.C., H.P. Fleming, and H.M. Hassan. 1990. Acid tolerance of *Leuconostoc mesenteroides* and *Lactobacillus plantarum*. Appl. Environ. Microbiol. 56:2120–2124.

Papadimitriou, K., Á. Alegría, P.A. Bron, M. de Angelis, M. Gobbetti, M. Kleerebezem, J.A. Lemos, D.M. Linares, P. Ross, C. Stanton, F. Turroni, D. van Sinderen, P. Varmanen, M. Ventura, M. Zúñiga, E. Tsakalidou, and J. Kok. 2016. Stress physiology of lactic acid bacteria. Microbiol. Mol. Biol. Rev. 80:837–890.

Sugimoto, S., A.-A.-Mahin, and K. Sonomoto. 2008. Molecular chaperones in lactic acid bacteria: physiological consequences and biochemical properties. J. Biosci. Bioeng. 106:324–336.

van de Guchte, M., P. Serror, C. Chervaux, T. Smokvina, S.D. Ehrlich, and E. Maguin. 2002. Stress responses in lactic acid bacteria. Antonic Leeuwenhoek. 82:187–216.

Wu, Q., H.M. Tun, Y.-S. Law, E. Khafipour, and N.P. Shah. 2017. Common distribution of *gad* operon in *Lactobacillus brevis* and its GadA contributes to efficient GABA synthesis toward cytosolic near-neutral pH. Front. Microbiol. 8:206.

Wu, R., X. Song, Q. Liu, D. Ma, F. Xu, Q. Wang, X. Tang, and J. Wu. 2016. Gene expression of *Lactobacillus plantarum* FS5-5 in response to salt stress. Ann. Microbiol. 66:1181–1188.

After fermentation, salt stock pickles can be held indefinitely in the brine. However, these pickles cannot be eaten directly, but rather must be de-salted by transfer to water. After several changes (a process called refreshing), the salt concentration is reduced to about 4%. They are then used primarily for relishes and other processed pickle products.

Defects

Among the microbial defects that occur in pickles, the most common are floaters and bloaters (Table 8.4). The defect is caused by excessive gas pressure that subsequently results in internal cavity formation within the pickles. The CO_2 gas is mainly produced by heterofermentative lactic acid bacteria, although some CO_2 can be produced via the malolactic fermentation. Coliforms and yeasts may also be responsible for CO_2 production (which would mean there are additional problems as well). Floaters and bloaters can still be used for some processed products (i.e., relish), however, they cannot be used for the whole or sliced pickle market. The most common way to control or minimize this defect is to remove dissolved CO_2 by flushing or purging the brine with nitrogen gas. Indeed, it has now become a rather standard practice to routinely purge fermentation tanks.

Destruction and softening of the pickle surface tissue is another serious defect. When this happens, the pickle loses its crispness and crunch and becomes slippery and soft. These pickles are neither edible nor can anything be done to salvage them. The defect is caused by pectinolytic enzymes produced by microorganisms that are part of the natural cucumber microbiota. The organisms responsible include mostly filamentous fungi, especially species of *Penicillium*, *Fusarium*, *Alternaria*, *Ascochyta*, and *Cladosporium*. Pectins are complex heteropolysaccharides that serve as the main structural component of plant cell walls. Their hydrolysis requires the concerted action of three different enzymes – pectin methylesterase, polygalacturonase, and polygalacturonate lyase. Although *Penicillium* and other fungi are capable of secreting these enzymes, they may also be produced by various yeasts, as well as by the cucumber flowers. Bacteria, however, do not appear to be a major source of pectin-degrading enzymes.

Another way to reduce problems caused by tissue softening is to add calcium chloride (about 1000–1500 ppm) to the brine. Calcium acts by neutralizing negative charges within the pectin polymer and by enhancing structural integrity of the pectin-containing cell walls. Recently, calcium has also been used to reduce the amount of sodium (i.e., salt) necessary to form a brine and initiate the fermentation.

The fungi responsible for the softening defect gain entry into the fermentation tank via their association with cucumber flowers. Thus, excluding these constituents from the fermentation may reduce or minimize this defect. Other preventative measures include maintaining sufficient salt concentrations, acidity, anaerobiosis, and temperature. Another method used to control adventitious microorganisms and to ensure a prompt fermentation (especially when the salt concentration is at the low end) is to partially acidify the cucumbers with acetic acid to about pH 4.5 to 5.0. This practice of chemical pasteurization is also used when starter cultures and controlled fermentation methods are used to produce pickles.

OLIVES: PRODUCTS AND MARKETS

Olives refer not only to the usually salty, acidic product known as table olives, but to the fruit from which they are made. As one might expect, the main use of raw olives is for olive oil. More than 90% of the total worldwide olive production is used for oil and only 7% to 10% are consumed as table olives.

Olive trees are native to the Middle East and, due to their hardy nature (some trees live as long as 1000 years), olives have long been a major agricultural crop throughout the region.

Olives and olive oil are among the most frequently mentioned foods in the Bible. Olive production subsequently spread from the Middle East across the Mediterranean, to Greece, Italy, France, Spain, and Northern Africa. Olives were not introduced to the Americas until the eighteenth century.

Currently (based on 2015–16 data from the International Olive Oil Council), five countries – Spain, Egypt, Turkey, Algeria, and Greece, are responsible for more than 70% of the total world-wide olive production (about 2.65 billion kg or 5.8 billion pounds). Not surprisingly, consumption of table olives in many of these countries is also high. For example, in Algeria and Turkey, per capita consumption is about 6.1 and 4.2 kg per person per year, respectively.

In contrast, the United States accounts for about 2% of total world table olive production, with nearly all 54 million kg (120 million pounds) being produced in California. Despite their popularity on pizzas and in cocktails, consumption in the US is less than 1 kg per person per year (Table 8.5). In fact, while table olive consumption has increased by about 40% in the past ten years (2005–2015), US consumption has stayed nearly the same. Moreover, while most of the olives grown in California had mainly been used in the manufacture of table olives, as of 2015, more than half of the olives are now used for olive oil.

Finally, as noted above, not all table olives are fermented. Most of the table olives produced and consumed in the United States are not fermented. More than 70% of the US olive market consists of olives that are simply brined and canned (hence, this type is referred to as California- or American-style olives). In contrast, fermented olives are much more common in Europe and other olive-producing regions.

There is also a small (but dedicated) market for fresh or raw tree-ripened olives. The famous Provence-style olives of France are this type. More than 30 cultivars are grown worldwide; however, fermented olives are usually produced using one of eight main varieties: Manzanillo, Gordal, Picholine, Rubra, Mission, Sevillano, Ascolano, and Barouni. These cultivars differ widely with respect to their composition, size, texture, color, and flavor. Olive properties also change during ripening; most olives are picked when they are still green, straw-yellow, or cherry-red, even if later they acquire a much darker appearance. Ultimately, their selection for table olive production is based on the style of the olive produced (discussed below).

Table 8.5 Leading producers and consumers of table olives.[1]

Country	Production (1000 metric tons)	Country	Consumption (kg per person per year)
Spain	602	Albania	10.7
Egypt	470	Algeria	6.1
Turkey	397	Syria	5.0
Algeria	234	Turkey	4.2
Greece	167	Spain	4.1
Syria	150	Lebanon	4.0
Morocco	120	Egypt	3.7
Iran	69	Jordan	3.3
Italy	66	Cyprus	3.1
USA	54	Israel	2.6

[1] Data (from 2015–16) courtesy of the International Olive Oil Council.

Composition

Phenolic and polyphenolic compounds are common to all olives. Some of these phenolic compounds are responsible for the color of olives, both as they ripen and during processing. Other phenolic compounds have antimicrobial activity against a wide variety of microorganisms (including lactic acid bacteria). The phenol-containing fraction of olives (and olive oil) may also have positive dietary and nutritional benefits, due to their antioxidant activities (Box 8.5). Perhaps the most important and certainly most abundant phenol is oleuropein (part of a class of compounds called secoiridoids).

Structurally, oleuropein is a glucoside ester of 3,4-dihydroxytyrosol and elenolic acid (Figure 8.6). Glucosidic phenols, and oleuropein in particular, are important in olives owing to the pronounced bitter flavor they impart. Some olive varieties can contain as much as 14%

Box 8.5 Olives, Mediterranean diet, and bioactive molecules

The Mediterranean diet has long been promoted as a model for healthy eating (Willet et al., 1995). This diet takes its name from the foods normally consumed by French, Greek, Italian, Spanish, and Middle East and North African populations that reside around the Mediterranean Sea. The diet advocates high consumption of fruits and vegetables, grains and pasta, legumes, olive oil, and fish; moderate intake of wine and dairy products; and low intake of meat products. Fermented foods, including olives, are also associated with this cuisine. The epidemiological association of this diet with reduced risks of cancer and heart disease and total mortality is highly significant (Martínez-González et al., 201; Sofi et al., 2016; Tong et al., 2016).

Perhaps the most prominent foods associated with the Mediterranean diet, and among those thought to be responsible for many of the health benefits, are olives and olive oil. Studies indicate that the phenolic fraction of olive and olive oil components is particularly important (Bulotta et al., 2014). As previously noted, phenolic compounds confer bitterness to olives (e.g., oleuropein), are involved in color development, and have antimicrobial activity. It now appears that some of these same phenolic compounds also have pharmacological activity and may be responsible, in part, for the health-promoting properties of the Mediterranean diet (Boskou, 2016; Bulotta et al., 2014).

The main biological activities associated with oleuropein, hydroxytyrosol, tryosol, verbascoside, and other phenolic compounds found in olives and olive oil are related to their antioxidant properties (Serreli et al., 2017). They have been found to inhibit low-density lipoprotein (LDL) oxidation and accumulation of oxidized end products. Oxidation of lipoprotein and other lipids is considered to be an important step in initiating coronary heart disease. Other cardioactive effects associated with these phenols include anti-arrhythmic and cardioprotective activities. Olive phenols have also been suggested to protect human cells against oxidative stress and membrane injury (Serreli et al., 2017). In addition, oxidative damage to DNA may be prevented by the free radical scavenging activity of olive phenols.

Most table olives are treated with lye as part of the de-bittering process (the exception being Greek-style, naturally black olives). This process hydrolyzes much of the bitter oleuropein and verbascoside, although appreciable amounts of the

hydrolysis products, including the glucosides and biophenols may remain (Boskou et al., 2015). Another group of bioactive molecules found in olives are the triterpenic acids. Two of these, maslinic and oleanolic acids are abundant in olives, but are also degraded by alkaline treatment (Alexandraki et al., 2014). Thus, alternative methods to debitter olives have been developed and rely on reducing the extent of alkali treatments (Romero et al., 2016) or on the use of "natural" processing methods (Ramírez et al., 2016). The latter approach relies on the natural enzymatic activities of the olives and requires more time, but yields olives with high phenol concentrations.

References

Alexandraki, V., M. Georgalaki, K. Papadimitriou, R. Anastasiou, G. Zoumpopoulou, I. Chatzipavlidis, M. Papadelli, N. Vallis, K. Moschochoritis, and E. Tsakalidou. 2014. Determination of triterpenic acids in natural and alkaline-treated Greek table olives throughout the fermentation process. LWT – Food Sci. Tech. 58:609–613.

Boskou, D. 2016. Mediterranean diet food: strategies to preserve a healthy tradition. J. Exp. Food Chem. 1:1.

Boskou, D., S. Camposeo, and M.L. Clodoveo. 2015. Table olives as sources of bioactive compounds. In: D. Boskou, ed. Olive and Olive Oil Bioactive Constituents. AOCS Press, Urbana, IL, pp. 179–215.

Bulotta, S., M. Celano, S.M. Lepore, T. Montalcini, A. Pujia, and D. Russo. 2014. Beneficial effects of the olive oil phenolic components oleuropein and hydroxytyrosol: focus on protection against cardiovascular and metabolic diseases. J. Transl. Med. 12:219.

Martínez-González, M.A., J. Salas-Salvadó, R. Estruch, D. Corella, M. Fitó, and E. Ros. 2015. Benefits of the Mediterranean diet: Insights from the PREDIMED study. Prog. Cardiovasc. Dis. 58:50–60.

Romero, C., P. Garcia-Garcia, and M. Brenes. 2016. Enrichment of black ripe olives in bioactive compounds using a single alkali treatment. J. Food Sci. 81:C2686–C2691.

Ramírez, E., P. García, M. Brenes, and C. Romero. 2016. Evaluation of chemical components of debittered olives undergone preservation and polyphenol oxidation. Int. J. Food Sci. Technol. 51:1674–1679.

Serreli, G., A. Incani, A. Atzeri, A. Angioni, M. Campus, E. Cauli, R. Zurru, and M. Deiana. 2017. Antioxidant effect of natural table olives phenolic extract against oxidative stress and membrane damage in enterocyte-like cells. J. Food Sci. 82:380–385.

Sofi, F., A. Fabbri, and A. Casini. 2016. Inflammation and cardiovascular disease and protection by the Mediterranean diet. In: Mediterranean Diet. D.F. Romagnolo and O.I. Selmin, eds. Springer International Publishing, New York, pp. 89–96.

Tong, T.Y.N., N.J. Wareham, K.-T. Khaw, F. Imamura, and N.G. Forouhi. 2016. Prospective association of the Mediterranean diet with cardiovascular disease incidence and mortality and its population impact in a non-Mediterranean population: the EPIC-Norfolk study. BMC Med. 14:135.

Willett, W.C., F. Sacks, A. Trichopoulou, G. Drescher, A. Ferro-Luzzi, E. Helsing, and D. Trichopoulos. 1995. Mediterranean diet pyramid: a cultural model for healthy eating. Am. J. Clin. Nutr. 61:Suppl 6:S1402–S1406.

oleuropein (on a dry basis) during the early stages of growth, although most contain less than 6%. As the olives mature, the oleuropein concentration decreases, and at maturation, only about 1 mg to 2 mg per g of pulp is present. Still, between the remaining oleuropein and related derivatives, the olives are generally too bitter to consume. Thus, manufacturing processes for most olives, including some fermented varieties, include an enzyme-mediated

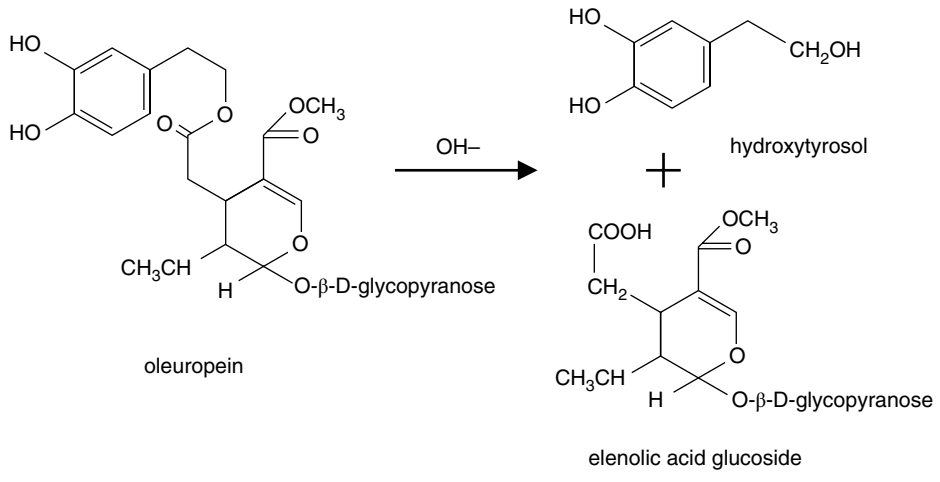


Figure 8.6 Alkaline hydrolysis of oleuropein.

step, or more commonly, a sodium hydroxide-treatment to hydrolyze and remove the bitter oleuropein fractions.

Olives, of course contain a significant amount of oil (12% to 30%, depending on cultivar). The fermentable carbohydrate concentration of ripe olives generally ranges from 2% to 5%. Most of this carbohydrate is glucose. However, when the olives are washed or treated to remove the bitter components, some of the sugars are also lost.

Manufacture of fermented olives

There are three main styles or types of table olives, based on their method of production (Figure 8.7). Spanish-style (or green Spanish-style) olives are treated with sodium hydroxide (lye) and fermented. Greek-style or naturally-black, ripe-style olives are not treated with lye, but are fermented. The fermentation for both types is mediated by the natural microbiota, much like that for other fermented vegetables (discussed below). The third type of olive is the ripe black- or green-style. They are lye-treated, but are not fermented. They may also undergo a special aeration treatment that promotes oxidation of pigments and conversion of a green color to black. This is the type referred to as California or American style olives. Their production, however is not limited to the US, as they are also produced in many other parts of the world.

Spanish-style

Spanish-style olives are harvested when the skin color is green or straw-yellow. They are then treated with a lye solution for four to 12 hours at 15°C to 20°C to de-bitter the olives via hydrolysis of oleuropein. The lye concentration may range from 0.5% to 3.5%, depending on the size and type of olive. Once the lye has penetrated to just outside the pit (about two-thirds of the way from the skin to the center), the olives are washed in one to three rinse cycles of water to remove the lye. The pH of the olives after washing should be less than 8.0. Although

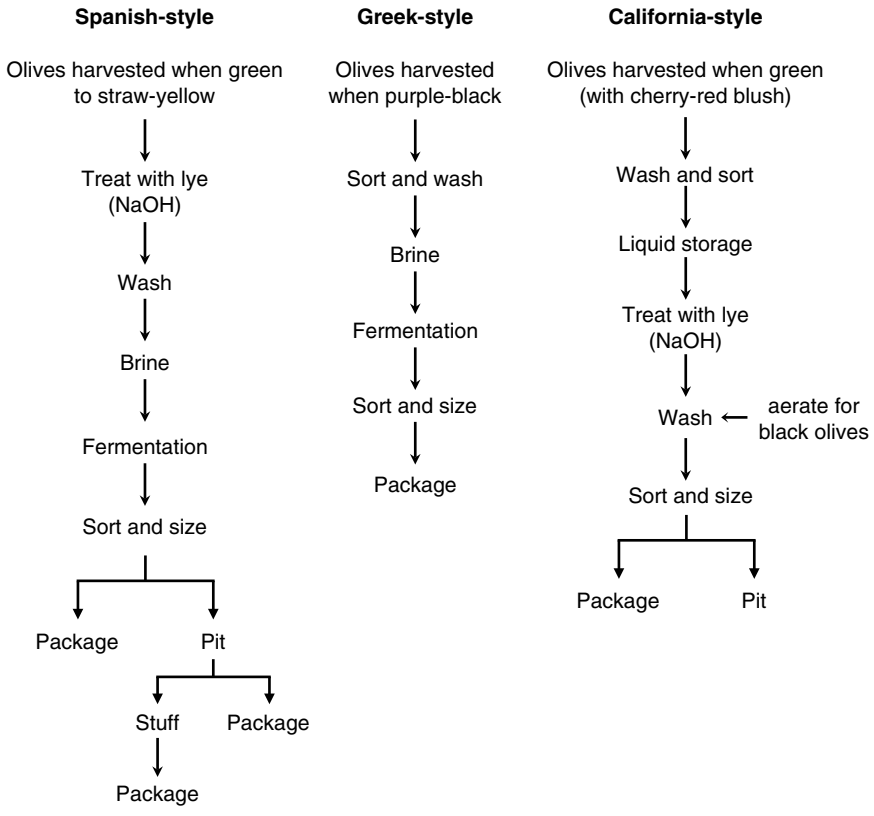


Figure 8.7 Manufacture of fermented and non-fermented olives. Adapted from Harris, 1998 and Romero et al., 2004.

there should be little residual lye remaining with the olives, a slight amount of bitterness may still be present, which is characteristic of these olives. Following the washing steps, the olives are moved to tanks or barrels and a brine of varying salt concentrations is added, at about a 1:1 volume ratio. For some olives, 10% to 15% salt brines are used (giving an actual concentration of 6% to 9%), whereas others start with lower salt brines (5% to 6%), and salt is added later to give comparable final concentrations. Glucose may be added to restore sugars lost during lye treatment and washing steps. The brined olives are subsequently held at 22°C to 26°C.

Although the endogenous microbial population is reduced by the lye and washing treatments, the olive production environment still contains a wide assortment of microorganisms. There is, in fact, an opportunity for growth of coliforms, *Pseudomonas*, *Bacillus*, *Clostridium* and other Gram-negative and Gram-positive bacteria during the first two to four days, especially when low salt brines are used, and before a lactic fermentation can begin in earnest. This first stage of the fermentation is then followed by a second stage, initiated primarily by *L. mesenteroides* and *Pediococcus* sp. Once the pH nears 5.0, non-lactic acid bacteria are inhibited, and the brine will be dominated by lactic acid bacteria. After two to three weeks, *L. plantarum*, *L. brevis, Lactobacillus fermentum*, *Lactobacillus pentosus*, and other lactobacilli displace the *Leuconostoc* population, and eventually *L. plantarum* dominates the third stage of the fermentation. The appearance of facultative yeasts at the end stage of the

fermentation, however, is not uncommon, nor undesirable, since they may produce ethanol, acetaldehyde, and other flavor compounds. In contrast, growth of *Propionibacterium acnes* is to be avoided, since this organism can raise the pH by fermenting lactic acid.

When the fermentation is complete, the final pH after three to four weeks (or longer for barrel-fermented olives) should be within a range of 3.5 to 4.2, with a titratable acidity (as lactic) of 0.8 to 1.0%. Following the fermentation, Spanish-style olives can be pitted, pitted and stuffed (e.g., with pimentos), or packed directly into jars. Pasteurization to promote extended shelf-life is optional.

Greek-style

There are several significant differences between Greek-style and other olive types. First, these olives are naturally black when they are harvested, in contrast to California-style black olives that rely on oxidation to generate black pigments. Second, Greek-style olives are not lye-treated, giving them a definite bitter flavor. Third, the fermentation is mediated not just by lactic acid bacteria, but also by yeast, non-lactic acid bacteria, and even fungi. Some of these non-lactic organisms (e.g., *Pseudomonas* and Enterobacteriaceae) may actually remain in the brine, albeit at low levels, for several weeks before they begin to decline. Thus, the fermentation end-products include not only lactic acid, but also acetic acid, citric acid, malic acid, CO_2, and ethanol. This mixed fermentation may result in a less acidic product with a final pH as high as 4.5 and an acidity less than 0.6%. Lower brine concentrations (5% to 10%) may contribute to the more diverse microbiota that develop in these olives.

Another type of fermented olive that is made in a very similar manner (i.e., no lye-treatment) is the Sicilian olive. The main difference between these olives and the Greek olives is that the Sicilian olives are green (like Spanish olives). Also, while lactobacilli are the main bacteria involved in the fermentation, the dominant species appears to be *Lactobacillus casei*, an organism not ordinarily associated with fermented olives.

California-style

California-style olives (sometimes referred to as black or green ripe olives) are the most popular olives consumed in North America. Both the black and green versions are produced from the same starting material: green olives (with a bit of cherry-red blush). For both types, the olives are lye-treated, as described above for Spanish olives, except that several applications are used. In the case of green olives, the lye is removed and the olives are washed in water, and then dilute brine is added. The olives are then canned (after a pitting step, if desired) and thermally processed as a low-acid canned food.

For black olives, the lye-treated olives are heavily aerated between the lye applications to promote darkening reactions. Aeration can be accomplished manually, by stirring, or mechanically, by direct injection of air into the tanks. The latter method is preferred because it is faster and reduces opportunities for spoilage. Air, or more specifically, oxygen, promotes a chemical oxidation reaction in which phenolic compounds are polymerized to form first brown, then black pigments. During air exposure, the concentration of two phenols, hydroxytyrosol and caffeic acid in particular, decreases, as the black color increases. Ionic iron can form complexes with the oxidized phenols. Thus, iron salts are usually added to these olives to fix the color and prevent fading.

Defects and spoilage

Olives are, generally, more susceptible to microbial spoilage than other fermented vegetables. Initially, there is a diverse microbiota present in raw olives that is well maintained during the early stages of the fermentation. Reflective of this heterogeneous mix, spoilage organisms include aerobic bacteria and fungi, facultative bacteria and yeasts, strict anaerobes, as well as spore-forming bacteria. If the lactic fermentation is delayed due to residual lye or limiting glucose, or if the pH remains high (i.e., above 4.5), organisms capable of causing spoilage may be given ample opportunity for growth and production of spoilage products.

Several of the spoilage defects that occur in olives are not unlike those that occur in pickles and other fermented vegetables (Table 8.4). Excessive gas production and tissue softening are major problems in olives, just as they are for pickles. In olive production, for example, gas produced by coliforms early in the fermentation can accumulate as gas pockets inside the olive surface, causing an unsightly blister-like appearance called "fish-eyes". Likewise, pectin hydrolysis by pectinolytic *Penicillium*, *Fusarium, Aspergillus*, and other fungi or, less frequently, by coliforms, results in a soft tissue defect.

Another defect that is mostly specific for olives is termed zapatera spoilage. This occurs late in the fermentation and is characterized by foul, fecal, cheesy-like odors. It is caused by *Clostridium sporogenes, Clostridium butyricum*, and other putrefactive clostridia whose presence in fermented foods is never a good thing. These bacteria, along with other anaerobes, produce butyric acid, sulfur dioxide, and other sulfur-containing compounds. Putrescine, cadaverine, and other putrefactive and malodorous end products may also be produced. *Propionibacterium* sp. are also associated with zapatera spoilage, although they may be indirectly involved. Specifically, growth of *Propionibacterium zeae* and other related species can occur at low salt concentrations (<5%), which leads to an increase in the brine pH (as lactic acid is consumed). If the pH is high enough, clostridia are no longer inhibited.

Control of spoilage organisms can be accomplished by making sure the salt concentration is sufficiently high (>7.5%) and the pH is sufficiently low (<4.0). Olives can be given a quick heat treatment or partially acidified with lactic acid (prior to fermentation) to reduce and control the background microbiota. Although seldom practiced, the addition of a lactic starter culture can provide additional assurance that a prompt fermentation will occur.

FERMENTED VEGETABLES AND FORMATION OF BIOGENIC AMINES

Nearly all fermented foods can potentially contain biogenic amines, and fermented vegetables are no exception. This is because the series of events that culminate in biogenic amine formation are essentially the same for fermented vegetables as they are for other fermented foods. Specifically, accumulation of amines occurs whenever there is an available pool of free amino acids and a source of amino acid decarboxylase enzymes. The amino acids are formed from protein in the food by the action of microbial proteinases and peptidases. The decarboxylases are also produced by microorganisms, including lactobacilli that may be present in fermented vegetables. Thus, the amount of biogenic amines that are actually produced depends on both the concentration of the amino acid substrates and the expression and activity of the relevant decarboxylases. The biogenic amines that have been found in fermented vegetables (mainly sauerkraut, kimchi, and fermented olives) include histamine

(from histidine), tyramine (from tyrosine), and putrescine and cadaverine (from lysine, arginine, and glutamine). In most cases, however, the concentrations that have been reported were less than the level ordinarily thought to cause food disease symptoms (1 g/kg).

BIBLIOGRAPHY

Breidt, F., R.F. McFeeters, I. Perez-Diaz, and C.-H. Lee. 2013. Fermented vegetables. In: Doyle, M.P. and R.L. Buchanan, eds. Food Microbiology: Fundamentals and Frontiers, 4th edn. ASM Press, Washington DC, pp. 841–855.

Buckenhueskes, H.J. 2015. Quality improvement and fermentation control in vegetables. In: Holzapfel, W., ed. Advances in Fermented Foods and Beverages. Woodhead Publishing, Cambridge, pp. 515–539.

Di Cagno, R., R. Coda, M. De Angelis, and M. Gobbetti. 2013. Exploitation of vegetables and fruits through lactic acid fermentation. Food Microbiol. 33:1–10.

Harris, L.J. 1998. The microbiology of vegetable fermentations. In: B.J.B. Wood, ed. Microbiology of Fermented Foods, Volume 1. Blackie Academic and Professional, Chapman and Hall, London, pp. 45–72.

Holzapfel, W., U. Schillinger, and H. Buckenhüskes. 2008. Sauerkraut. E.R. Farnworth, ed. Handbook of Fermented Functional Foods, 2nd edn. CRC Press, Boca Raton, FL.

Hurtado, A., C. Reguant, A. Bordons, and N. Rozès. 2012. Lactic acid bacteria from fermented table olives. Food Microbiol. 31:1–8.

Jung, J.Y., S.H. Lee, and C.O. Jeon. 2014. Kimchi microflora: history, current status, and perspectives for industrial kimchi production. Appl. Microbiol. Biotechnol. 98:2385–2393.

Leff, J.W. and Fierer, N. 2013. Bacterial communities associated with the surfaces of fresh fruits and vegetables. PLoS One 8, 59310.

Lu, Z., F. Breidt, V. Plengvidhya, and H.P. Fleming. 2003. Bacteriophage ecology in commercial sauerkraut fermentations. Appl. Environ. Microbiol. 69:3192–3202.

Mataragas, M., K. Rantsiou, and L. Cocolin. 2017. Impact of fermentation on the microbial ecology of foods. In: A. de Souza Sant'Ana, ed. Quantitative Microbiology in Food Processing: Modeling the Microbial Ecology. John Wiley, Oxford, pp. 226–240.

Nout, M.J. and Rombouts, F.M. 1992. Fermentative preservation of plant foods. J. Appl. Microbiol. 73:S136–S147.

Park, J. and H.-J. Lee. 2017. Shifts in kimchi consumption between 2005 and 2015 by region and income level in the Korean population: Korea National Health and Nutrition Examination Survey (2005, 2015). Korean J. Community Nutr. 22:145–158.

Pérez-Díaz, I.M., F. Breidt, R.W. Buescher, F.N. Arroyo-Lopez, R. Jimenez-Diaz, J. Bautista-Gallego, A. Garrido-Fernandez, S. Yoon, and S.D. Johanningsmeier. 2014. Fermented and acidified vegetables. In: F. Pouch Downes, K.A. Ito, eds. Compendium of Methods for the Microbiological Examination of Foods, 5th edn. American Public Health Association.

Pérez-Díaz, I.M., J. Hayes, E. Medina, K. Anekella, K. Daughtry, S. Dieck, M. Levi, R. Price, N. Butz, Z. Lue, and M.A. Azcarate-Peril. 2017. Reassessment of the succession of lactic acid bacteria in commercial cucumber fermentations and physiological and genomic features associated with their dominance. Food Microbiol. 63:217–227.

Saha, B.C. and F.M. Racine. 2010. Biotechnological production of mannitol and its application. Appl. Microbiol. Biotechnol. 86:1003–1015.

9 Bread

> So today I have already been to a bakery… the thing that I am obsessed with is carbohydrates. I feel that I'm now living in an age where there's the best bread we have ever had in the history of the world. There has never been more bread that is good out there. So it seems to me a shame not to eat some of it.
>
> Nora Ephron, American essayist and screenwriter, from a 2006 interview.

INTRODUCTION

The fermentation that occurs during bread manufacturing is different from most other food fermentations in several obvious, but important respects. First, the very purpose of the bread fermentation is not to extend the shelf-life of the raw materials, per se, but rather to convert the grain or wheat into a more functional and consumable form. In fact, in contrast to dairy, meat, vegetable, or wine fermentations, where the starting material is more perishable than the finished product, the raw material for bread-making, i.e., cereal grains, are better preserved than the bread that is ultimately produced. It is also interesting to note that in the bread fermentation, again in contrast to the lactic acid or ethanolic fermentations, essentially none of the primary fermentation end products remain in the food product.

Worldwide, bread manufacture occurs in the home, in small bakeries, and in large factories. Collectively, on a global basis, bread manufacturing is about a $200 billion industry. Despite its commercial and dietary importance, however, per capita consumption of bread was substantially higher a century ago than it is currently, especially in the US. At the end of the nineteenth century, for example, Americans consumed more than 100 kg of wheat flour (used mostly for bread) per person per year. Starting early in the 1900s, consumption of wheat began to decline steadily for nearly a hundred years, reaching an all-time low of just 50 kg per person per year in the 1960s. Although per capita grain consumption slowly increased somewhat, to nearly 70 kg in 1980 (Figure 9.1), it decreased again in 2000 to just under 65 kg, where it has remained. Several reasons likely account for this low level of wheat (and bread) consumption in the US. First, low-carbohydrate and gluten-free diets have become popular among many consumers for perceived health reasons. Similarly, consumer surveys indicate that more than one-third of Americans think that bread contributes to weight

Microbiology and Technology of Fermented Foods, Second Edition. Robert W. Hutkins.

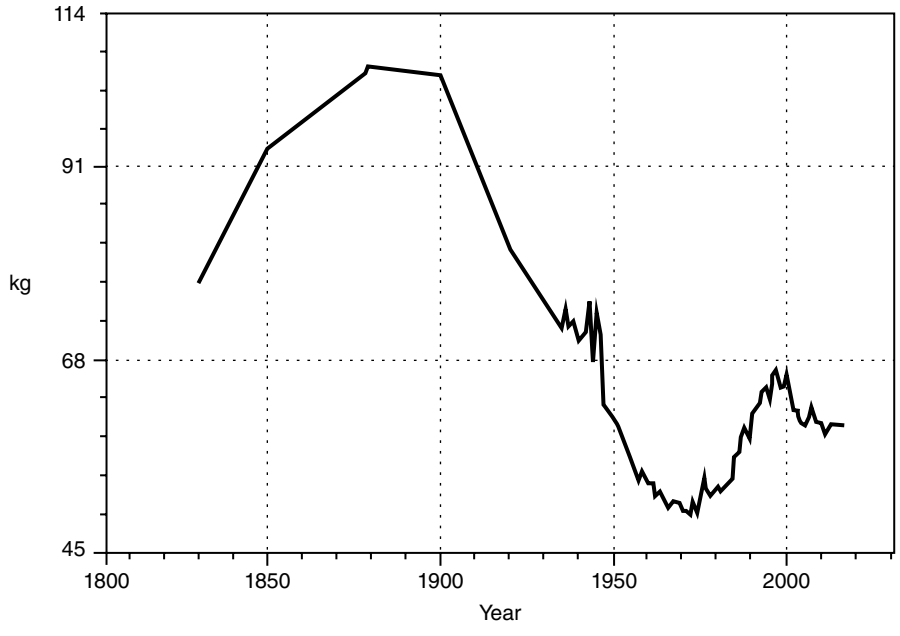


Figure 9.1 Wheat flour consumption (per person per year) in the United States. Adapted from the USDA Economic Research Service.

gain. Low bread consumption could also be due to other cultural changes, including interest in Asian, Latino, and other ethnic cuisines for which bread is not typically consumed.

These trends have occurred despite the recommendations for increased consumption of whole grains made in the USDA's 2015–2020 Dietary Guidelines. Among western countries, the United States ranks near the bottom for bread consumption, at only 17 kg per person per year (about 1.5 slices per day). This is in contrast to much of Europe and the Middle East, where per capita consumption (per person per year) averages about 60 kg (Figure 9.2). Turkey, the worlds' largest consumer, consumes more than 100 kg of bread per person (more than 0.6 pounds per day).

HISTORY

Ancient artifacts and writings discovered in the Middle East suggest that bread-making had its origins in the eightieth century BCE, although it is possible that bread may have been produced even several thousands of years earlier. The first breads were probably flat breads, with little or no leavening (i.e., fermentation), similar to those consumed even today in many parts of the world. It seems likely that humans began making leavened bread at very near the same time they began to make other fermented products. Leavening must certainly have been an accidental discovery made when a flour–water mixture became contaminated with wild airborne yeasts, leading to a spongy dough that was transformed by baking into a light and airy, aromatic, and flavorful product. Perhaps more so than any other fermented food, bread has played a major role in the social history and culture of human civilization (Box 9.1).

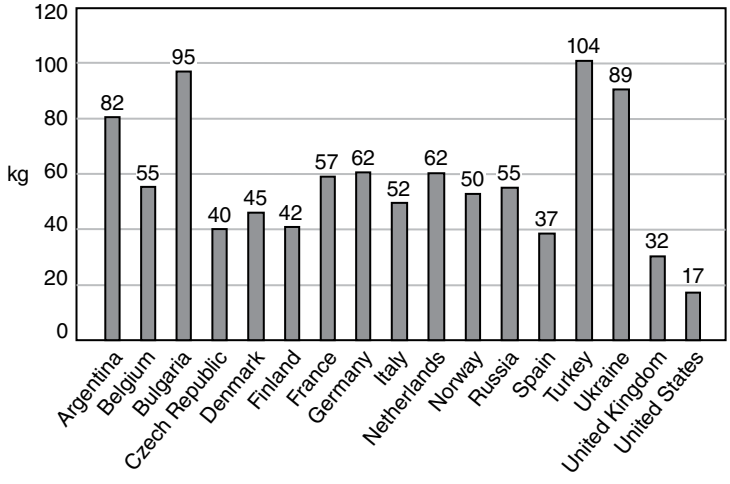


Figure 9.2 World-wide consumption of bread (per person per year). Adapted from Association Internationale de la Boulangeri Industrielle (www.aibi-online.org), Germany Trade and Invest, and the USDA Foreign Agricultural Service. Data is from 2013–2015.

Box 9.1 Bread and the rise and fall of civilizations

It is neither an exaggeration nor a coincidence that the history of bread making parallels the history of human civilization. Bread, the oft-quoted "staff of life" is still one of the principle staple foods throughout the world, and has sustained human beings for thousands of years. While bread has provided the nourishment for building civilizations, its absence has contributed to the collapse of some of the world's strongest empires.

The importance of grain and bread during the course of human history is evident in the Bible, in literature and mythology, and throughout the historical record. Osiris, the Egyptian god of the underworld, was also responsible for vegetation and agriculture, and Neper was the Egyptian god of grain. During the ancient Greek spring festival of Thargelia, bread is offered to the mythological gods Artemis and Apollo, thanking them for providing fertile soil.

According to the Old Testament story, when Joseph, son of the Hebrew patriarch Jacob, predicted that a famine was soon to occur in Egypt, the Pharaoh ordered that surplus grain be stored. This ensured a sufficient supply of wheat when the famine eventually occurred. Generations later, when Hebrew slaves fled Egypt, their hasty exodus left no time for their dough to rise. Instead, they ate unleavened bread, a form of which is eaten even today as a symbol of the Jewish holiday of Passover. The Eucharist, one of the sacraments of Christianity, requires consecration and consumption of bread during Holy Communion. A blessing over bread is commonly recited as part of many religious rites.

During the Roman era, bakers had an elevated status and were widely respected. Bread was so important to the citizenry that it was provided free or was subsidized. Roman soldiers were even equipped with portable bread-making equipment. In the

Middle Ages, baker guilds, the forerunners of trade unions, were formed in Britain and Europe, and quality standards were established to protect their overall interests. At the same time, laws were enacted that set prices and weights for bread.

Because bread is such an important food, wheat has become one of the most important agricultural crops produced on the planet. For thousands of years, and even today, when wheat harvests are poor – due to weather, plant diseases, or famines – starvation conditions often result. Political unrest, trade embargos, and other economic factors have also contributed to shortages of flour and bread. History books are replete with instances in which rebellions and uprisings, and even revolutions, occurred when a steady supply of affordable bread became unavailable.

In pre-revolution France, during the aptly named Flour War, there were hundreds of food riots (Bohstedt, 2014). Ultimately, Queen Marie Antoinette's apocryphal remark about the lack of bread for the hungry children of France ("let them eat cake") proved to be her undoing. A similar fate fell to tsarist Russia, following years of bread and other food shortages starting in 1915 (Engel, 1997). In the modern era (as recently as the 1980s), citizens of the former Soviet Union had to wait in long lines just to buy a loaf of bread. This situation led to significant public discontent and a loss of confidence in the government, both of which may have even contributed to the fall of the USSR.

Bread riots also occurred in Egypt in the 1970s following subsidy cuts that led to shortages and high prices (Bohstedt, 2014). Bread shortages in other Middle East countries, including Jordan, Morocco, and Lebanon were also reported in the 1980s (Wilson, 1994). Riots spread throughout Egyptian society in the 2000s, culminating in the overthrow of the government. Indeed, the slogan of Arab Spring protesters was "bread, freedom and social justice" (Bohstedt, 2014). As recently as spring, 2017, cuts to bread subsidies led again to "we want bread" chants.

Bread riots are not unique to Europe or Asia. The Boston Bread Riot in the early 1700s was reportedly caused by merchants diverting flour to export markets, at the same time when Bostonians were going hungry. During the US Civil War, shortages and high prices in Virginia led to the Richmond Bread Riot in 1863, initiated mainly by women (Titus, 2011). In recent years, long bread lines in Venezuela have led to food riots.

Food, and wheat in particular, has long been used as a weapon in the form of trade embargoes. Grain embargos are still in place even today. Grain bans have also been threatened in Europe, Asia, and Africa if genetically-engineered varieties were to be introduced (Lusk and Miller, 2014). Ironically, not only do grain shortages exist in many of these countries, GMO wheat varieties are not even available.

References

Bohstedt, J. 2014. Food Riots and the Politics of Provisions in World History. IDS Working Paper 444.

Engel, B.A. 1997. Not by bread alone: subsistence riots in Russia during World War I. J. Mod. Hist. 69:696–721.

Lusk, J. and H.I. Miller. 2014, "We Need G.M.O. Wheat", New York Times, Feb. 2, 2014.

Titus, K.R. 2011. The Richmond Bread Riot of 1863: class, race, and gender in the urban Confederacy. The Gettysburg College Journal of the Civil War Era 2:86–146.

Wilson, M.C. 1994. Jordan: bread, freedom, or both? Curr. Hist. 93:87–90.

Despite obvious increases in the size and scope of the bread-baking industry, the historical record has revealed that ancient bread-making was probably not all that different from modern bread manufacturing practices. Given the simplicity of the bread manufacturing process, perhaps this observation is not surprising. After all, bread-making requires only a few ingredients, a few simple mixing and incubation steps, and a baking oven or device. In this chapter, the chemical, physical, and biological properties of these ingredients and the processes used for converting ingredients into doughs and breads will be described.

WHEAT CHEMISTRY AND MILLING

The most common starting material for bread is wheat flour. Breads are also commonly made from a wide variety of other cereal grains, including rye, barley, oats, corn, sorghum, and millet. However, as will be discussed in more detail below, gluten, a protein complex that gives bread its structure and elasticity and is necessary for the leavening process, is poorly formed or absent in most non-wheat flours. Although accommodations can sometimes be made to account for the absence of gluten in rye or other wheat-free doughs, most commercial breads contain at least some wheat.

Wheat is one of several cereal grasses in the family Poaceae (or Gramineae). The main variety used for bread is *Triticum aestivum*. The wheat plant contains leaves, stems and flowers. It is within the flowers (referred to as spikelets) that the wheat kernels are formed. The kernels are essentially seeds that are surrounded by a hard covering material designed to protect the would-be plant from the external elements. The original or wild wheat seed that grew many thousands of years ago had a tough, hard-to-break husk which made it difficult to use. However, the crossing of different wheat varieties led to the "domestication" of wheat with more manageable properties. Wheat breeding continues to be very important in the baking industry. There are dozens of commercially available cultivars, with both agronomic properties and functional traits. Note however, that none of the wheat currently grown or cultivated is genetically modified.

The wheat kernel consists of three main constituents: the germ, bran, and endosperm (Figure 9.3). The germ portion contains the embryonic plant, along with oils, vitamins, and other nutrients. It represents 2–3% of the total kernel weight. The bran portion, or coat, represents 12–13% of the kernel, and is comprised of multiple distinct layers. The bran contains mostly cellulose and other fibrous carbohydrates, along with proteins, minerals, and vitamins. The remaining portion, about 85%, is called the endosperm and is the main constituent of wheat flour. It consists mostly of protein (9–12%) and starch (75–80%), along with some water (12–14%) and a small amount of lipid (1%). Kernels that are crushed or milled will yield "whole" wheat flour that contains all of these fractions, i.e., the germ, bran, and endosperm. Usually, however, these three constituents are separated, either partially or nearly completely, in the milling step, yielding refined flours containing the endosperm fraction, with little or no germ or bran (Box 9.2).

Flour composition

The specific composition of flour is critically important because it has a major influence on the fermentation as well as the physical structure of the dough and finished bread. Wheat flour, after all, is the primary ingredient in most varieties of bread. As noted above, however, most of the flour used for bread manufacture in the United States is refined white flour,

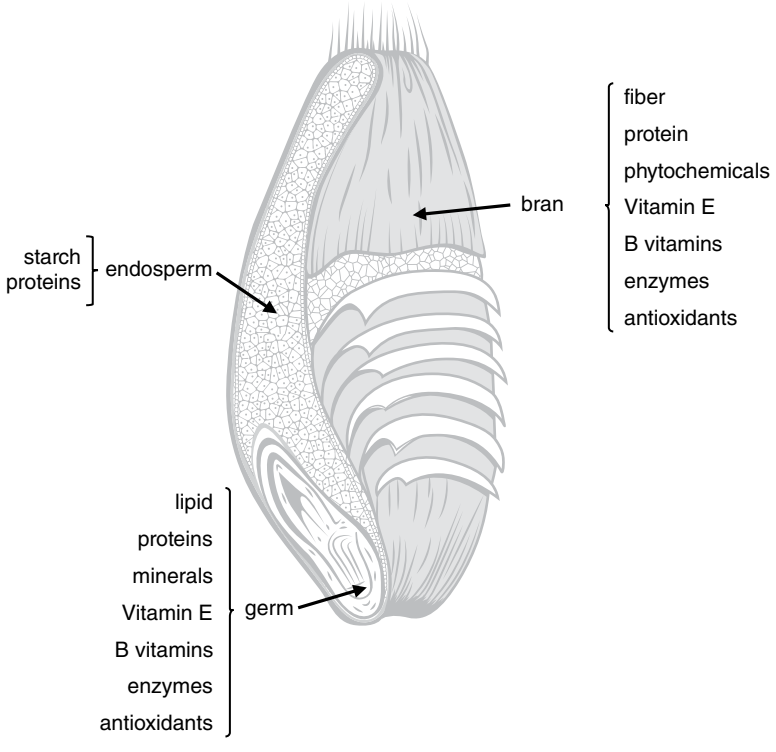


Figure 9.3 Structure of a wheat kernel. Adapted from the Montana Wheat & Barley Committee.

Box 9.2 Principles of milling wheat

The goal of wheat milling is to extract the flour from the wheat kernel. This is such an integral step in the manufacture of bread that a sophisticated and highly automated milling industry has developed whose purpose is to do just that – convert wheat into flour.

Originally, some thousands of years ago, milling was done in a single step, as the kernels were ground or pounded manually by a crude mortar and pestle or between stones. Even today, single-pass milling is performed to make whole wheat flour, although milling technologies are now highly automated. Still, stone-ground flours are widely available and are valued for their quality. Functionally, however, stone-grinding has no advantages over other size reduction techniques, such as a hammer mill.

The modern milling operation begins with various wheat handling steps, including sampling, inspection, and testing; screening; and conditioning. Except for whole wheat flours produced by a single-pass grinding step, most breads are made using flours that contain reduced levels (or even none) of the germ and bran. It is the job of the wheat miller to separate these wheat constituents from the main component, the endosperm.

In principle, this can be done simply by successive "gradual reduction" flour milling steps (Cauvain, 2015). These include grinding, sieving, and separation operations, such that the resulting fractions can either be removed or further processed. In actual practice, the wheat kernels are passed through a series of paired-rollers or stones

whose gap distances between each pair is successively narrower. Modern grinding devices consist of corrugated cast iron break rollers, followed by smooth rolls called reduction rolls. The former are constructed with flutes or ridges to enhance the grinding or shearing action (Figure 9.2.1). The rolls run at different speeds to give a shearing, rather than a crushing, action. In between each so-called break steps, the ground wheat is passed through sieves that retains the coarse material (containing bran still adhering to the endosperm), while permitting the fine flour to pass. Along the way, an air purifying device is also used to remove fine bran particles that are lighter than the flour. Eventually, white flour is produced.

The milling operation actually yields dozens of different product streams (Clavel, 2001). Flour removed from the early and late separations is called clear or common flour. It is generally less refined, higher in protein (>14%), and somewhat darker in color. Patent flour is obtained from the intermediate separation steps. It has the least amount of germ and bran, and, therefore has the whitest appearance. Patent flour is still high in protein (13%) and is considered to have the best bread-making quality (at least in the United States and Canada).

In many parts of the world, it is a standard practice to re-combine the different flour fractions to give flours of varying composition, ranging from a straight flour containing all of the separated fractions, to different patent flours containing 60% to 80% of the original wheat. This allows bakers to choose a flour based on desired strength (i.e., protein content) or functional properties (i.e., dough elasticity, bread flavor, and oven spring).

It is important that a high yield be achieved during the milling process. The yield is given as the extraction rate, and is expressed as the amount of flour that is obtained from

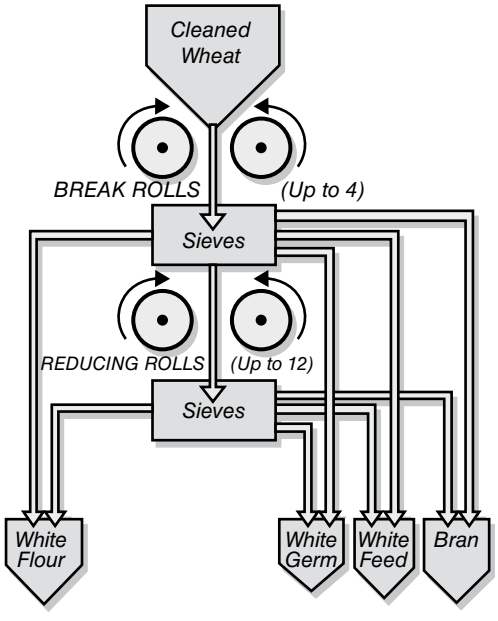


Figure 9.2.1 A simplified diagram of wheat milling. From Hazel and Patel, 2004, with permission.

the wheat. In general, extraction rates of about 70–80% are achieved, meaning that about 72 kg of straight flour can be obtained from 100 kg of wheat. The balance (about 28%) includes mainly bran and germ. However, since 85% of the wheat is endosperm, some of the remaining material consists of endosperm that adheres to the bran layers.

Finally, despite its ancient history, flour milling remains an area of active research (Doblado-Maldonado et al., 2012). In large part, this is because of changing consumer preferences regarding color, flavor, and nutritional properties. Shelf-life is also an important attribute, especially for whole wheat flours. In particular, the current popularity of whole wheat products not only dictates the type of wheat a miller may use, but also the manner in which the wheat is milled (Doblado-Maldonado et al., 2012; Heiniö et al., 2016). For example, bitterness, due mainly to peptides and phenolics, and rancidity, due to lipolysis and oxidation, may appear in flour as a result of the milling and fractionation techniques.

References

Calvel, R. 2001. The Taste of Bread. (R.L. Wirtz, translator). Aspen Publishers, Inc. Gaithersburg, MD.

Cauvain, S. 2015. Wheat milling and flour testing. In: Technology of Breadmaking. Springer International Publishing, New York, pp. 339–375.

Doblado-Maldonado, A.F., O.A. Pike, J.C. Sweley, and D.J. Rose. 2012. Key issues and challenges in whole wheat flour milling and storage. J. Cereal Sci. 56:119–126.

Hazel, C.M., and S. Patel. 2004. Influence of processing of trichothecene levels. Toxicol. Lett. 153:51–59.

Heiniö, R.L., M.W.J. Noort, K. Katina, S.A. Alam, N. Sozer, H.L. de Kock, M. Hersleth, and K. Poutanen. 2016. Sensory characteristics of wholegrain and bran-rich cereal foods: a review. Trends Food Sci. Technol. 47:25–38.

containing only the endosperm portion. It consists of two main components – protein and starch, with a small amount of hemicellulose and lipid.

1. Wheat protein

About 8% to 15% of wheat flour is protein. This wide range reflects the various types or classes of wheat used as a source of the flour (Table 9.1). It is the protein content, as well as protein quality that dictate the use of that flour. High protein flours, derived from hard wheat, generally contain more than 11% protein and are best used for bread. In contrast, low-protein flours, derived from soft wheat, contain 9% or less protein, and are most often used for

Table 9.1 Major types of wheat and their applications.

Type	% Protein	Application
Hard winter	10–13	Bread
Hard spring	13–16	Bread
Soft winter	8–10	Cakes, cookies, pastries
Durum	12–14	Pasta

cakes, cookies, and pastries. Protein is important nutritionally, but in bread making, it also provides essential functional properties. Specifically, wheat proteins form the matrix necessary to retain the carbon dioxide made during fermentation. Therefore, protein content has a major impact on dough expansion and loaf volume during bread making.

The protein fraction actually contains several different proteins. The two most important of these are gliadin and glutenin, accounting for 85% of the total protein. When gliadin and glutenin are hydrated and mixed, they form a complex called gluten, which is a key component of bread dough. The remaining proteins (15% of the total) consist of other globulins and albumins. Several enzymes are also present, in particular, α-amylase and β-amylase that play an important role in the fermentation (discussed below).

2. Carbohydrates

Carbohydrates represent the main fraction of flour, accounting for up to 75% of the total weight. This fraction is largely comprised of starch, although other carbohydrates are also present, including a small amount of simple sugars, fructans and cellulose and other fibers. Wheat starch consists of amylose, an α-1,4 glucose linear polymer (about 4000 glucose monomers per molecule) and amylopectin, an α-1,4 and an α-1,6 glucose branched polymer (about 100,000 glucose monomers per molecule). About 20% to 25% of the starch fraction is amylose and 70 to 75% is amylopectin. Properties of these two fractions are described in Table 9.2.

In its native state (i.e., before milling or processing), wheat starch exists as starch granules. The amylose and amylopectin are contained within these spherical granules in a rigid, semi-crystalline network. Native starch granules are insoluble and resist water penetration. However, some of the starch granules (3% to 5%) are damaged during milling, which enhances absorption of water and exposes the amylose and amylopectin to hydrolytic enzymes, including α-amylase.

3. Yeast cultures

The yeast used for bread manufacture throughout the world is *Saccharomyces cerevisiae*, often referred to as simply baker's yeast. As discussed later, ale (Chapter 10), wine (Chapter 11), and several distilled alcoholic beverages are also made using *S. cerevisiae*. However, the actual strains used for each of these products are not interchangeable. Baker's yeast strains of *S. cerevisiae* clearly have properties and performance characteristics

Table 9.2 Physical and chemical properties of amylose and amylopectin.

Property	Amylose	Amylopectin
Monomers per molecule	300–1000	>5000
Linkage	α-1,4	α-1,4 and α-1,6
Conformation	Linear	Branched
% in wheat starch	25	75
Retrogradation rate	Fast	Slow
Products of:		
α-amylase	Dextrins	Limit dextrins
β-amylase	Maltose	Maltose, limit dextrins

Table 9.3 Desirable properties of baker's yeasts.

• Gassing power
• Flavor development
• Stable to drying
• Stable during storage
• Easy to dispense
• Ethanol tolerant
• Cryotolerant

especially suited for bread manufacture (Table 9.3). For example, bread strains are selected, in part, on their ability to produce CO_2 – their so-called gassing rate. It is the CO_2, evolved during fermentation that is responsible for leavening. Other properties are also important, including the obvious ability to produce good bread flavor. In addition, yeasts should be able to grow well in fermenters during production, be stable during drying, and maintain viability during storage. Other specific properties are discussed below.

Yeast manufacture

Like for other starter cultures, modern industrial production of baker's yeast starts with a pure stock culture, which is then scaled up through a series of fermentors until a large cell mass is produced (Figure 9.4). The culture is propagated initially in small (1 L to 5 L) seed flasks, and then in progressively larger fermentors of about 250 L to 1000 L. Eventually the culture is inoculated into large (200,000 L) production fermentors.

The growth medium usually consists of molasses or another inexpensive source of sugar and various ammonium salts (e.g., ammonium hydroxide, as a cheap source of nitrogen). Other yeast nutrients include ammonium phosphate, magnesium sulfate, calcium sulfate, and lesser amounts of trace minerals, zinc and iron. Since cell mass is the goal (rather than metabolic products), growth occurs under optimized conditions. In particular, highly aerobic conditions are created by providing agitation and oxygen (or air). In addition, the yeast propagation step is performed at optimum temperature and under pH control, usually around 30 C and at a pH of 4.0 to 5.0, with nutrients provided on a continuous basis. At the end of the growth fermentation, the yeast is collected by centrifugation, resulting in a yeast "cream" that contains about 20% total yeast solids and a yeast concentration of about 10^{10} cells/g.

Yeast forms

Baker's yeast preparations are available in several forms. The yeast cream can be used directly, although this form is highly perishable. Still, the cells are highly active and therefore may be practical for high volume bread manufacturers. Many commercial bakers prefer compressed yeast cultures. These are produced by pumping the yeast cream through a filtration press or vacuum filter to remove more of the water. The yeast is collected in the form of moist cakes, separated by wax paper. Compressed yeast cakes (about 30 cm × 30 cm × 2 cm) still have high moisture content (70% to 75%), require refrigeration, and will last a few weeks. Because the cells are metabolically active, once they are introduced into the dough, fermentation can occur very quickly.

Compressed yeast can be further dried to about 90% solids to provide dry active yeast. This is the form that is familiar to consumers who make homemade bread, but small manufacturing operations or those located where compressed yeast is not available also use dry yeast preparations. Dry active yeast preparations last six months or longer, even at room temperature. They do require a hydration step, and in general, are not as active as compressed

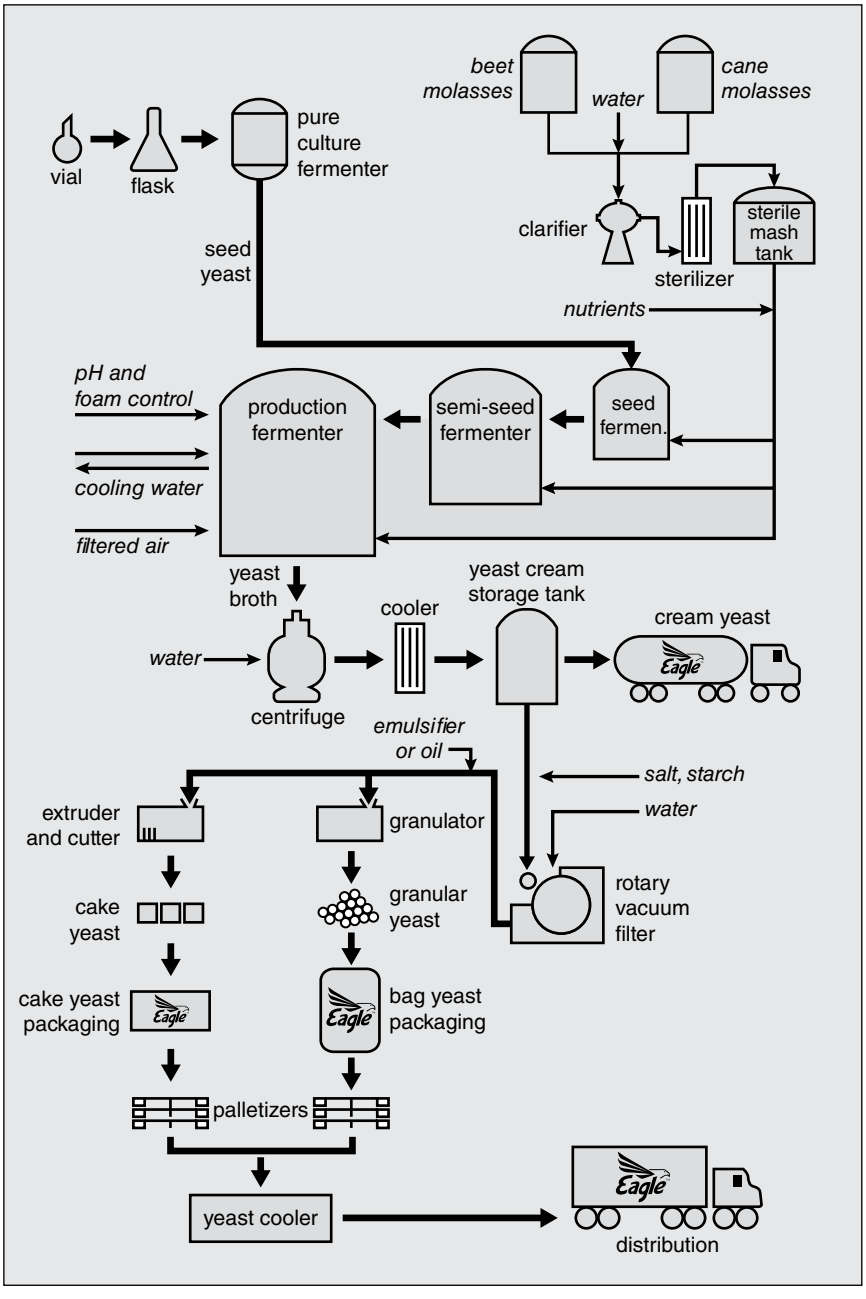


Figure 9.4 Industrial production of baker's yeast. From Lallemand Baking Update, Volume 1/Number 9 (with permission).

yeast, although improved drying technologies have greatly enhanced the activity of dried yeast. In addition, dry active yeasts can be "instantized" such that they rehydrate quickly. This form of yeast is popular for use in bread machines, since they can also be added directly to the dough.

BREAD MANUFACTURING PRINCIPLES

As noted previously, bread manufacture can be as simple as just a few ingredients and a few steps. First, the ingredients are assembled, weighed, and mixed to make a dough. This "bulk" dough is then allowed to ferment. The fermented dough is portioned and shaped and given a second opportunity to ferment. Finally, the dough is baked, cooled, and packaged. Of course, the actual procedures used by modern industrial bakeries are often considerably more involved. Likewise, modern bread making also relies on a number of additional ingredients.

Ingredients

A typical ingredient statement on a loaf of commercial bread will be a paragraph long and list 20 or more ingredients. However, only four ingredients – flour, water, salt, and baker's yeast – are actually required to make perfectly acceptable, if not exceptional, bread. That being said, the other added ingredients provide many important functional properties, not only in the finished product, but also during dough development steps. Still, technically speaking, these ingredients are optional. Of course, one could argue that consumer preferences for inexpensive breads with a soft crumb texture, long, mold-free shelf-life, and resistance to staling have led to such widespread use of some of these ingredients that they can hardly be considered "optional". Moreover, large scale manufacturing operations that demand high throughput could not function nearly as well without these extra ingredients and process aids.

Wheat flour is the main ingredient of bread, representing about 60% to 70% of a typical formulation. Note that bread formulations are based on "baker's math", where the flour is set at 100% and the remaining ingredients are calculated as percent of the flour. As noted above, flour contains two main constituents – the proteins that are essential for dough formation and the starch that absorbs water and serves as an energy source for the yeasts. Water is added at about 60% to 80% (i.e., of the flour weight) and acts as the solvent necessary to hydrate the flour and other ingredients. Salt is added at 1% to 2% to control the fermentation and provide flavor. It is also thought to toughen the gluten. Finally, the yeast is added at about 1% to 2% (depending on the form) to ferment sugars to CO_2 and provide leavening. Yeast growth during dough formation and the leavening step also contributes to flavor formation.

As noted above, many optional ingredients are often included in bread formulations, depending on the preferences of the manufacturer. These ingredients and their functions are described below.

1. Sugars

In the United States, many large-scale bakeries add either sucrose or glucose (about 2% to 3%) as an additional source of readily fermentable sugars. They also supply flavor and, when the dough is baked, color. Wheat flour contains only modest amounts of maltose and glucose, and although yeasts express invertases and maltases that over time can release fermentable sugars, sugar availability may still be growth-limiting.

2. Enzymes

Another way to increase the amount of free sugars in the dough is to add α- and β-amylases, enzymes that specifically hydrolyze the α-1,4 glucosidic bonds of amylose and amylopectin (Figure 9.5). Flour naturally contains both of these enzymes, but α-amylase, in particular, is

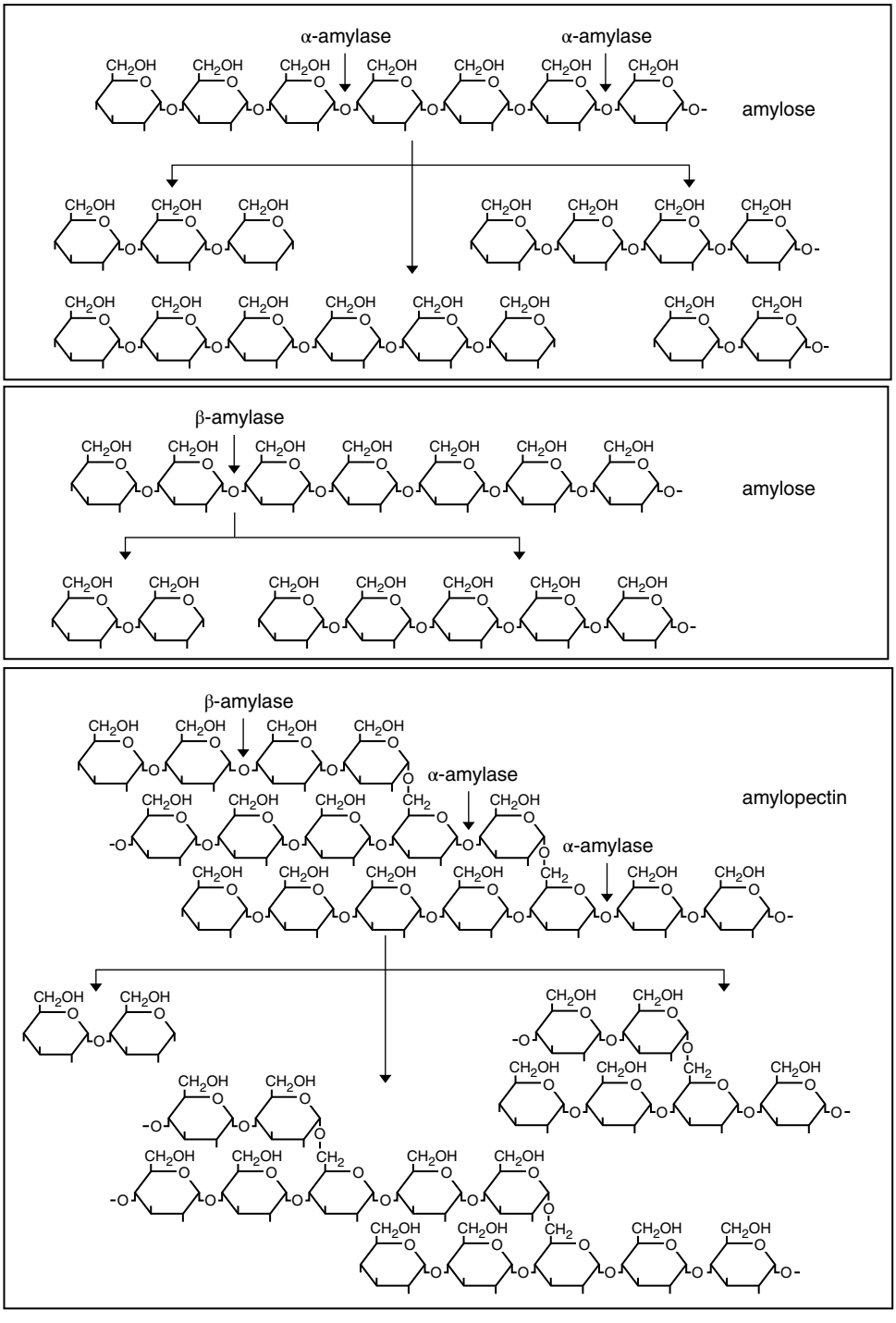


Figure 9.5 Hydrolysis of amylose and amylopectin by α- and β-amylases. The top and middle panels show reaction products of α-amylase and β-amylase on amylose. The bottom panel shows reaction products of β-amylase and α-amylase on amylopectin.

present at very low levels. These enzymes are also available in the form of microbial preparations or in a more natural form as malt. Not only do these enzymes collectively increase the free sugar concentration by hydrolyzing amylose and amylopectin to maltose, they also can enhance bread quality. Controlled hydrolysis of starch – especially the amylopectin portion by α-amylase tends to increase loaf volume. Also, by virtue of softening the crumb texture, staling is delayed (more on the staling phenomenon is described below). Of course, if too much enzyme is added, the dough will be sticky and unmanageable and the excess free sugars that are formed could contribute to over-browning during baking.

Another important factor that must be considered when enzymes are used as process aids is their temperature inactivation profile. Heat-resistant amylases, such as those produced by bacteria, may survive baking and continue to hydrolyze starch. This could result in soft and sticky bread. Fungal enzymes are generally heat labile and are inactivated during baking.

Increasing the amylase activity in dough, and thereby increasing the free sugar concentration, can also be accomplished by adding malt powder. Malt, an essential ingredient in beer manufacture, is prepared from germinated grains, usually barley or wheat that are dried and ground into a powder. Malt contains both α- and β-amylases with temperature profiles similar to the microbial sources.

Finally, the addition of proteolytic enzymes has been advocated as a means of achieving partial hydrolysis of the gluten. Proteases decrease elasticity, increase extensibility, and make the dough easier to sheet or roll out. In theory, this would reduce mixing time and would also make a softer dough.

3. Fat

The addition of 0.1% to 0.2% fat, as either a shortening or oil, is now common in most commercial breads. Whereas a generation or two ago, animal fats with excellent "shortening" properties were used in breads, the use of such fats has essentially disappeared in the United States and in most of the world. These fats were subsequently replaced with partially hydrogenated vegetable oil. The composition of these oils is not that different from animal fats, in that they contain mostly long chain, saturated fatty acids, with a high melting point. Not surprisingly, they impart similar properties as the animal fats, giving a soft, cake-like texture.

Because partially hydrogenated vegetable oils contain trans fatty acids, which have been associated with heart disease, even these oils have been largely phased out of most commercial bread formulation. Tropical- and other non-hydrogenated oils have been developed that provide similar functionality in the dough.

4. Yeast nutrients

Various nutrients can be added to the dough mixture to enhance growth of the yeast, including ammonium sulfate, ammonium chloride, and ammonium phosphate, all added as sources of nitrogen. Phosphate and carbonate salts may also be added to adjust the acidity or alkalinity, and calcium salts can be added to increase mineral content and water hardness.

5. Vitamins

Flour and bread have been enriched in the United States for more than sixty years. Flour currently is fortified with four B vitamins – thiamine, riboflavin, niacin, and folic acid, and

one mineral, iron. However, non-enriched flour is also available and bread manufacturers can enrich bread by adding these nutrients directly to the dough. Enrichment and fortification of flour are now common throughout the world.

6. Dough improvers

Dough mixing can be one of the more-time consuming steps, as mixing the ingredients to make an elastic dough is often a slow process. In part, this is because of the sticky gluten protein that forms when the flour is hydrated. Reducing agents, such as cysteine, decrease the number of disulfide cross-links that make gluten so elastic and sticky. In other words, these agents function by weakening the dough structure and thereby decreasing mixing times. Other bread dough improvers work in the opposite manner. These include oxidizing agents, such as ascorbic acid and potassium bromate that improve dough structure by increasing the number of disulfide bonds (and cross-linkages) in the gluten network. Their effect is to increase elasticity and gas retention. Bromates have long been considered as the most effective oxidizing agent; however, recent questions regarding their safety has led some government authorities to discourage or prohibit the use of this additive in bread manufacture.

7. Biological preservatives

Biological spoilage of bread (in contrast to staling or other chemical-physical causes) is invariably caused by fungi. Thus, mold inhibitors are routinely added to bread. By far, the most common anti-fungal agents are the salts of weak organic acids; they include potassium acetate, sodium diacetate, sodium propionate, and calcium propionate. The latter is more effective and is the most widely used bread preservative in the United States. As illustrated in Box 9.3, the mode of action of these agents against target cells is similar and depends on the form of the acid and the pH of the food. They are typically added at 0.15% to 0.2% of the flour weight; the maximum amount allowed for acetates and propionates in the United States is 0.32% and 0.40%, respectively (less in Europe). It is important to note that, despite their effectiveness against fungi, these agents are only inhibitory and are not cidal. They are not inhibitory to baker's yeast, however, and have no effect on fermentation.

Box 9.3 Bread preservation – propionates and beyond

Unfortunately, bread is one of the most wasted foods, with nearly 30% of purchased bread tossed away by consumers. This is true not only in Europe, the United Kingdom, and the United States, where bread is relatively cheap, but also in less well-off countries that depend on bread as a staple food (Capone et al., 2016; Neff et al., 2015). The main reason why bread spoils is due to fungal growth. Accordingly, the main preservative used in bread is the anti-mycotic agent, calcium propionate.

Although it is the propionic acid moiety that is the active agent, the calcium from is used commercially since it is readily soluble and is easier to handle. Calcium propionate is a GRAS substance (generally recognized as safe), and even occurs naturally (e.g., Swiss cheese). Its inhibitory activity against fungi is due to its ability to penetrate

the cytoplasmic membrane and accumulate inside the cell. However, propionate can only cross the membrane when it is in the acid form. This is because lipid-rich, non-polar, hydrophobic cytoplasmic membranes are impermeable to charged, highly polar molecules, such as the anion or salt form of organic acids. Only the uncharged, undissociated, lipophilic acid form can diffuse across the membrane.

How then, does calcium propionate exert its anti-fungal activity? Recall that weak organic acids can exist in either the acid (undissociated) or salt (dissociated) form (Figure 9.3.1). When the pH is below the pKa, the undissociated form predominates. It is this uncharged and lipophilic form of the acid that diffuses across the cytoplasmic membrane. When the propionic acid reaches the more neutral cytoplasmic environment, it dissociates, releasing a proton. Not only does the organism have to spend ATP to expel protons, but intracellular pH may decrease to a point that is inhibitory to the cell.

There is now considerable interest among bakeries and bread manufacturers for propionate alternatives (Axel et al., 2017). This has less to do with the proven effectiveness of propionates, and everything to do with clean or friendly labels. Many consumers expect bread to contain only "natural" ingredients. Thus, bread manufacturers are under pressure to provide products to consumer that are resistant to fungal spoilage and have good shelf-life, but without synthetic chemical preservatives.

Accordingly, the ingredients industry has developed so-called natural anti-fungal agents that would presumably be considered as label-friendly. In general, these products consist of spent culture medium following growth of an organism that had produced the antimicrobial agent, *in situ*. When that medium is concentrated by spray

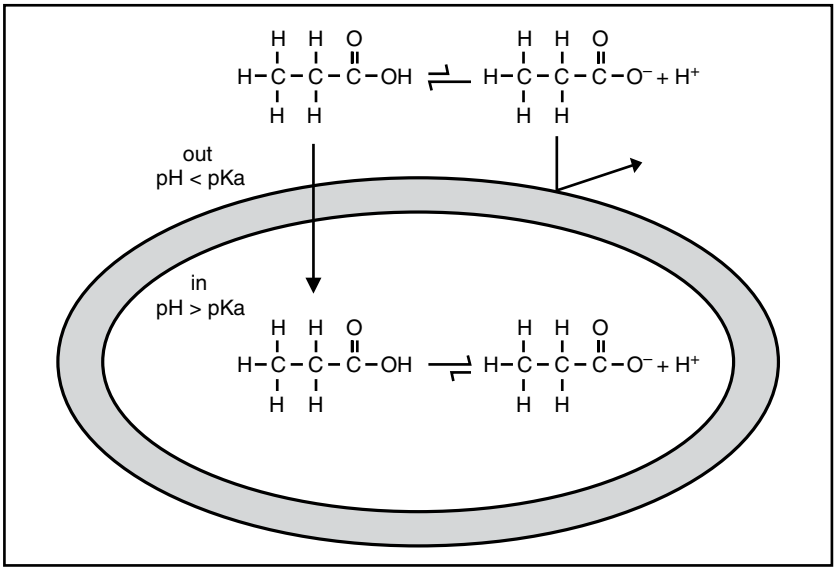


Figure 9.3.1 Mode of action of propionate.

drying, the agent remains active and can be added to bread or other foods. These "fermentate" products can carry a label that simply declares "cultured dextrose" or whatever the fermentation medium had been.

One anti-fungal agent produced in this manner is natamycin (also known as pimaricin). It is produced by *Streptomyces natalensis* and is lethal against a range of bread molds. Another type of fermentate is made by culturing lactic or propionic acid-producing bacteria (Le Lay, et al., 2016). The active agents in the latter products are organic acids (e.g., lactic, acetic, or propionic). Interestingly, although a given fermentate could contain propionic acid that would be indistinguishable from chemically-derived propionic acid, the manner in which they were produced would make all the difference.

References

Axel, C., E. Zannini, and E.K. Arendt. 2017. Mould spoilage of bread and its biopreservation: a review of current strategies for bread shelf life extension. Crit. Rev. Food Sci. Nutr. 57:3528–3542.

Capone, R., H. El Bilali, P. Debs, F. Bottalico, G. Cardone, S. Berjan, G.A.G. Elmenofi, A. Abouabdillah, L. Charbel, S.A. Arous, and K. Sassi. 2016. Bread and bakery products waste in selected Mediterranean Arab countries. Am. J. Food Nutr. 4:40–50.

Le Lay, C., E. Coton, G. Le Blay, J.-M. Chobert, T. Haertlé, Y. Choiset, N.N. Van Long, L. Meslet-Cladière, and J. Mounier. 2016. Identification and quantification of antifungal compounds produced by lactic acid bacteria and propionibacteria. Int. J. Food Microbiol. 239:79–85.

Neff, R.A., M.L. Spiker, and P.L. Truant. 2015. Wasted food: U.S. consumers reported awareness, attitudes and behaviors. PLoS ONE, 10, 2015.

8. Emulsifiers

Although bread (and bread dough specifically) does not require emulsification, emulsifying agents do have several functional properties. They can increase water absorption and gas retention in the dough, decrease proofing times, and reduce the staling rate. As such, bread emulsifiers are perhaps best considered as dough conditioners. By far, the most commonly added emulsifiers are mono- and diglycerides, derived from hydrogenated vegetable oils. In the United States, they are added at a concentration of 0.5% (flour weight).

9. Gluten

Gluten, as discussed above, is the main protein in bread dough and is formed naturally during hydration and mixing steps. However, it is also a common practice to supplement flour with additional gluten, in the form of vital wheat gluten, as an ingredient in the dough mixture. Not only does the added gluten increase the protein content of the bread, but it also increases loaf volume and extends shelf-life. In addition, high gluten breads may have improved texture properties by virtue of strengthening the dough and bread structure. In general, vital gluten is most commonly added to flour in crop years when protein quality or quantity are low; it is also added to whole grain and specialty breads to increase loaf volume.

Hydration and mixing

Depending on the nature of the ingredients (solid or liquid), they are either weighed or metered into mixing vats. After all of the ingredients are combined, they are vigorously mixed. This is a crucial step because the flour particles and starch granules are hard and dense and water penetrates slowly. Therefore, mixing is the primary driving force for the water molecules to diffuse into the wheat particles. It is also by mixing that the yeast cells, yeast nutrients, salt, air, and other ingredients are distributed throughout the dough. Mixing, in other words, is absolutely necessary to develop a proper dough.

In the home, a good strong pair of hands can do the mixing and kneading, but commercial bread manufacturers rely on large mixers that knead and tumble the dough. There are actually many different types of mixers, ranging from low- or slow-speed mixers to high-speed devices that require as little as five minutes to complete the job. The latter are used for processes, such as "no-time" bread manufacturing methods that rely on mechanical development of the dough and shorter fermentations. This is in contrast to traditional low-speed mixing methods in which dough development depends on an extended period of yeast activity and fermentation.

Several important physical-chemical events occur during mixing. First, as hydration occurs, gliadin and glutenin form a visco-elastic gluten complex. The sulfur-containing amino acid cysteine is present in both gliadin and glutenin, and disulfide bonds will cross-link the poypeptide chains. In gliadin the disulfide crosslinks occur within a single protein molecule, whereas the disulfide crosslinks in glutenin form between different protein strands. The more crosslinking, the more elastic is the dough. Dough mixing and elasticity are affected by oxidizing and reducing agents, as described above. Eventually, as the dough is mixed and the gluten complex is formed, a continuous network or film will surround the starch granules. Even during this early stage, naturally occurring or exogenous amylases can begin to hydrolyze the starch, generating maltose, maltodextrins, and other sugars.

FERMENTATION

Yeast growth is initiated as soon as the flour, water, yeasts, and other ingredients are combined and the dough is adequately mixed. A lag phase usually occurs, but the duration depends on the form of the yeast and the availability of fermentable sugars. Cream yeasts, for example, require little time to begin their work, and even compressed yeast do not take long before log growth occurs. Baker's yeast (i.e., *S. cerevisiae*) has a facultative metabolism, meaning that it can use glucose by either aerobic or anaerobic pathways. The former pathway (i.e., via the tricarboxylic acid or TCA cycle) yields much more cell mass and more ATP per glucose than the anaerobic pathway. However, despite the incorporation of oxygen into the dough during the mixing step, oxidative metabolism of carbohydrates occurs only briefly, if at all. Instead, carbohydrates are metabolized by the glycolytic fermentative pathway. This is due, in part, to the presence of glucose, which inhibits synthesis of TCA cycle enzymes via catabolite repression, but also because the dough quickly becomes anaerobic due to the evolved CO_2.

Sugar metabolism by baker's yeast

Because several sugars are ordinarily present in the dough (mainly glucose and maltose), and others may be added (sucrose, high fructose corn syrup), the yeast has a variety of metabolic

substrates from which to choose. In addition, more maltose may be formed in the dough during the fermentation step via the successive action of α- and β-amylases that act on damaged starch granules. Recall that these amylases can either be endogenous (i.e., present in the flour) or added in the form of enzyme preparations or malt. Ultimately, a range of carbohydrates may be available to the yeast, including glucose, fructose, maltose, and maltotrioses. The order in which these different carbohydrates are fermented by *S. cerevisiae* is not random, but rather is based on a specific hierarchy, with glucose being the preferred sugar.

Regulation is mediated by catabolite repression, acting at early steps in various catabolic pathways. Thus, in most strains of *S. cerevisiae,* glucose represses genes responsible for maltose transport and hydrolysis, as well as the invertase that hydrolyzes sucrose to glucose and fructose (Figure 9.6). Consequently, in dough containing glucose, sucrose, and maltose, the disaccharides will be fermented only when the glucose is consumed. Moreover, maltose represses invertase expression, so sucrose would ordinarily be the last sugar fermented in this mixture.

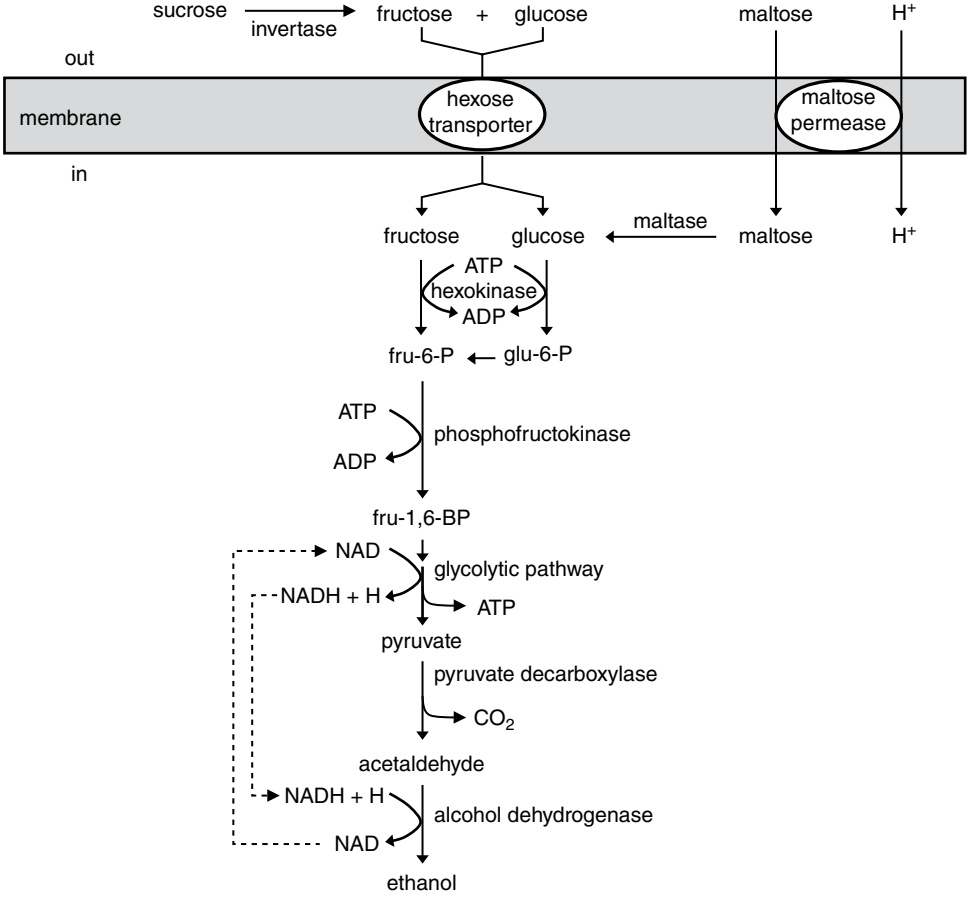


Figure 9.6 Transport and metabolism of sugars by *Saccharomyces cerevisiae*. Fructose and glucose are transported by one of several different hexose transporters via facilitated diffusion, whereas sucrose is first hydrolyzed by a secreted invertase. Maltose transport occurs via a proton symport-mediated maltose permease. Once inside the cell, the accumulated monosaccharides are phosphorylated and eventually fed into the Embden-Meyerhof-Parnas glycolytic pathway.

Depending on how the cells had previously been grown, however, it is possible that invertase had already been induced, resulting in rapid formation of sucrose hydrolysis products. Finally, there are strains of *S. cerevisiae* whose expression of catabolic genes is constitutive, meaning that they are not subject to catabolite repression. Such strains may be particularly useful, since constitutive expression of relevant genes means that these strains will ferment maltose even in the presence of glucose. In most doughs, however, glucose is metabolized rather quickly, and the maltose utilization genes would soon be expressed, even in non-constitutive strains.

Sugar transport

Glucose and fructose are both transported by common hexose transporters (Figure 9.6). Genetic evidence has revealed that there may actually be as many as 18 to 20 of these transporters in *S. cerevisiae.* Despite differences in the structure of these transport proteins, they all transport their substrates via facilitated diffusion. No energy is required and instead the driving force is simply the osmotic gradient. It is also clear that they vary with respect to their substrate affinities. The presence of high-affinity and low-affinity systems provides the cell with the versatility necessary to transport sugars under a wide range of available concentrations. Thus, yeasts are especially suited to grow in environments containing high concentrations of glucose and/or fructose, since their uptake costs are cheap (in terms of energy expenditure). But they can also manage when substrates are not plentiful, such as in lean doughs where no sugar had been added.

Glycolysis

Following transport, the accumulated monosaccharides are rapidly phosphorylated by hexokinases or glucokinases. Similar to the hexose transport systems, hexokinase enzymes have broad substrate specificity and are able to phosphorylate glucose and fructose, as well as mannose. The sugar phosphates then feed into the glycolytic or Embden-Meyerhof-Parnas pathway, with the eventual formation of pyruvate. Reducing equivalents, in the form of NADH, are formed from oxidized NAD by the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase. To maintain glycolytic metabolism during active growth, therefore, it is necessary to restore the NAD.

Recall that lactic acid bacteria reduce pyruvate and re-oxidize NAD via the lactate dehydrogenase reaction. In contrast, in *S. cerevisiae*, a similar process occurs, but in a two-step series of reactions. First, pyruvate is decarboxylated by pyruvate decarboxylase, generating acetaldehyde and CO_2. Next, the acetaldehyde is reduced by one of several NADH-dependent alcohol dehydrogenases to form ethanol and oxidized NAD. The net effect of glycolytic metabolism by baker's yeast is the formation of two moles of CO_2 and two moles of ethanol per mole of glucose fermented. The oxidized and reduced forms of NAD are in balance, and the cell earns itself a net gain of two moles of ATP. Yeast growth itself is quite modest during glycolysis, because most of the glucose carbon is used for generating energy rather than for producing cell mass. Therefore, the initial population will increase only a few generations during the entire fermentation process.

End products

The most obvious and most important end product of the bread fermentation is CO_2. It is, after all, the CO_2 that is mainly responsible for transforming the stiff, heavy, dough into a

spongy, elastic, and airy material. Despite its low solubility, some of the CO_2 will dissolve in the water phase, causing the pH to decrease. Rather quickly, the CO_2 saturates the aqueous environment surrounding the yeast micro-colonies. It is at this point that the CO_2 evolves into the dough. The CO_2 causes the gluten proteins to stretch, and although some CO_2 escapes into the atmosphere, most of the gas is retained and is trapped within the matrix. This process is known as leavening.

The retained CO_2 ordinarily collects in the dough at rather random locations to form large irregular gas cells that are dispersed in a non-uniform manner. This is one reason why doughs are "punched down" during the course of the fermentation. This step re-disperses the gas, forming smaller, more regular, and evenly dispersed gas cells. About 45% of the gas is lost. However, re-mixing causes the yeast cells and sugars to be re-distributed in the dough such that more substrate is made available to the yeast and additional CO_2 can be formed.

Other metabolic products are also produced during the yeast fermentation, including various organic acids that give the dough a slightly acidic, but pleasant flavor and aroma. The yeast can form other organic compounds, including those that contribute flavor (more on that later). Lactic acid bacteria, which are inevitably present, either in the yeast cake or in the flour, also begin to grow, ferment sugars, and produce acids. Ultimately, the pH of the dough will drop from about 6.0 to 5.0. This decrease in pH, however, has little effect on yeast growth and metabolism. In fact, pH 5 is very near the optimum for *S. cerevisiae*. Only when there is enough acid produced in the dough to lower the pH to 4.0 or less (as is the case for sourdough breads) will inhibition of baker's yeast strains occur. Despite their modest accumulation in dough, the acids produced by yeasts and lactic acid bacteria make important contributions to the flavor, as well as rheology of the dough. For example, low pH improves the water-binding capacity and swelling of gluten, making it more extensible and pliable.

Factors affecting growth

Temperature and relative humidity have an important effect on yeast growth and fermentative capacity. Most strains of *S. cerevisiae* used for bread manufacture have an optimum temperature of about 36°C to 39°C, although seldom are such high temperatures reached during dough fermentations. Rather, doughs are ordinarily held at temperatures of about 25°C to 28°C (and sponges slightly lower). Although higher temperatures can accelerate fermentation and gassing rates (i.e., the amount of CO_2 produced per unit time), elevated temperatures also can enhance growth of microbial contaminants, including wild yeasts. Also, the temperature at which yeast activity and fermentation rate declines is only a few degrees higher than the optima. Relative humidity must also be controlled, usually at about 70% to 80%. Lower levels of moisture in the air may cause the dough to dry at the surface, leading to formation of a crust-like material, as well as inhibiting the fermentation within the areas near the surface.

Dividing, rounding, and panning

The fermentation described above for straight dough or bulk fermentation processes (described below) usually occurs in troughs. Once the fermentation is considered complete, the dough is then ready to be divided, on a volumetric basis, into loaf-sized portions. Dividing is usually a simple process that involves cutting extruded dough at set time intervals, such that each piece has very near the same weight. Other devices, driven by suction, are also common. Despite the differences in design, the main requirement of the dividing step is that

it be done quickly, since the fermentation is still on-going and the weight-to-volume ratio of the dough pieces may change.

The divided dough is then conveyed to a rounding station, where ball-shaped pieces are formed. At this point the dough is given a short opportunity (less than twenty minutes) to recover from the physical strains and stresses caused by being cut, compressed, and rounded, bounced about. A portion of the gas is also lost during the dividing and rounding steps. Thus, not only does the dough have a chance to rest (structurally, that is), but the fermentation also continues, adding a bit more gas into the dough. Following this intermediate proofing step, the dough pieces are delivered into a molding system that first sheets the dough between rollers, then rolls the dough into a cylinder shape via a curling device, and finally, shapes the dough into the desired final form. The shaped loaves are then transferred to pans for proofing and baking.

Proofing

Most of the CO_2 is expelled during the sheeting step, and it is during the final proofing step where the dough is re-gassed and the fermentation is completed. For some bread production systems, such as the no-time processes, the entire fermentation takes place during the proofing step. Proofing is usually done in cabinets or rooms between 38°C and 42°C, dough temperatures near the optimum for *S. cerevisiae* (36°C to 39°C). Proofing rooms are also maintained at high relative humidity (>85%). Total proofing times vary, depending on temperature, but are usually about one hour. The increase in dough volume, measured as the height of the loaf, is often used to determine when the dough is sufficiently proofed.

BAKING

A remarkable confluence of physical, chemical, and biological events occurs when the dough loaves are placed into a hot oven. Into the oven goes a glutinous, sticky, spongy mass, with a pronounced yeasty aroma and inedible character, and out comes an airy, open-textured material, with a unique aroma and complex flavor. It is a transformation like none other in food science.

Smaller bakeries may use conventional ovens or reel ovens having Ferris wheel-like configurations. However, most modern commercial bakers use continuous conveyer-type ovens, rather than batch-type, constant temperature ovens. Most of these tunnel-like ovens are configured as single or double-lap types, in which the dough loaves are loaded and unloaded (after baking) at the same end. Alternatively, in non-lap type ovens, the dough is fed at one end and the baked breads are discharged from the other end. It usually takes about 25 to 28 minutes for a loaf to traverse through the oven.

The temperature within these ovens is not constant, but rather increases in several stages along the route, according to the conditions specified by the manufacturer. Typically, they start at about 200°C for six to eight minutes, and then increase to about 240°C for the next twelve to fourteen minutes. Finally, the temperature is reduced slightly, to about 220°C to 235°C for the remaining four to eight minutes. It should be evident that the temperature of the dough itself will always be less than the oven temperature. The actual temperature of the loaf during baking depends on heat transfer kinetics and all of the factors that affect these kinetics. Thus, dough composition, moisture content, loaf size and shape, and the type or

form of heating applied all affect the temperature within the dough and at the surface. Although ovens may transfer heat by convection currents, via the air and water vapor, heat transfer within the dough occurs via conduction. Thus, there is a decided temperature gradient within the loaf, with the interior being much cooler than the surface. Moreover, many of the readily observed physical changes that occur in the bread during heating are localized, the formation of crust color being the most obvious example.

Several events occur rapidly when the dough is placed into the first stage or zone of the oven. First, as the interior dough temperature increases (about 5°C per minute), there is an immediate increase in loaf volume or "oven spring" due to expansion of the heated CO_2. When the dough temperature reaches 60°C, all of the dissolved CO_2 is evolved, causing further expansion of the dough. Ethanol also is volatilized and lost to the atmosphere. At very near the same time, there is a transitory increase in yeast and amylase activity, at least up to dough temperatures of 55°C to 60°C, at which point yeasts begin to die. Starch swelling and gelatinization increases, and the gluten begins to dehydrate and denature, causing the bread to become more rigid and more structured. All of these events occur within about six to seven minutes.

In the meantime, as the surface temperature reaches close to 100°C, the early stages of crust formation will have begun. During the next heating stage, evaporation of water, gelatinization of starch, and denaturation and coagulation of gluten continue. The remaining enzymes and microorganisms are inactivated as the internal temperature approaches 80°C. Finally, when the temperature reaches 95°C, the dough takes on a crumb-like texture and the crust become firmer, due to dehydration at the surface. A brown crust color is formed as a result of caramelization and Maillard non-enzymatic browning reactions. These reactions also generate volatile flavor and aroma compounds.

Cooling and packaging

Once out of the oven, the baked bread is susceptible to microbial spoilage. Therefore, cooling must be performed under conditions in which exposure to airborne microorganisms, particularly mold spores, is minimized. The bread must also be sufficiently cool prior to packaging such that condensate will not form inside the package, a situation that could also lead to microbial problems. Various cooling systems are used, including tunnel-type conveyers in which slightly cool air passes counter-current to the direction of the bread, as well as forced air, rack-type coolers. For sandwich breads, the cooled loaves are sliced by continuous slicing machines. Ultimately, most commercial breads are packaged in moisture impermeable polyethylene bags.

MODERN BREAD TECHNOLOGY

The very general process described above covers all of the essential bread-making steps, but how they are specifically conducted in actual practice depends on several factors, including the scale of the operation and the style of bread being produced. Although hundreds of variations exist, most leavened breads are manufactured by one of four general processes (Figure 9.7). These processes vary in several respects, including how the ingredients are mixed, where and for how long the fermentation occurs, and the overall time involved in the manufacturing process. Of course, how the bread is made also has a profound effect on the quality characteristics of the finished product.

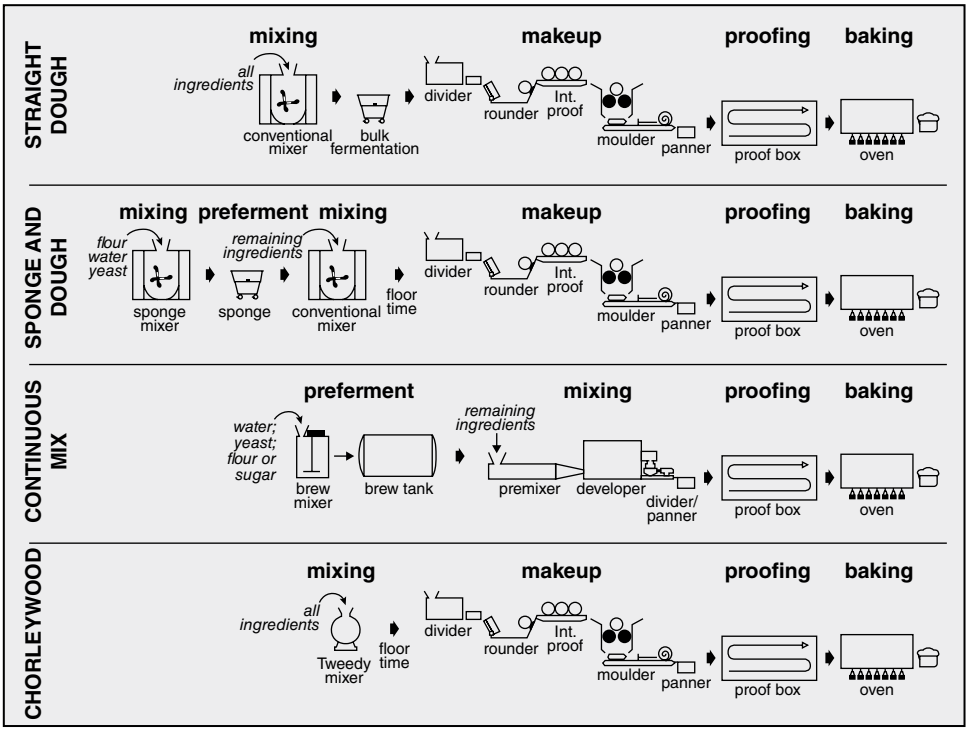


Figure 9.7 Major bread manufacturing processes. From Lallemand Inc., with permission.

Straight dough process

The homemade, one-batch-at-a-time method described in the above section is generally referred to as the straight method or straight dough process. Basically, the procedure involves mixing all of the ingredients and then allowing the dough to ferment for several hours (with intermittent punching down). The developed dough is then divided, formed into round balls, given a brief or intermediate proof, shaped into loaves, and placed into baking pans. Finally, after a final fermentation or proof, the bread is baked. Except for small or specialty bakeries, the straight dough method is little used by the bread industry.

The straight dough process results in breads that are chewy, with coarse cell structure and moderate flavor. Although the quality of these breads is quite good, it is a rather laborious process. Another limitation of this process is that it lacks flexibility and is sensitive to time. In other words, when the fermentation is complete, the dough must soon be baked. Otherwise, if fermentation is prolonged, the bread will be yeasty, excess air cells will be formed, and the structure will be weak.

Sponge and dough process

A more commonly used method in the bread industry is the sponge and dough process. It is used by small, medium, and even some large bakeries in the United States, the United Kingdom, and Europe. The basic principle of this process relies on the use of a "sponge," a partially concentrated portion of a flour-water dough that is allowed to ferment and then is

mixed with the remaining dough ingredients. The main advantage of this method is it that it is tolerant to time. In other words, once the sponge is developed, it does not have to be used immediately, but rather can be used over a period of time. Also, bread made by this method has a fine cell structure and well developed flavor.

The process starts by making a sponge that consists of part of the flour (60%–70%), part of the water (40%), and all of the yeast and yeast nutrients. The sponge is mixed and allowed to ferment for three to six hours at 16°C to 18°C (80°F). The remaining ingredients are then added and mixed, and the dough is allowed to develop. After a brief floor time period, the dough is then divided, molded, panned, given a final proof, and baked.

Liquid sponge process

The traditional methods of bread making, as described above for the straight dough and sponge and dough methods, require sufficient time for the initial or bulk fermentation to occur and for the dough to develop. In the past several decades, an emphasis on speed and economy of scale has led to newer methods of bread manufacture. These methods rely less on bulk fermentation and natural dough development, and more on mechanical dough development and a relatively short fermentation period. The straight dough method, for example, can be shortened considerably by adding more yeast and giving the dough just a few minutes of floor time, or perhaps even eliminating the bulk fermentation step altogether. The sponge and dough method can be similarly modified by reducing the flour-to-water ratio in the sponge (i.e., adding more water), thereby shortening the pre-fermentation time and producing a more pumpable sponge.

One of the best examples of the quick type of process is the continuous bread-making procedure, sometimes called the liquid sponge process. Developed in the 1960s, it was adopted by large-scale bakeries in the United States to enhance throughput and to mass produce loaves in as short a time as possible. The method saved time and labor and was very economical. As much as 35% to 50% of the industry used this method, but its popularity eventually waned, and now more bread is probably made by other methods. In principle, the process is simply an extension of the sponge-and-dough rationale.

Instead of making a thick sponge (i.e., mostly flour), a thin, liquid sponge (mostly water) is made. This mixture of water, sugar, yeast, yeast nutrients, and a very small portion of the flour is allowed to ferment in a tank to form a liquid "pre-ferment." This pre-fermented material is then metered into high speed mixers, where it is rapidly mixed with the remaining dough ingredients. The dough is then divided, panned, given a final proof, and baked.

Although the process is fast – three hours versus up to six hours for sponge and dough methods, and produces bread with a uniform structure and texture, many experts have argued that bread quality is not nearly as good as that made by other methods. By growing mainly in the liquid material and not in the actual dough, the yeasts have less time to generate flavor compounds or to react with flour components. Moreover, yeasts perform other functions in the dough that are minimized by limiting contact time. For example, they have an oxidizing effect on gluten proteins during fermentation in the straight dough or sponge and dough methods, leading to increased elasticity and stronger dough structure. This is lost in the continuous mix system. Thus, these breads are typically softer and spongy, less elastic, and have a weak, cake-like texture. The flavor is rather bland and not well-developed. One noted bread researcher suggested that these breads are simply wrappers for meats, cheeses, and jams. Today, continuous mix systems are used almost exclusively for hot dog and hamburger buns.

Chorleywood process

In the United Kingdom, a similar technology, called the Chorleywood process was developed in the 1970s. It is a rapid, high throughput method and is probably the main commercial system for bread manufacture. This is a no-time process in which rapid mixing is a critical feature. Whatever fermentation there is, prior to the final proof, occurs during the brief floor time (at temperatures near 40°C). Thus, the start-to-finish process takes only two hours.

SOURDOUGH FERMENTATION

Most yeast breads have a slightly acidic pH, due, in part, to organic acids produced by the yeast and, to a lesser extent, to dissolved CO_2 in the dough. In addition, acids also are produced by lactic acid bacteria that ordinarily are present in the flour, in the yeast preparations, or that simply naturally contaminate the dough. However, the presence of endogenous lactic acid bacteria usually has only a modest effect on dough and bread acidity. The perception of sourness for most consumers does not occur unless the pH is near 4.0, which is far lower than the normal pH range of 5.0 to 6.0 for most yeast breads. However, if lactic acid bacteria are deliberately added and allowed to grow, enough acids can be produced to lower the pH to 4.0 or below. This results in a distinct, but appealing sour flavor. Not only do these breads have a unique flavor, but they are also better preserved due to the presence of organic acids and low pH. In fact, there are several functional advantages to the sourdough fermentation, particularly for breads made from rye and other flours (Box 9.4).

Box 9.4 Beyond sourness: Functional advantages of sourdoughs

Although a desirable sour flavor is likely the most noticeable property of sourdough breads, the use of sourdough cultures in bread manufacture offers other important advantages (Gobbetti et al., 2014; Table 9.4.1). Arguably, the most important benefit of the sourdough fermentation is due to the organic acids that are produced by the sourdough bacteria and the ensuing low pH that results in enhanced preservation and increased shelf-life of sourdough breads. However, the sourdough fermentation also has a major impact on dough functionality, in particular, when rye or other whole grain flours are used as an ingredient.

Next to wheat, rye is the second most common cereal grain used to make bread. Rye, however, has properties that pose particular challenges when used in bread making. First, rye contains a high concentration of pentosans, a heterogenous mixture of pentose-containing polysaccharides consisting mostly of xylose and arabinose. They constitute as much as 10% of rye flour, which is four to five times more than that found in wheat flour.

In bread making, the pentosans may have both positive and negative effects. For example, pentosans have high water-binding capacity and may, therefore, decrease retrogradation and delay staling. On the other hand, pentosans may interfere with gluten formation, giving an inelastic dough that retains gas poorly. Perhaps the major problem with rye is that the rye proteins do not form a viscoelastic dough. As a result, breads made with rye as the main grain typically have a small loaf volume and a dense crumb texture. In addition, rye flour contains more α-amylase than is present in wheat,

Table 9.4.1 Functional advantages of sourdough cultures.

Property	Examples
Reduce anti-nutritional factors	Increased phytase activity
Enhanced nutritional value	Digest proteins
	Synthesize GABA
	Increased antioxidant activity
	Increased mineral availability
	Increased gluten tolerance
	Lower glycaemic index
Sensory properties	Unique sour flavor
	Increased loaf volume
	Improved crumb softness
	Improved texture
Shelf-life	Anti-fungal
	Reduced staling rate

Adapted from Arendt and Moroni 2013; Gobbetti et al., 2014; and Corsetti, 2013.

and this amylase is particularly active at the temperature at which starch gelatinizes. This results in excessive starch hydrolysis in the dough and bread, giving a poor texture and further reducing loaf volume.

The addition of sourdough cultures to rye doughs can compensate, to a large extent, for these complications. First, as the pH decreases due to the lactic fermentation, the pentosans become more soluble. They also begin to swell and form a gluten-like network that enhances dough elasticity and gas retention. In other words, at low pH, pentosans assume the role normally performed by gluten. In addition, the sourdough organisms are stimulated in rye flours by the availability of free sugars liberated from starch via the α-amylase. Moreover, this enzyme begins to lose activity at the low pH values achieved during the sourdough fermentation, so excessive hydrolysis is avoided. Some sourdough lactobacilli also have the ability to ferment pentoses released from pentosans, producing heterofermentative end products, including acetic acid (Gänzle, 2014). Finally, the subsequent acidic conditions enhance the water-binding capacity of the starch granules, which further decreases the rate at which staling occurs.

Another potential problem in breads made from rye and other whole grain flours is due to the presence of phytic acid. Phytic acid (also called myoinositol hexaphosphate) is a highly phosphorylated, negatively charged molecule that is capable of binding zinc, iron, calcium, and other divalent metal cations, preventing their absorption. Thus, phytic acid, by reducing the bioavailability of essential minerals, has "anti-nutritional" properties. Cereal grains can contain as much as 4% phytic acid, and although levels in whole grain bread are usually less than 0.2%, this is still enough to be a nutritional concern.

Degradation of phytic acid in bread ordinarily occurs via the enzyme phytase, which is present in flour and may also be produced by yeasts. Sourdough bacteria also produce this enzyme (Mamhoud et al., 2016; Nuobariene et al., 2015). Moreover, many of the phytases produced by sourdough lactobacilli have high activity at low pH

(De Angelis et al., 2003). Thus, the sourdough fermentation may enhance the nutritional quality of rye and other phytic acid-containing whole grain breads.

References

Arendt, E.K. and A.V. Moroni. 2013. Sourdough and gluten-free products. In: M. Gobetti and M.G. Gänzle, eds. Handbook on Sourdough Biotechnology. Springer, New York, pp. 245–264.

Corsetti, A., 2013. Technology of sourdough fermentation and sourdough applications. In: M. Gobetti and M.G. Gänzle, eds. Handbook on Sourdough Biotechnology. Springer, New York, pp. 85–103.

De Angelis, M., G. Gallo, M.R. Corbo, P.L.H. McSweeney, M. Faccia, M. Giovine, and M. Gobbetti. 2003. Phytase activity in sourdough lactic acid bacteria: purification and characterization of a phytase from *Lactobacillus sanfranciscensis* CB1. Int. J. Food Microbiol. 87:259–270.

Gänzle, M.G., 2014. Enzymatic and bacterial conversions during sourdough fermentation. Food Microbiol. 37:2–10.

Gobbetti, M., C.G. Rizzello, R. Di Cagno, and M. De Angelis. 2014. How the sourdough may affect the functional features of leavened baked goods. Food Microbiol. 37:30–40.

Mamhoud, A., L. Nionelli, T. Bouzaine, M. Hamdi, M. Gobbetti, and C.G. Rizzello. 2016. Selection of lactic acid bacteria isolated from Tunisian cereals and exploitation of the use as starters for sourdough fermentation. Int. J. Food Microbiol. 225:9–19.

Nuobariene, L., D. Cizeikiene, E. Gradzeviciute, Å.S. Hansena, S.K. Rasmussen, G. Juodeikiene, and F.K. Vogensen. 2015. Phytase-active lactic acid bacteria from sourdoughs: isolation and identification. LWT – Food Sci. Technol. 63:766–772.

Despite the considerable rather recent academic and commercial interest in sourdough breads, they have been made for many centuries. In fact, it seems rather certain that the very first breads made thousands of years ago were sourdough breads. It is also probable that even as recently as 100 or so years ago sourdough breads were the main type of bread consumed throughout Europe and North America. This is partly because baker's yeast cultures were only developed and introduced commercially only in the early 1900s. This necessarily meant that most bread was made by natural fermentations using wild cultures that inevitably contained lactic acid bacteria. Among the strains likely present would have been heterofermentative strains that could produce sufficient CO_2 to cause the dough to rise. In addition, most wild sourdough cultures would also have contained wild yeasts that would further contribute to the leavening process. These mixed bacteria-yeast wild type cultures could easily be maintained and used for many years, if not decades.

Only in the past 40 years, however, have the lactic acid bacteria and yeasts that participate in the sourdough fermentation been identified. In the early 1970s, researchers at the USDA Western Regional Research Laboratory in Albany, California (located about ten miles from San Francisco) isolated a strain of *Lactobacillus* from a commercially-manufactured, locally-produced sourdough sponge. The isolate appeared to be a unique species, based on its physiological properties and DNA–DNA hybridization, leading the discoverers to propose the name, *Lactobacillus sanfrancisco* (later changed to *Lactobacilllus sanfranciscensis*). This organism, which became known as the sourdough bacterium, was also present in sourdough sponges from other area bakeries. Of course, many other lactic acid bacteria have since been isolated from sourdough sponges. Interestingly, most of the isolated sourdough lactobacilli are functionally similar to one another, even if they represent different species and were isolated from different regions.

In addition to having isolated *L. sanfranciscensis* from sourdoughs, the same USDA researchers discovered that the sourdough sponge also contained a unique yeast strain. This strain was identified as *Torulopsis holmii*, the imperfect form of a *Saccharomyces* species that was once classified as *Saccharomyces exiguus*. This strain was later re-classified as *Candida milleri*. This yeast was found in other *L. sanfranciscensis*-containing sourdough sponges from the local area, and nearly always in a yeast:bacteria ratio of about 1:100. Similar yeast-bacteria duos have subsequently been isolated from sourdoughs from around the world. That these organisms are found consistently in sourdough breads from distant geographical locations reflects their remarkable adaptation to the dough environment (Box 9.5). This also accounts for the durability and stability of sourdough sponges, some of which have reportedly been carried for more than 100 years.

Box 9.5 The sourdough fermentation – it takes a two to tango

Almost all wild sourdough cultures, especially those that have been maintained for a long time, contain both lactic acid bacteria and yeasts. Although many species of lactic acid bacteria have been isolated from sourdoughs, the most common are species of *Lactobacillus,* including *Lactobacillus sanfranciscensis, Lactobacillus brevis,* and *Lactobacillus plantarum* (De Vuyst et al., 2009). Likewise, most sourdough yeasts belong to the genera, *Saccharomyces*, *Candida,* and *Kazachstania*. It is rather remarkable that the interaction between wild bacteria and wild yeast is not only stable but also repeatable. Perhaps even more interesting from an ecological perspective is that they also appear to observe a non-compete clause, sharing the available resources present in the dough.

Both physiological processes and biochemical events are responsible for the stable, cooperative relationship between the sourdough lactic acid bacteria and yeasts (Kline and Sugihara, 1971; and Sugihara et al., 1971). First, the *Lactobacillus* spp. are usually heterofermentative, producing acetic and lactic acids, as well as CO_2. The pH of the dough can drop to as low as 3.5, a pH that is inhibitory to competing organisms, including traditional baker's yeast strains of *Saccharomyces cerevisiae*. In contrast, sourdough yeasts (e.g., *Candida humilis* and related species) is acid-tolerant and benefits by the lack of competition from other acid-sensitive yeasts.

There is also a fascinating metabolic component to the *Lactobacillus*–yeast partnership (Gobbetti, 1998). Most of the fermentable carbohydrate found in sourdough is in the form of maltose. Whereas *S. cerevisiae*, the ordinary baker's yeast, readily ferments maltose, *Candida* and other sourdough yeast are unable to ferment this sugar, leaving this organism without an obvious substrate for growth. In contrast, *L. sanfranciscensis* and other sourdough bacteria not only prefers maltose as a carbohydrate source, but ferments this sugar in a particularly relevant manner.

One might expect that metabolism of the disaccharide maltose would result in both glucose moieties being fermented. However, when *L. sanfranciscensis* grows on maltose it uses the cytoplasmic enzyme maltose phosphorylase to hydrolyze accumulated maltose, yielding free glucose and glucose-1-phosphate. The latter is isomerized to glucose-6-phosphate, which then feeds directly into the phosphoketolase pathway. The glucose generated by the reaction is not further metabolized, but rather is released

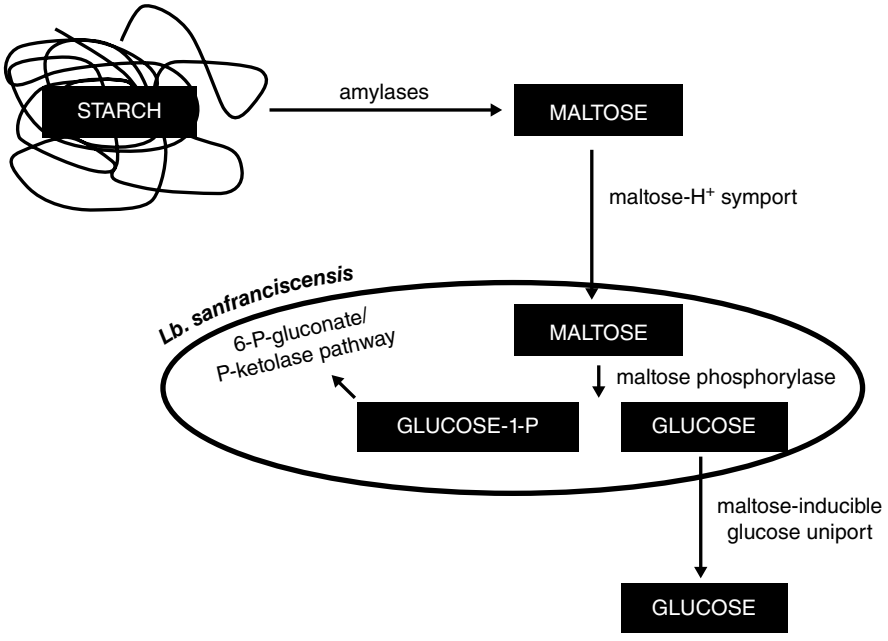


Figure 9.5.1 Maltose utilization by *L. sanfranciscensis*. From Corsetti and Settanni, 2007, with permission.

into the extracellular medium, or in this case, the dough (Figure 9.5.1). The net effect of this unique metabolic situation is that maltose is available for the sourdough bacteria, glucose is available for the sourdough yeast, and neither organism competes against each other for fermentation substrates.

References

Gobbetti, M., 1998. The sourdough microflora: interactions of lactic acid bacteria and yeasts. Trends Food Sci. Technol. 9:267–274.

Corsetti, A. and L. Settanni. 2007. Lactobacilli in sourdough fermentation. Food Res. Int. 40:539–558.

De Vuyst, L., G. Vrancken, F. Ravyts, T. Rimaux, and S. Weckx. 2009. Biodiversity, ecological determinants, and metabolic exploitation of sourdough microbiota. Food Microbiol. 26:666–675.

Kline, L. and T.F. Sugihara. 1971. Microorganisms of the San Francisco sour dough bread process. II. Isolation and characterization of undescribed bacterial species responsible for the souring activity. Appl. Microbiol. 21:459–465.

Sugihara, T.F., L. Kline, and M.W. Miller. 1971. Microorganisms of the San Francisco sour dough bread process. I. Yeasts responsible for the leavening action. Appl. Microbiol. 21:456–458.

Many traditional sourdough bread manufacturers continue to maintain their own particular sponge, which contains a mixture of undefined, yet distinctive strains. In fact, some artisanal bakeries consider their sourdough starters to be uniquely responsible for the quality of their bread and guard their cultures as valued assets. Analyses of these cultures have shown that

Table 9.4 Microorganisms isolated from sourdoughs[1].

Bacteria		Yeast
Lactobacillus alimentarius	*Lactobacillus plantarum*	*Saccharomyces cerevisiae*
Lactobacillus brevis	*Lactobacillus pontis*	*Candida milleri*
Lactobacillus buchneri	*Lactobacillus panis*	*Candida humolis*
Lactobacillus casei	*Lactobacillus reuteri*	*Debaryomyces hansenii*
Lactobacillus curvatus	*Lactobacillus sakei*	*Issatchenkia orientalis*
Lactobacillus delbrueckii	*Lactobacillus sanfranciscensis*	*Kazachstania exiga*
Lactobacillus fermentum	*Leuconostoc mesenteroides*	*Pichica kudriavzevii*
Lactobacillus helveticus	*Pediococcus acidilactici*	*Torulaspora delbrueckii*
Lactobacillus hilgardii	*Pediococcus pentosaceus*	
Lactobacillus paracasei	*Weissella ciberia*	
Lactobacillus pentosus	*Weissella confusa*	

[1] Data adapted from de Vuyst, 2014.

they contain many different species of *Lactobacillus* and other lactic acid bacteria, as well as several species of yeast (Table 9.4). This diversity of sourdough starter strains likely accounts for the variety of sourdough breads produced by different bakeries.

Sourdough cultures, comprised of one or more defined strains, are now commercially available. In addition to *L. sanfranciscensis*, available strains include *L. brevis*, *L. delbrueckii*, and *L. plantarum*. Some of these strains are obligate homofermentors (e.g., *L. delbrueckii*), and although the sourdough breads made from these cultures are indeed sour, they may lack the characteristic acetic acid flavor and have a somewhat less complex flavor profile.

In actual practice, authentic sourdough breads are almost always made via a sponge and dough process. The initial sponge is maintained by regular (daily or even more frequent) additions of flour–water mixtures. Portions are removed and are successively built up by adding water–flour mixtures to the sponge followed by an incubation period, until the appropriate size is achieved. Incubations can be long (24 hours) or short (three hours), depending on the incubation temperature, type of flour, culture activity, and the presence of salt. The sponge can be held for a period of time, and is eventually mixed with the remaining dough ingredients at a rate of about 10% to 20% of the total dough mixture. The conditions for sponge preparation can have a profound effect on the types and proportions of microorganisms present within the sponge as well as the properties of the dough and quality of the bread.

It should be noted that so-called sourdough bread can be made simply by adding sourdough concentrates, vinegar, or combinations of acetic and lactic acids directly to the dough. This eliminates the need for an actual sourdough fermentation step. Although such breads are quite common in the retail market, they certainly lack the complex flavor and functional characteristics associated with authentic sourdough bread. Alternatively, some baker's may add baker's yeast to the sourdough to accelerate production of CO_2. While this practice may shorten the time necessary for dough development and leavening, it likely results in bread with less sourdough flavor.

BREAD SPOILAGE AND PRESERVATION

Despite the fact that bread has a moderately low water activity (about 0.95) and pH (about 5.0-6.0) and contains few if any viable microorganisms when it leaves the oven, it is still,

relative to other fermented foods, a highly perishable product. This is because the shelf-life of bread depends not only on microbial activities, but also on physical–chemical changes in the bread. Specifically, it is the phenomenon called staling that most frequently causes consumers to reject bread products. For some fresh baked products stored under ambient conditions, shelf-life can be as short as just two or three days, due to the onset of staling. However, the longer the bread is held, the more likely it is that microorganisms, and fungi, in particular, will grow and produce visible and highly objectionable appearance defects. Thus, maintaining bread in a fresh condition remains a major challenge for the bread industry.

Staling

To say that staling is a complicated phenomenon would be an understatement. Entire books and extensive review articles have been written on this topic alone. Recently, however, thanks to the application of modern molecular methods, including infrared, near infrared, and nuclear magnetic resonance spectroscopy, X-ray crystallography, and various microscopic techniques, a clearer understanding of the details involved in staling has emerged.

Simply stated, staling refers to the increase in crumb firmness that makes the bread undesirable to consumers. Staling is also associated with an increase in crust softness (almost "rubbery") and a decrease in fresh bread flavor. Despite the complicated physical and chemical reactions occurring during staling, the staling phenomenon is essentially a starch structure and moisture migration problem. The reactions that eventually lead to staling actually start when the bread is baked, as starch granules in the dough begin to adsorb water, gelatinize, and swell. The amylose and amylopectin chains separate from one another and become more soluble and less ordered. Then, when the bread is cooled, these starch molecules, and the amylopectin, in particular, slowly begin to re-associate and re-crystallize (see below). This process, called retrogradation, results in an increase in firmness due to the rigid structures that form. Amylose retrogrades rapidly upon cooling, while amylopectin retogrades slowly. It is the slow retrogradation of amylopectin that is now thought to be primarily associated with staling of the crumb. Furthermore, moisture migration from starch to gluten and from crumb to crust make the crumb dryer and more firm. Although staling is an inevitable process, a number of strategies have been adopted to delay these reactions and extend the shelf-life of bread (Box 9.6).

Box 9.6 Fresh ideas for controlling staling of bread

Fresh bread reaches the end of shelf-life for two main reasons (Cauvain, 2015). One is microbiological, due mainly to fungal spoilage. Perhaps equally common is the complex physical-chemical phenomenon known as staling. So important is this staling phenomenon to the bread industry that numerous reviews, chapters, and entire books have been written to describe the mechanisms responsible for bread staling (Fadda et al., 2014).

Staling is now known to occur as a result of starch retrogradation (Goesaert et al., 2009). When the dough is first heated, the starch gelatinizes and becomes unstructured (Figure 9.6.1). Upon cooling, however, the starch (mainly amylopectin) reorganizes or retrogrades, resulting in rigid, crystalline structures. Intramolecular hydrogen bonding

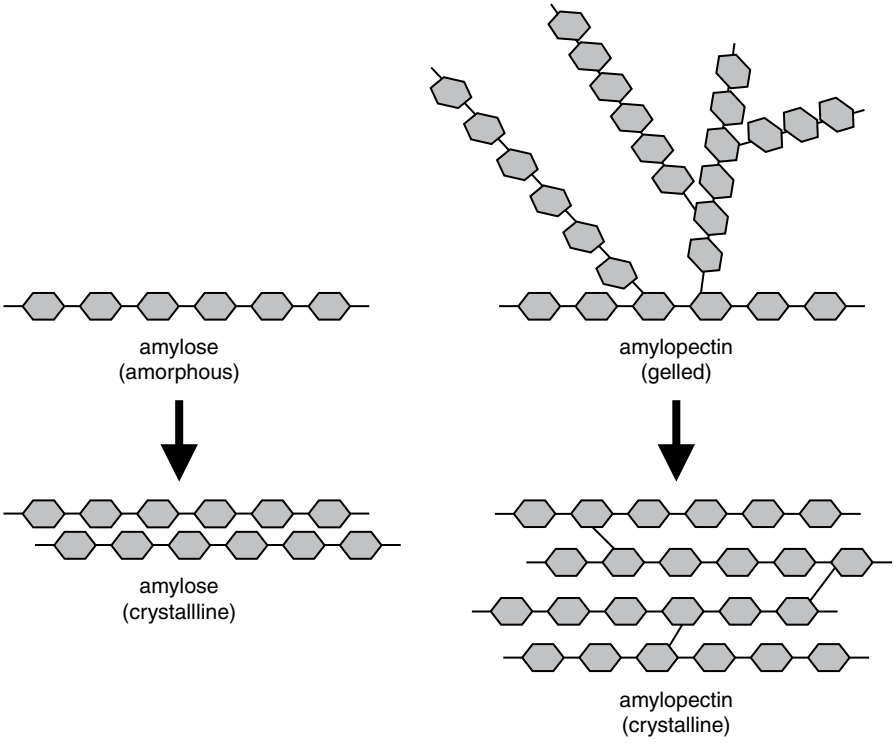


Figure 9.6.1 Model of bread staling. In fresh bread, amylose and amylopectin exist in amorphous or gelled forms. During storage, moisture is lost, the amylose and amylopectin retrograde, and crystalline forms appear, leading to firmness and staling. Adapted from Goesaert et al., 2009 and Lallemand, Baking Update, Volume 1/Number 6.

between amylopectin branches also occurs, causing additional structural rigidity. In addition, the outer chains of adjacent amylopectin molecules may form double helices. Efforts to reduce staling, therefore, necessarily involve reducing the rate at which these crystallization and hydrogen-binding reactions occur.

Because staling is influenced by many factors (Table 9.6.1), no single approach aimed at delaying or preventing staling is likely to be completely effective. Rather, bread manufacturers have adopted a more multifaceted strategy that considers the entire bread-making process, from ingredient selection and product formulation to processing and packaging steps (Fadda et al., 2014). In the discussion below, the role of ingredients, enzymes, and processing conditions on controlling staling are described.

Ingredients. Anti-staling ingredients are generally intended to either retard retrogradation rates and/or soften bread crumb and crust. Included are surfactants and other emulsifying agents, as well as various carbohydrates. Mono- and diglycerides are probably the most common surfactant-type agents used by the bread industry and are among the most effective anti-staling agents. They function by binding to amylose and

Table 9.6.1 Factors affecting staling.

Factor	Effect
Ingredients	
Flour	High protein flours maintain crumb softness
Fats	Delays staling by increasing loaf volume
Surfactants	Increases crumb softness
Enzymes	Amylases (mainly α) hydrolyze starch and reduce retrogradation
Moisture	Low moisture increases staling
Packaging	Prevents moisture loss and crumb firming
Manufacturing	Long fermentation times increase loaf volume and softness
Baking temperature	High temperature, short time baking increases staling rates
Storage temperature	Refrigeration increases staling, freezing decreases staling rates

amylopectin and forming complexes with lipids, thereby reducing starch retrogradation (Knightly, 1996). In contrast, hydrocolloids and other carbohydrate-based agents act by binding water and thereby maintaining moisture content (Fadda et al., 2015).

Enzymes. Enzymes are widely used by the bread industry. Amylases, in particular, are often used to hydrolyze starch and to increase the concentration of fermentable sugars in the dough. These sugars can also contribute to desirable flavor and color changes via the Maillard reaction. Amylases are also used to improve functional properties of dough and bread. In particular, amylases reduce dough viscosity during starch gelatinization and delay crumb firming (Goesaert et al., 2009). Importantly, specific α-amylases also reduce staling by their ability to hydrolyze amylopectin and limit formation of the crystalline network that would otherwise form. Similarly, water immobilization is also reduced.

Most anti-staling α-amylases are derived from either bacterial or fungal sources (produced by species of *Bacillus* and *Aspergillus*). These enzymes have optimum activity within a moderately high temperature range (50°C to 70°C), and are most active during the early stages of baking. However, bacterial α-amylases are generally thermal-resistant and are inactivated only at high baking temperatures (>80°C).

If too much hydrolysis occurs during dough formation and baking, the dough will be gummy and sticky, and the baked bread may also have a similar texture. Depending on the specific application, residual activity may remain in the bread even after baking, resulting in an increase in starch hydrolysis and free sugars during storage. Thus, there is interest in identifying α-amylases that are less thermal-resistant. Although the fungal-produced α-amylases are more heat-labile (or have intermediate heat tolerance) than bacterial enzymes, they typically have less activity at the baking temperatures normally used.

Processing. The manner in which the dough is mixed and fermented has considerable impact on bread staling. In general, bread freshness may be poorly retained during storage if the dough is over-mixed or under-mixed. Likewise, if the

fermentation time is too short or too long, bread freshness is reduced. Thus, all other factors being equal, bread made from no-time doughs or long-time (i.e., straight) doughs usually will be less soft than bread made using continuous or sponge and dough processes.

References

Cauvain, S., 2015. Bread spoilage and staling. In: Technology of Breadmaking. Springer International Publishing, New York, pp. 279–302.
Fadda, C., A.M. Sanguinetti, A. Del Caro, C. Collar, and A. Piga. 2014. Bread staling: updating the view. Comp. Rev. Food Sci. Food Safety 13:473–492.
Goesaert, H., L. Slade, H. Levine, and J.A. Delcour. 2009. Amylases and bread firming – an integrated view. J. Cereal Sci. 50:345–352.
Knightly, W.H. 1996. Surfactants. In: Hebeda, R.E. and H. F. Zobel, eds. Baked goods freshness. Marcel Dekker, Inc. New York, pp. 65–103.

Biological spoilage

Microbiological spoilage of bread is most often associated with fungi, and occurs when fungal mycelia are visible to the consumer. In addition, some strains of *Bacillus subtilis, Bacillus mesentericus*, and *Bacillus licheniformis* can spoil high-moisture breads via production of an extracellular capsule material that gives the infected bread a mucoid or ropy texture. There are also wild yeasts capable of causing flavor defects in bread after baking. However, bacterial and yeast spoilage of bread are both relatively rare. Fungi are, by far, the most common microbial cause of bread spoilage (Table 9.5). In large part, this is because the

Table 9.5 Spoilage organisms of bread.

Organism	Appearance or defect
Fungi	
Aspergillus niger	Black
Aspergillus glaucus	Green, Grey-green
Aspergillus flavus	Olive green
Penicillium sp.	Blue-green
Rhizopus nigricans	Grey-black
Mucor sp.	Grey
Neurospora sitophila	Pink
Yeasts	
Saccharomyces cerevisiae	Alcoholic-ester off-odors
Pichia burtonii	White, chalky appearance
Bacteria	
Bacillus subtilus	Ropy
Bacillus licheniformis	Ropy
Bacillus mesentericus	Ropy

Adapted from Pateras, 2007.

water activity of bread is usually less than 0.96, which is below the minimum for typical spoilage bacteria. In addition, baking ordinarily kills potential spoilage bacteria, the exception being spore-forming bacilli mentioned above.

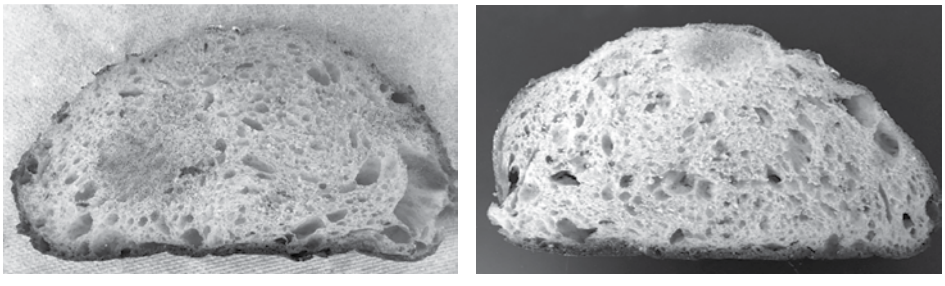
The baking process also kills fungi and their spores. Thus, when molds are present in bread, it is invariably a result of post-processing contamination. Fungal spores are particularly widespread in bakeries due to their presence in flour and their ability to spread throughout the production environment via air movement. When the baked breads leave the oven, their transit through the cooling, slicing, and packaging operations provide ample opportunity for infection, either indirectly by airborne spores or directly by contact with contaminated equipment. Fungi are aerobic and ordinarily grow only on the surface of loaf bread. However, slicing exposes the internal surfaces to mold spores, enabling growth within the loaf. Once packaged, moisture loss in the bread is negligible; thus, the water activity remains well within the range necessary for growth of fungi. Bread that is packaged while still warm is very susceptible to mold growth, due to localized areas of condensate that form within the package.

Although there are many different fungi associated with bread spoilage, the most common are genera of *Penicillium*, *Aspergillus*, *Mucor*, and *Rhizopus*. Bread serves as an excellent growth substrate for these fungi; visible mold growth may appear within just a few days. What the consumer actually sees is a combination of vegetative cell growth (the mycelia), along with sporulating bodies. The latter are responsible for the characteristic blue-green or black color normally associated with mold growth (Figure 9.8).

The ability of fungi to grow on bread and which species predominate depend on several factors, including bread pH, water activity, storage temperature, and atmosphere. Finally, it is important to recognize that some mold strains not only can grow on bread, causing spoilage and economic loss, but, under certain conditions, specific strains can also produce mycotoxins. Fortunately, visible mold growth ordinarily precedes mycotoxin formation, so in the very unlikely event that a mycotoxin were present, most consumers would reject the product before ingesting it. There have been, however, occasions where mycotoxin-contaminated breads were consumed. These historically documented cases were caused by the use of moldy grains and not bread that later became contaminated.

Controlling mold

Given that biological spoilage of bread is caused primarily by molds, it is not surprising that preservation strategies have focused on controlling fungi, both in production environments



(A) (B)

Figure 9.8 Spoilage of bread by fungi. Shown are Rhizopus (A) and Penicillium (B). Photos courtesy of Heather Hallen-Adams, University of Nebraska.

and in the finished product. As noted previously, mold and mold spores are present in flour and other raw materials and may be widespread in bakeries. Therefore, rigorous attention to plant design and sanitation is essential. The post-production environment (i.e., baked products) should be separated from pre-production environments. Air handling systems should be designed (including the use of filters or ultraviolet lamps and positive air pressure) such that airborne mold spores cannot gain entry to the product side. Water can also spread mold spores, so keeping the environment dry is also warranted.

Several different approaches have been adopted to control mold growth in bread. In the United States, the most common means of bread preservation is to use propionate salts. Calcium propionate is especially effective against most of the molds associated with bread, and is widely used in commercially-produced products. Sorbate and acetate salts are also used as anti-mycotic preservatives in bread. However, these acids also have inhibitory activity against baking yeasts, whereas propionates are much less inhibitory. Moreover, calcium propionate has no flavor or toxicity, is active even against rope-forming bacteria, and is effective in most varieties of bread. There are, however, some fungal strains (e.g., *Penicillium roquefortii*) that are associated with rye breads and that are insensitive to propionates.

Due to interest in chemical additive-free breads, alternative approaches for bread preservation have been considered. Bread can be exposed to ultraviolet, infrared, or microwave radiation to inactivate mold and mold spores or packaged in modified or vacuum atmospheres to inhibit their growth. These methods, however, are not widely used. In contrast, one effective method used for bread preservation that is very popular and does not involve addition of chemical preservatives instead relies on growth of lactic acid bacteria in the dough. As discussed above, these bacteria produce lactic and acetic acids and lower the pH to levels inhibitory to most fungi. Sourdough breads, especially those that have pH values near 4.0, rarely develop mold problems.

Another indirect way to extend the shelf-life of bread is via freezing. Many bread manufacturers freeze the baked and packaged breads as a means of preserving the bread prior to delivery. One popular freezing method that is to freeze un-baked or par-baked (i.e., half-baked) breads (Box 9.7). This process is gaining popularity, since it allows retailers to complete the baking and then sell "fresh"-baked breads (aroma and all) at the retail level.

Box 9.7 Some like it hot – the challenge of frozen bread doughs

Although artisanal, made-from-scratch bakeries appear to be everywhere, making bread this way is quite a challenge. It is a time-consuming job, requiring specialized equipment, as well as know-how, skill, and patience. For these reasons, frozen bread doughs have become hot sellers. These doughs are especially appealing to small retail bakery operations, grocery stores, and restaurants. This is because they eliminate the need for dough production equipment and labor, while at the same time making it possible to offer fresh-baked bread products to customers. Note that these breads are not quite the same as so-called par-baked bread. The latter are fully-proofed breads that are partially baked, frozen, and then re-baked to doneness by the end-user.

The actual handling of frozen doughs is quite simple for the end-user. The frozen dough is simply removed from the freezer, thawed overnight, given an all-important

final proof, and baked. Despite these simple instructions, however, the quality of breads made from frozen doughs can be quite variable.

Several reasons exist for the inconsistent quality of frozen doughs, including physical-chemical and rheological changes to the dough and gluten structure (Meziani et al., 2012; Öhgren et al., 2016). Another important factor affecting frozen dough quality is biological, specifically the decreased viability of yeast during frozen storage. Often, frozen doughs may require longer proof times, due to the cold-sensitivity of the yeast and extended lag times during proofing. However, the worst case scenario occurs when the yeasts are so cryosensitive that they fail to provide any leavening or gassing power at all following the dough-thawing step.

What makes some yeast strains sensitive to the effects of freezing? And what can be done to improve cryotolerance? These questions are now receiving much research attention, due to their practical implications (Ballester-Tomás et al., 2016; Randez-Gil et al., 2013).

One of the main reasons why viability of *Saccharomyces cerevisiae*, as well as other microorganisms, is reduced by freezing is due to ice crystal formation. Large ice crystals are particularly damaging to the physical integrity of the cells. At a molecular level, large ice crystals poke holes in cells causing irreparable damage. Crystal size is mainly a function of freezing rate, with slow freezer resulting in larger ice crystals. Ice crystal formation occurs both intracellularly and extracellularly and is also affected by storage temperature and time (Jia et al., 2016). Importantly, frozen doughs are often inadvertently exposed to freeze-thaw cycles, which can significantly increase crystal size and cause severe damage to cells. Ultimately, it should be clear that controlling ice crystal formation would be a potential strategy for maintaining yeast viability.

In general, two approaches have been applied to address this challenge. One is to add stabilizers, such as gums and other hydrocolloids, to the dough (Maity and Saxena, 2016; Selomulyo and Zhou, 2007). These agents bind water, depress freezing point, and reduce extracellular crystal formation and growth. They are commonly used in other frozen foods, especially ice cream, where ice crystal formation is also deleterious to product quality. In bread dough, therefore, hydrocolloids serve two functions – they retain physical integrity to gluten structure and they maintain yeast viability.

The other approach is based on the use of cryotolerant strains that are less prone to suffer damage during freezing and thawing of bread dough. In general, microorganisms protect themselves against freeze damage via the intracellular accumulation of cryoprotectant solutes. These molecules can be transported from the extracellular environment across the cytoplasmic membrane or they can be synthesized directly within the cytoplasm. Cryoprotectant solutes, much like osmoprotectants, are able to accumulate to high intracellular concentrations, without causing detrimental effects on the enzymatic or reproductive machinery within the cell. Ultimately, they act much like the stabilizers noted above, in that they re-structure bound water in the cytoplasm, such that ice crystal formation is reduced.

Microbial cryoprotectants are typically small, polar molecules and include several amines (e.g., betaine, glutamine, and proline), as well as glycerol and various sugars. In *S. cerevisiae*, the disaccharide trehalose (which consists of two glucose moieties linked α-1,1; Figure 9.7.1) is one of the more common cryoprotectants (Gerardo-Rodríguez et al., 2016; Rubio-Texeira et al., 2016; Sasano et al., 2012). When the temperature

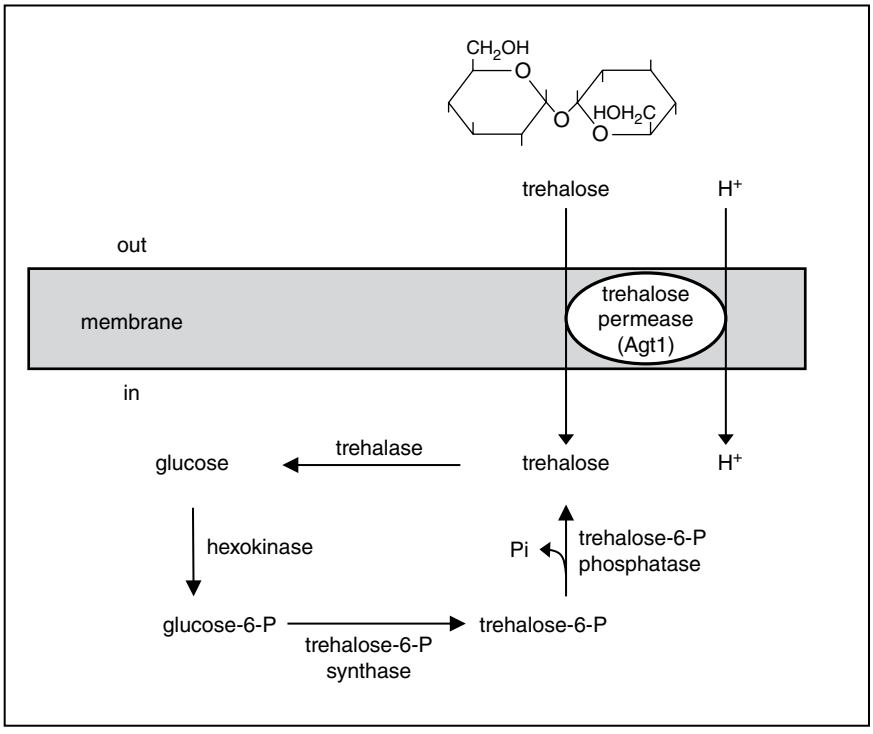


Figure 9.7.1 Metabolism of trehalose (α-D-glucosyl-1, 1-α-D-glucose) by *Saccharomyces cerevisiae*. Transport is mediated by a symport system, but biosynthesis from glucose may be the major route for intracellular accumulation. Trehalose not only alleviates cryo-stress, but it can also serve as a carbon source following its hydrolysis to glucose by the enzyme trehalase.

decreases (or when other environmental stresses are applied), the cell responds by increasing *de novo* synthesis of trehalose (Figure 9.7.1). Interestingly, trehalose synthesis and freeze-tolerance can also be increased by overexpression of an α-glucosidase (MAL26) that presumably generates free glucose from maltose (Sun et al., 2016).

Extracellular trehalose, if available, may also be transported via a high affinity trehalose transport system (Agt1). This symport system is essential for *Saccharomyces* to adapt to stress (Eleutherio et al., 2015; Gibney et al., 2015). Ultimately, intracellular levels can reach up to 20% of the total weight of the cell (on a dry basis).

Despite its importance in cryotolerance, trehalose is not the only cryoprotectant molecule accumulated by *S. cerevisiae.* As noted above, glycerol and proline can also serve this role. Thus, approaches similar to that described for trehalose have been used to enhance accumulation of these molecules and increase crytolerance in baker's yeast strains of *S. cerevisiae* (Ballester-Tomás et al., 2016; Dong et al., 2016; Kaino et al., 2008). Recently, a polymeric amine called γ-polyglutamic acid was tested as a baker's yeast cryoprotectant (Jia, et al., 2016). This is the same molecule synthesized by *Bacillus* in the manufacture of natto (Chapter 14). The authors suggested that in addition to its water holding capacity and anti-freeze activity, it also enhanced oxidation resistance.

Of course, it is also possible to compensate for any loss of activity due to freeze damage by simply increasing the amount of yeast. However, there is no assurance that this will be consistently successful.

References

Ballester-Tomás, L., R. Pérez-Torrado, S. Rodríguez-Vargas, J.A. Prieto, and F. Randez-Gil. 2016. Near-freezing effects on the proteome of industrial yeast strains of *Saccharomyces cerevisiae*. J. Biotechnol. 221:70–77.

Dong, J., D. Chen, G. Wang, C. Zhang, L. Du, S. Liu, Y. Zhao, and D. Xiao. 2016. Improving freeze-tolerance of baker's yeast through seamless gene deletion of NTH1 and PUT1. J. Ind. Microbiol. Biotechnol. 43:817–828.

Eleutherio, E., A. Panek, J.F. De Mesquita, E. Trevisol, and R. Magalhães. 2015. Revisiting yeast trehalose metabolism. Curr. Genet. 61:263–274.

Gerardo-Rodríguez, J.E., B. Ramírez-Wong, A.I. Ledesma-Osuna, C.L. Medina-Rodríguez, R. Ortega-Ramírez, and M.I. Silvas-García. 2016. Management of freezing rate and trehalose concentration to improve frozen dough properties and bread quality. Food Sci. Technol. (Campinas).

Gibney, P.A., A. Schielera, J.C. Chena, J.D. Rabinowitza, and D. Botstein. 2015. Characterizing the in vivo role of trehalose in *Saccharomyces cerevisiae* using the AGT1 transporter. PNAS 112:6116–6121.

Goesaert, H., L. Slade, H. Levine, and J.A. Delcour. 2009. Amylases and bread firming – an integrated view. J. Cereal Sci. 50:345–352.

Knightly, W.H. 1996. Surfactants. In: Hebeda, R.E. and H.F. Zobel, eds. Baked Goods Freshness. Marcel Dekker, Inc. New York, pp. 65–103.

Jia, C. W. Huang, X. Tang, S. Ding, W. Yang, Z. Li, G. Fu, and P. Rayas-Duarte. 2016. Antifreeze activity of γ-polyglutamic acid and its impact on freezing resistance of yeast and frozen sweet dough. Cereal. Chem. 93:306–313.

Kaino, T., T. Tateiwa, S. Mizukami-Murata, J. Shima, and H. Takagi. 2008. Self-cloning baker's yeasts that accumulate proline enhance freeze tolerance in doughs. Appl. Environ. Microbiol. 74:5845–5849.

Maity, T. and A. Saxena. 2016. Use of hydrocolloids as cryoprotectant for frozen foods. Crit. Rev. Food Sci. Nutr. DOI: 10.1080/10408398.2016.1182892.

Meziani, S., J. Jasniewski, P. Ribotta, E. Arab-Tehrany, J.-M. Muller, M. Ghoul, and S. Desobry. 2012. Influence of yeast and frozen storage on rheological, structural and microbial quality of frozen sweet dough. J. Food Eng. 109:538–544.

Öhgren, C., N. Fabregat, and M. Langton. 2016. Quality of bread baked from frozen dough - effects of rye, and sugar content, kneading time and proofing profile. LWT – Food Sci. Techn. 68:626–633.

Randez-Gil, F., I. Córcoles-Sáez, and J.A. Prieto. 2013. Genetic and phenotypic characteristics of baker's yeast: relevance to baking. Annu. Rev. Food Sci. Technol. 4:191–214.

Rubio-Texeira, M., G. Van Zeebroeck, and J.M. Thevelein, 2016. Trehalose Metabolism: Enzymatic Pathways and Physiological Functions. In Biochemistry and Molecular Biology. Springer International Publishing, 2016. 191–277.

Sasano, Y., Y. Haitani, K. Hashida, I. Ohtsu, J. Shima, and H. Takagi. 2012. Simultaneous accumulation of proline and trehalose in industrial baker's yeast enhances fermentation ability in frozen dough. J. Biosci. Bioeng. 113:592–595,

Selomulyo, V.O., and W. Zhou. 2007. Frozen bread dough: effects of freezing storage and dough improvers. J. Cereal Sci. 45:1–17.

Sun, X., C.-Y. Zhang, M.-Y. Wu, Z.-H. Fan, S.-N Liu, W.-B. Zhu, and D.-G. Xiao. 2016. MAL62 overexpression and NTH1 deletion enhance the freezing tolerance and fermentation capacity of the baker's yeast in lean dough. Microb. Cell Fact.15:54.

BREAD QUALITY

Assessing the quality of bread is a very subjective process. Cultural, ethnic, and personal attitudes certainly influence the sort of bread an individual prefers. For example, the hard, crusty baguette eaten in France bears little resemblance to the soft, doughy, plastic-wrapped French bread seemingly preferred by many US consumers. Whole grain peasant breads – so-named because centuries ago expensive refined flours went to the upper classes, while the poor were left with unrefined, whole flours – are now popular in specialty bakeries serving an affluent, upscale clientele.

The criteria commonly used to assess bread quality are based primarily on appearance, texture, and flavor attributes. Appearance refers both to external (i.e., crust), as well as internal appearance (i.e., crumb). Depending on the type of bread, the intact loaf should have a particular volume, or in some cases, height and length. The color of the crust is also an important property, and is judged mainly on the basis of the expected color established by the manufacturer. Internal appearance is also based, in part, on color, specifically, the absence of streaks or uneven color. The size, frequency, and distribution of air cells are important determinants of bread quality, especially if there are large, irregular-shaped holes that are often considered as defects.

In contrast to the appearance properties, which are difficult to assess objectively, methods do exist to measure texture properties. Instrumental devices, such as the Instron Universal Testing Machine, can be used to measure crumb softness, resistance, and compressibility. Other compression-type instruments also exist, such as the Baker Compressimeter and the Voland Stevens Texture Analyzer, which can be used to measure firming changes and staling rates. Various spectrophotometric methods, including near infrared and infrared spectroscopy can detect changes in starch crystallization and conformation, and are especially useful for measuring retrogradation and staling rates. However, X-ray diffraction is now generally considered the most definitive technique for detecting changes in crystallization.

Bread flavor is no less easy to describe or quantify. It can also be difficult to separate flavor from texture and other sensory attributes. In general, bread should have a fresh, somewhat yeasty flavor and aroma, with acidic and wheaty notes. Salt also provides an important flavor in bread, as do the alcohols, aldehydes, and other organic end products produced during yeast metabolism.

Many of the important flavor compounds of bread are formed during baking. The Maillard reaction alone results in more than 100 volatile aroma compounds. Importantly, these reactions also generate pigments and are responsible for crust color formation. Although both caramelization and Maillard reactions require heat, are non-enzymatic, and use sugars as the primary reactant, they differ in that caramelization reactions occur at higher temperatures than Maillard reactions. Also, only reducing sugars (and not sucrose) react in the Maillard reaction. The latter also requires primary amino acids. The Maillard reaction is probably predominant during baking, since the temperatures achieved generally are not high enough to induce extensive caramelization (except in the crust, which can exceed 300 C).

BIBLIOGRAPHY

Axel, C., E. Zannini, and E.K. Arendt. 2017. Mould spoilage of bread and its biopreservation: a review of current strategies for bread shelf life extension. Crit. Rev. Food Sci. Nutr. 57:3528–3542.

Calvel, R. 2001. The taste of bread. (R.L. Wirtz, translator). Aspen Publishers, Inc. Gaithersburg, Maryland.

Cauvain, S.P. 2007. Bread – the product. In: S.P. Cauvain and L.S. Young, eds. Technology of Breadmaking, 2nd edn. Springer, New York, pp. 1–19.

Cauvain, S.P. 2007. Breadmaking processes. In: S.P. Cauvain and L.S. Young, eds. Technology of Breadmaking, 2nd edn. Springer, New York, pp. 21–49.

De Vuyst, L., S. Van Kerrebroeck, H. Harth, G. Huys, H.-M. Daniel, and S. Weckx. 2014. Microbial ecology of sourdough fermentations: Diverse or uniform? Food Microbiol. 563:11–29.

Fadda, C., A.M. Sanguinetti, A. Del Caro, C. Collar, and A. Piga. 2014. Bread staling: updating the view. Compr. Rev. Food Sci. Food Saf. 13:473–492.

Gänzle, M. and V. Ripari. 2016. Composition and function of sourdough microbiota: from ecological theory to bread quality. Int. J. Food Microbiol. 239:19–25.

Gobbetti, M. and M. Gänzle. 2013. Handbook on Sourdough Biotechnology. Springer, New York.

Hui, Y.H., ed. 2008. Bakery Products: Science and Technology. John Wiley & Sons, Oxford.

Kline, L. and T.F. Sugihara. 1971. Microorganisms of the San Francisco sour dough bread process. II. Isolation and characterization of undescribed bacterial species responsible for the souring activity. Appl. Microbiol. 21:459–465.

Marques, W.L., V. Raghavendran, B.U. Stambuk, and A.K. Gombert. 2016. Sucrose and *Saccharomyces cerevisiae*: a relationship most sweet. FEMS Yeast Res. 16.

Mondal, A. and A.K. Datta. 2008. Bread baking – A review. J. Food Eng. 86:465–474.

Pateras, I.M., 2007. Bread spoilage and staling. In: S.P. Cauvain and L.S. Young, ed. Technology of Breadmaking, 2nd edn. Springer, New York, pp. 275–298.

Randez-Gil, F., I. Córcoles-Sáez, and J.A. Prieto. 2013. Genetic and phenotypic characteristics of baker's yeast: relevance to baking. Annu. Rev. Food Sci. Technol. 4:191–214.

Roberts, D. 1995. After 4500 years rediscovering Egypt's bread-baking technology. National Geogr. 187:32–35.

Sugihara, T.F., L. Kline, and M.W. Miller. 1971. Microorganisms of the San Francisco sour dough bread process. I. Yeasts responsible for the leavening action. Appl. Microbiol. 21:456–458.

Wood, E. 1996. World Sourdoughs from Antiquity. Ten Speed Press, Berkeley, CA.

10 Beer

> Beer and other alcoholic beverages may have played a pivotal role in cementing human societies through the social act and rituals of drinking and by providing a source of nutrition, medicine, and uncontaminated water.
>
> Libkind et al., 2011, Proc. Natl Acad. Sci. 108:14539–14544.

> Groucho and Chico step up to the bar.
> Groucho: Two beers, bartender.
> Chico: I'll take two beers, too.
>
> From the 1935 Marx Brothers' film, "A Night at the Opera"

INTRODUCTION

The relationship between humans and their beer goes way back. It's not only sociologists and anthropologists who have been interested in this relationship. Indeed, since the early 1990s, biologists and other scientists have also sought to identify when, where, and how brewing science began. One of the first research teams to address these questions was from the Applied Science Center for Archaeology at the University of Pennsylvania (led by Patrick McGovern). Using sensitive analytical methods, they examined organic residues from inside a 5000 year old pottery vessel that had been retrieved from the Zagros Mountains of Western Iran. The results revealed the presence of oxalate ion. Since oxalate, and calcium oxalate in particular, accumulate in only a few places, this finding could mean only one thing – the pot had been used to brew beer. The archaeologists concluded that this was the earliest chemical evidence for the origins of beer in the world. Researchers further suggested that beer drinking, then, as now, was also an important social activity for early humans.

While it is impossible to know precisely when humans first began to make and consume beer, it is certain that it soon became a staple. Historians have even suggested that beer, rather than bread, was the real "staff of life". Moreover, although the discovery of the first beer vessel attracted its share of headlines, it was but one of many events in the remarkable historical record of beer making (Figure 10.1).

From the early days of the Mesopotamian civilizations, beers were widely prepared and consumed. Barley was domesticated in this so-called Fertile Crescent region, providing an ample source of this essential beer ingredient. According to one historical account

Microbiology and Technology of Fermented Foods, Second Edition. Robert W. Hutkins.

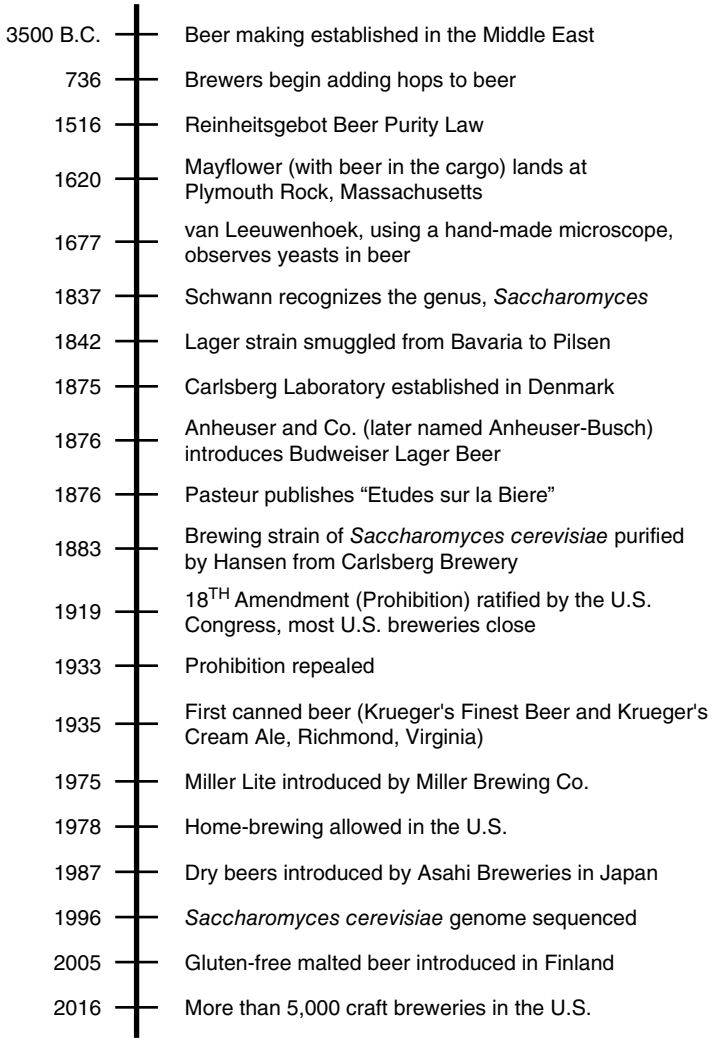


Figure 10.1 Milestones in the history of beer.

(see Rasmussen, 2014), the popularity of beer among local populations extended across class and gender. Eventually, beer technology spread west into Europe and later to the British Isles. While wine technology had also been developed and had gained many converts, beer remained popular, especially in Bavaria and Western Europe. Despite the introduction of Islam in the eighth century, and its prohibition against alcohol consumption, beer making and consumption continued to grow during the Middle Ages.

From early on, beer making (like baking and wine-making) was considered an art, performed by skilled craftsmen. Many of the early breweries were located within European monasteries, which created a tradition of brewing expertise and innovation. The use of hops in beer as a flavoring agent, for example, was first practiced by monks, as was the use of bottom-fermenting yeasts (discussed below). Although beer manufacture was practiced throughout Europe, it was of particular cultural and economic importance in Great Britain, Czech Republic, and Germany, which became the epicenters for brewing technology.

By the late eighteenth century, and especially by the mid-1800s, beer making was one of the first food processes to become industrialized. Although monastery-based breweries were still common, many small breweries began to form, serving their product directly on the premises (much like the brew pubs that are popular even today). Eventually, some of the larger breweries contained not only production facilities, but also the beginning of what we might now consider to be quality control laboratories. And although these breweries were located mainly in Europe and England, beer making had also spread to North America and the American colonies.

The history of the American beer experience begins with the Pilgrims, as documented by written logs and diaries. Indeed, English ale-style beer had been among the provisions carried on the Mayflower. However, as the ship began to run low on beer, the passengers became worried, with one Pilgrim writing: "We could not now take time for further search…our victuals being much spent, especially our beer…" Upon landing in Plymouth Rock, one of the settlers' first efforts was to construct a brew house to replenish their beer supply.

Why was beer so important during the Mayflower voyage? Considering that that the trans-Atlantic trip took more than 2 months, having enough safe potable water on board was critical. Water, however can spoil, so the Pilgrims relied on beer as a well-preserved form of liquid nourishment. In fact, for hundreds of years, beer was often safer to drink than water, was less likely to cause water-borne disease, and was less susceptible to spoilage. Of course, we now know that the microbiological stability and "safety" of beer is due, in part, to its ethanol content, as well as other anti-microbial constituents and properties (discussed, in more detail, later in this chapter).

Soon, brewing spread across the Americas as it had done in Europe and the United Kingdom. By the latter half of the nineteenth century, there were 1300 breweries in the United States. Most produced German lager-style beer, reflecting the huge immigrant population from Germany during that era, including the brewers themselves, (e.g., Adolphus Busch, Eberhard Anheuser, Adolph Coors, Frederick Miller, Joseph Schlitz).

BEER, BEER SPOILAGE, AND THE ORIGINS OF MODERN SCIENCE

In addition to the remarkable cultural and economic history of beer, the origins of microbiology as a science in the nineteenth century also coincide with an emerging beer industry. As beer manufacturing in Europe grew from small, local, and craft-oriented production into a large brewing industry in the middle of the nineteenth century, the prevention of beer spoilage became an important goal of industrial brewers. Despite the inherent stability of beer noted above, it is still vulnerable to spoilage and quality defects. Thus, brewers were often troubled by inconsistencies in beer quality. These difficulties attracted the attention of chemists and other scientists, who were enlisted to solve some of these technical problems. Thus, beer manufacturing was one of the first industrial fermentations to be studied and characterized, and was the subject of scientific inquiry by early microbiologists and biochemists. In particular, the science of beer making was revolutionized in 1876 by Pasteur, who not only showed that yeasts were the organisms responsible for the fermentation, but also that the presence of specific organisms were associated with specific types of spoilage (Box 10.1). Pasteur also developed processes to reduce contamination and preserve the finished product. It is worth noting that even today, preventing beer spoilage by microorganisms is still an important challenge faced by the brewing industry.

Scientific interest in brewing extended throughout Europe, leading to establishment of research laboratories in Copenhagen (the Carlsberg Laboratory) and Bavaria (the Faculty of

Brewing), as well as laboratories located within several breweries. Perhaps the most noteworthy discoveries of the late nineteenth and early twentieth centuries were made by Emil Christian Hansen at the Carlsberg Laboratory. He developed pure culture techniques for yeasts that eventually led to methods (still in use today) for propagation and production of yeast starter cultures free of contaminating bacteria and wild yeasts.

Box 10.1 Pasteur, the origins of microbiology, and beer

It is hard to imagine, given the current age of scientific specialization, that one person could have been as accomplished in so many fields as was Louis Pasteur in the latter half of the nineteenth century. He was trained as a chemist and, at the age of only 26, made important discoveries in stereochemistry and crystallography, specifically, while examining tartaric acid crystals in wine (Berche, 2012). Pasteur then became interested in the not yet named field of microbiology, and devoted nearly two decades of his life disproving the theory of spontaneous generation and establishing microorganisms as the causative agent of fermentation and putrefaction.

His work on vaccines against animal anthrax and human rabies, as well as the establishment of the germ theory of disease, brought Pasteur worldwide recognition and fame (Schwartz, 2001). During his career, Pasteur addressed practical agricultural, food, and medical problems, but did so by relying on basic science. The famous quote, "There is no such thing as applied science, only applications of science," perfectly captures this sentiment (Baxter, 2001). He was a meticulous researcher, who rigorously defended the scientific method, saying, "In the field of observation, chance favors only the prepared mind."

There were certainly other well-known scientists of his era, including Schwann, Koch, and Lister, whose works also helped establish the field of microbiology. But the collective contributions of Pasteur led to his recognition as one of the "most distinguished microbiologists of all time" (Barnett, 2000), or as stated by Krasner (1995), the "high priest of microbiology."

Pasteur was already an accomplished scientist, having completed his work on the stereochemistry of tartaric acid, when he turned his attention to solving spoilage problems plaguing the French wine industry. In reality, he was "commissioned" by Emperor Napoleon III in 1863 to serve as a "consultant" and to study these so-called wine diseases. Pasteur correctly diagnosed the spoilage conditions, which he called tourne (mousy), pousse (gassy), and amertume (bitter), as being caused by bacteria. He then showed that the responsible organisms could be killed, and the wine stabilized, by simply heating the wine to 50°C to 60°C. These findings were published in 1866, as "Eudes sur le Vin" or Studies about Wine.

Nearly ten years later, Pasteur began studying the fermentation of beer, another industrially-important product that was also suffering from spoilage defects. This work led to a second treatise on alcoholic fermentations, "Eudes sur la biere," published in 1876 (the English-translated version is available and well worth reading). His motivation this time had less to do with his interest in beer (he apparently was not a beer drinker), than it did for his animosity for all things German (Baxter, 2001).

In the early 1870s, France and Germany were at war (the Franco-Prussian War). The outcome resulted in France having to cede the hop-producing region of Alsace-Lorraine to Germany. Already Germany produced the best beer and was the dominant producer in Europe. German beers were better from most English and other beers for several reasons, but mainly because these beers were fermented at a low temperature (6°C to 8°C). The resulting "low beers" (so-named because of the low fermentation temperature, but also because the yeast would sink to the bottom of the tank) were lighter-colored and less heavy than "high beers", but most importantly, they were also better preserved.

Low beers (which we now refer to as lagers) could be produced in the cool winter months, and, provided they were stored cold (in cellars or by ice), be consumed during the summer. High beers (or ales) had to be consumed shortly after manufacture and did not travel well. This situation, in which French beers were at a commercial disadvantage, was intolerable to Pasteur and he was determined to improve French-made beers or as he himself stated, to make the "Beer of the National Revenge".

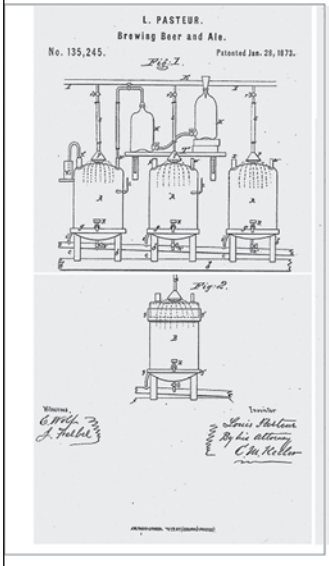
Note the wording of his preface to "Studies on Fermentation":

> Our misfortunes inspired me with the idea of these researches. I undertook them immediately after the war of 1870, and have since continued them without interruption, with the determination of perfecting them, and thereby benefiting a branch of industry herein we are undoubtedly surpassed by Germany.

Pasteur started out by building a brewing "pilot plant" in the basement of his laboratory and by visiting commercial breweries. Then, using microscopic techniques, he showed that specific yeasts were necessary for a successful beer fermentation. He also observed that, based on microscopic morphology, certain contaminating microorganisms were associated with specific types of spoilage conditions or "diseases". For example, long rod-shaped or spherical-shaped organisms in chains (probably lactic acid bacteria) were responsible for a sour defect. In Pasteur's own words, "Every unhealthy change in the quality of beer coincides with a development of microscopic germs which are alien to the pure ferment of beer" (Pasteur, 1879). The corollary was also true: "The absence of change in wort and beer coincides with the absence of foreign organisms." These foreign organisms had gained entrance into the beer either by virtue of their presence in the production environment or via the yeast (which were used repeatedly and were subject to contamination).

To solve these problems, Pasteur offered several recommendations. He showed that a modest heating step (55°C to 60°C) would destroy spoilage bacteria and render a beer palatable even after nine months. He emphasized that elimination of contaminants, both environmental and within yeast preparations, would also be effective. Oxygen, Pasteur realized, enhanced growth of spoilage organisms, thus, he devised a brewing protocol that precluded exposure of the wort to air (described in a US patent; Figure 10.1.1).

Pasteur demonstrated through actual industrial scale experiments that these strategies would work, and indeed these practices were readily adopted. And although the German beer industry was hardly affected by his personal "revenge", Pasteur's influence on brewing science and microbiology is considerable even today, nearly 150 years later.



L. PASTEUR.

Brewing Beer and Ale.

No. 135,245. Patented Jan. 28, 1873.

Fig. 1.

Fig. 2.

Witnesses: C. Wolf, J. Fiebel

Inventor: Louis Pasteur, By his attorney, C. M. Keller

UNITED STATES PATENT OFFICE.

LOUIS PASTEUR, OF PARIS, FRANCE.

IMPROVEMENT IN BREWING BEER AND ALE.

Specification forming part of Letters Patent No. 135,245, dated January 28, 1873.

To all whom it may concern:

Be it known that I, LOUIS PASTEUR, of the city of Paris, France, have invented certain new and useful Improvements in the Process of Making Beer, for which Letters Patent were granted to me in France on the 28th day of June, 1871; and I do hereby declare the following to be a full, clear, and exact description thereof, reference being had to the accompanying drawing making part of this specification and the letters of reference marked thereon.

Previous to my invention in the process of making beer it has been customary to permit the exposure of the "wort"—that is, the boiled extract of malt or other material seasoned with hop or other qualifying ingredient—to the action of atmospheric air. I have discovered that by contact in the usual way with air during the process not only is the quality of the beer produced much impaired, but also that a less quantity is made from a given amount of wort than can be otherwise produced.

Based upon this discovery and the idea of performing the process of brewing without the presence in the wort of atmospheric air, my invention has for its object to produce a better quality and greater quantity of beer from the same quantity and quality of wort, and to afford a beer which shall also embody the quality of greater degree of unalterableness during time and changes of climate, &c., in transportation and use; and to these ends my invention consists in expelling the air from the boiled wort while confined in a closed vessel or closed vessels, and then cooling it by the application of sprays of water to the exterior of such vessel or vessels, as will be hereinafter more fully explained.

To enable those skilled in the art to fully understand and practice my improved process for the manufacture of beer, I will proceed to more fully describe it, referring at the same time by letters to the accompanying drawing, in which I have shown an apparatus adapted to carry on my said improved process.

At Figure 1, A A A represent three casks or tanks, which may be made of galvanized iron, wood, or other suitable material, and which are supported on suitable stands b, as represented. Above the series of cylinders or vessel A is arranged a water-supply pipe, B, from which depend branch pipes, (one over each of the vessels A,) provided with cocks *r*, and having attached to their lower ends flexible tubes or hose *s*, which in turn carry at their lower extremities spray-nozzles F. Upon a suitable stand or shelf, T, is located an apparatus, M M, for the generation of carbonic-acid gas, which is to be supplied therefrom to the vessels A for purposes to be presently explained, and by means of tubes connected at or to the said vessels. The escape of the gas is permitted through exit or escape tubes at *x*, which extend siphon-like into water cups or chambers *v* from whence the gas may be collected in a gasometer.

I have shown the connection of the gasgenerator with only one of the vessels; but it will be understood that the others may be similarly connected.

The spray-nozzles F are located about centrally over each of the vessels A, which should be made slightly convex or dome-shaped on top, and so that the jets of water discharged therefrom will fall like rain on the tops of the said vessels and trickle down their sides, as illustrated by the dotted lines in Fig. 1. Around the base of each vessel A is arranged a circular trough, which catches the water and from which the water is led off by a tube, *i*, into a conductor or discharge-trough, *o*, which carries it to any desired destination. B' are cocks through which the contents of the vessels may be discharged into other vessels for the permanent retention of it, and R are funnels, which are used to draw off the beer for use, when it shall have been left or allowed to remain in the vessels A, as will be presently explained.

At Fig. 2 is illustrated a modification of the vessel or cask, in which, in lieu of being closed permanently at the top, said vessel B is made with a removable top, and is provided with thermal and necessary water-gages, thermometers, man-holes, &c., common to such contrivances.

The following explanation in connection with the foregoing description of apparatus will suffice to convey a full exposition of my improved process: The wort prepared in the usual manner, and while yet boiling hot, is introduced into the vessel A, into which a current of carbonic-acid gas is then conveyed for the purpose of expelling all contained air, and the water-spray is then let on to the vessels

to cool them and their contents. As soon as the temperature of the charge has been reduced to about from 10° to 15° Reaumur, the yeast or pure ferment is added to provoke or induce fermentation. After this fermentation, or the first fermentation, the contented the vessels A may be drawn off through the cocks B' into casks or barrels for future use, and in which the usual and further fermentation goes on, from which the beer becomes both clear and bright.

In lieu of drawing off the beer thus into barrels it may be allowed to remain, when the apparatus is not needed further, in the vessels A, and therein complete its fermentation, and be drawn for use through the faucets R; but in this case, or where it is desired to accelerate or make more complete the first fermentation, it may be found desirable to introduce a small quantity of air into the vessel, first, however, passing the air through a hot tube, or at least filtering it through cotton, for the purpose of either killing or extracting any germs which it may contain.

The apparatus which I have shown is adapted to the working of small quantities —say about one barrel; but it is obvious that the capacity of the apparatus may be varied at pleasure to manufacture more or less extensively.

In conducting my new mode of manufacture or process, the carbonic-acid gas generated from the fermentation of the wort may be collected properly in a gasometer, of course, and employed in lieu of or in connection with that derived from a generator, such as shown, and which is necessary for the first operation, it being important always to effect a thorough penetration of the mass by the carbonic-acid gas to expel all contained air.

It will be understood that by my improved process not only are the usual cooling-vessels dispensed with and all loss by evaporation prevented, but that the quality of the beer and its alcoholic gradation are improved and a larger quantity produced from a given supply of material.

I have found that by my new process the beer produced possesses in an eminent degree the capacity of unchangeableness, and can be transported without detriment or deterioration; and that in the use of my process, by which I am enabled to brew in all seasons and in most any climate successfully, the product is more aromatic and is perfectly limpid.

What I claim as new in the process of brewing or in the manufacture of beer is—

Subjecting the wort to a process for the expulsion of the air and cooling it off, substantially as and for the purposes set forth.

In testimony whereof I have hereunto set my hand this 6th day of December, 1871.

LOUIS PASTEUR.

Witnesses:
CATON,
GUERET FILS.

Figure 10.1.1 "Improvement in brewing beer and ale" a US patent (No. 135,245) issued to Louis Pasteur of Paris, France in 1873. The patent is not for "Pasteurization", but rather describes a method for excluding air from the boiled wort prior to inoculation with yeast. The resulting beer has improved stability, or, according to the patent, "possesses in an eminent degree the capacity for unchangeableness".

References

Barnett, J.A., 2000. A history of research on yeasts 2: Louis Pasteur and his contemporaries 1850–1880. Yeast. 16:755–771.

Baxter, A.G. 2001. Louis Pasteur's beer of revenge. Nature Rev. 1:229–232.

Berche, P. 2012. Louis Pasteur, from crystals of life to vaccination. Clin. Microbiol. Infect. 18:1–6.

Krasner, R.I. 1995. Pasteur: high priest of microbiology. ASM News. 61:575–579.

Pasteur, L. 1879. Studies on fermentation. The diseases of beer, their causes, and the means of preventing them. A translation, made with the author's sanction, of "Etudes sur la biere" with notes, index, and original illustration by Fran Faulkner and D. Constable Robb. Macmilan and Co. 1879, London and Kraus Reprint Co., 1969, New York.

Schwartz, M. 2001. The life and works of Louis Pasteur. J. Appl. Microbiol. 91:597–601.

THE MODERN BEER INDUSTRY

Today, beer has one of the largest dollar values of all fermented food products, with US retail sales of over $110 billion (according to 2017 Beer Institute data). In 2017, US consumers drank nearly 200 million barrels (1 barrel = 117 L = 31 gallons), with per capita consumption of 75 L or 20 gallons per person per year (or more than 35 six-packs per man, woman, and child!). That's also more, on a volume basis, than wine (11 L), juice (50 L), and milk (68 L). Although one might assume this is a lot of beer, beer consumption in the United States is far less than several European countries (Figure 10.2). For example, per capita consumption (for 2015) in Germany and Austria was 105 L per person per year, with Ireland (98 L) was not far behind. The Czech Republic, however, is way ahead, with per capita consumption of 142 L. As for many other fermented foods, however, on an overall consumption basis, China is by far, the top beer consuming country in the world. Indeed, it's been so for more than a decade. Nearly 25% of the total beer produced in the world (about 2 billion barrels) is consumed in China.

The importance of beer to the world economy is also considerable. In the United States alone, the overall economic impact is estimated to be more than $350 billion dollars (according to the US Beer Institute). Beer is heavily taxed – 40% of the retail cost of beer is taxes, generating over $60 billion in revenue to federal and state government.

The beer industry, like many other segments of the food industry, both in the United States as well as internationally, has become highly consolidated. In the past 60 years, for example, many medium sized breweries were either acquired by larger breweries or were unable to compete and went out of business. Where there were once several hundred small, privately owned breweries operating in the United States as recently as 1950, now more than 80% of US beer is produced by just three companies. The same trend has occurred in other large beer markets in Europe, Australia, Canada, and Japan.

Despite this centralization of the beer industry, there has been a remarkable increase in the number of small or craft or micro-breweries. Defined generally as a brewery with annual production of less than 15,000 US barrels (about 1.8 million liters), microbreweries are now operating in all regions of the United States. In 1970, microbreweries existed only in Europe and the UK, and literally none operated in the US By 2017, however, there were more than 5300 (as reported by the US Brewers Association). Some microbreweries have become so successful they have exceeded the 15,000 barrel definition for "micro".

These breweries are important to mention here, in part because they have captured a substantial share of the total US beer market. According to 2016 data, craft beers accounted for

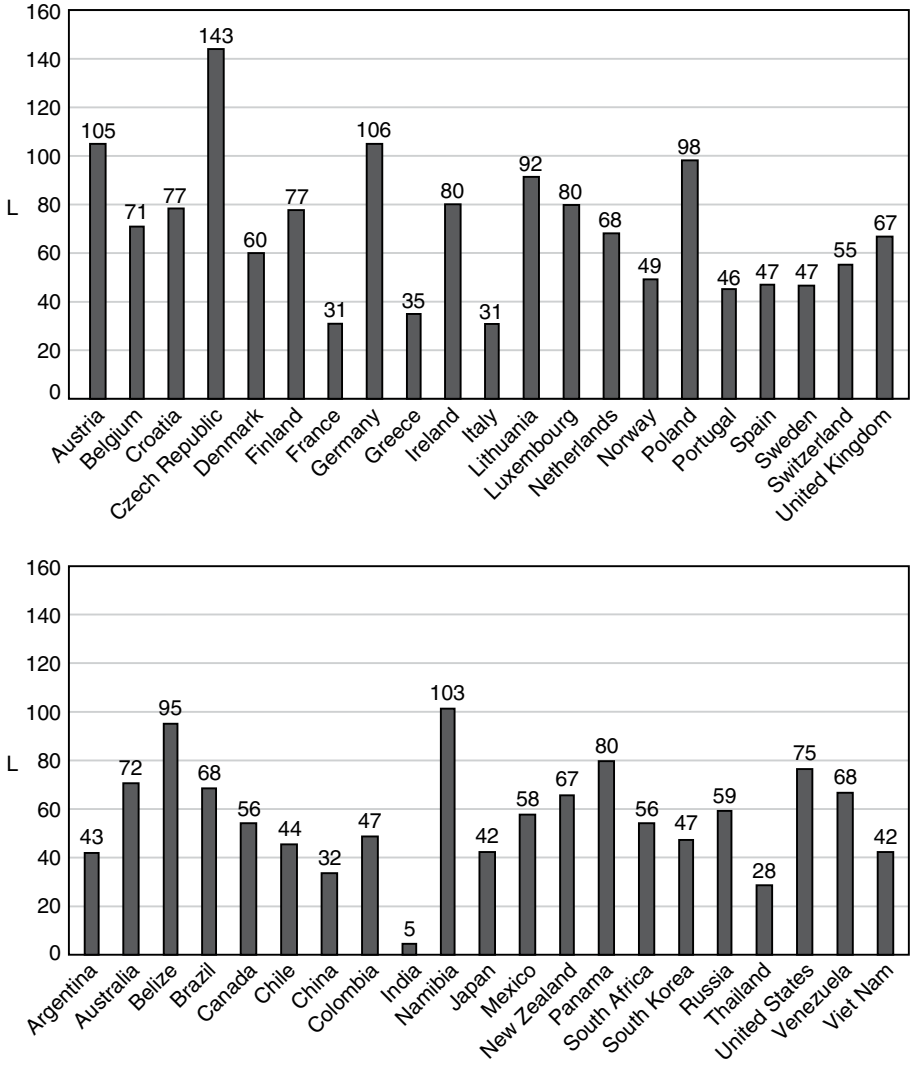


Figure 10.2 World-wide per beer consumption (L per person per year), 2015.

nearly 12% by volume of beer consumed in the US and more than 22% by sales. However, craft or microbreweries are also important because they have adopted many of the traditional brewing practices that will be described later in this chapter.

BEER MANUFACTURING PRINCIPLES

One of the recurring themes in this book has been how the manufacture of most fermented foods requires only three or four simple ingredients. Thus, only water, malt, hops, and yeast are necessary to make beer. In fact, the requirement that beer be made with just a few ingredients was codified 500 years ago, in the famous Bavarian law called the Reinheitsgebot (Box 10.2). However, despite its ancient origins and long history, and this seemingly short

Box 10.2 The Reinheitsgebot – a 500-year-old law

Among the most ancient of all laws related to foods is the German Beer Purity Law known as Reinheitsgebot. These brewing laws were established in 1516 by Bavarian dukes Wilhelm IV and Ludwig X in response to the frequent occurrences of what we would now call adulteration. During this time, brewers had begun to add questionable, if not dangerous, substances to beer in an effort to disguise defects and deceive consumers as to the quality. Brewers had been known to add tree bark, various grains, herbs, and spices to make the beer more palatable. These unscrupulous beer traders were giving the legitimate brewers of Bavaria a rather bad name. Thus, these laws were written to protect the brewing industry and perhaps to protect consumers. It is interesting to note that the ingredients clause is but a part of the Reinheitsgebot; most of the law deals with the price that brewers can charge for beer.

Below is an English translation of the Reinheitsgebot (from the article "History of German Brewing" by K.J. Eden, published in Zymurgy, Vol. 16).

> We hereby proclaim and decree, by Authority of our Province, that henceforth in the Duchy of Bavaria, in the country as well as in the cities and marketplaces, the following rules apply to the sale of beer: From Michaelmas to Georgi, the price for one Mass or one Kopf, is not to exceed one Pfennig Munich value, and From Georgi to Michaelmas, the Mass shall not be sold for more than two Pfennig of the same value, the Kopf not more than three Heller. If this not be adhered to, the punishment stated below shall be administered.
>
> Should any person brew, or otherwise have, other beer than March beer, it is not to be sold any higher than one Pfennig per Mass. Furthermore, we wish to emphasize that in future in all cities, markets and in the country, the only ingredients used for the brewing of beer must be Barley, Hops and Water. Whosoever knowingly disregards or transgresses upon this ordinance, shall be punished by the Court authorities' confiscating such barrels of beer, without fail. Should, however, an innkeeper in the country, city or markets buy two or three pails of beer (containing 60 Mass) and sell it again to the common peasantry, he alone shall be permitted to charge one Heller more for the Mass of the Kopf, than mentioned above. Furthermore, should there arise a scarcity and subsequent price increase of the barley (also considering that the times of harvest differ, due to location), we, the Bavarian Duchy, shall have the right to order curtailments for the good of all concerned.

Note that, in contrast to the often-quoted statement that only four ingredients are permitted, the Reinheitsgebot actually restricts beer making to just three ingredients: barley, hops, and water. This is because the fourth ingredient, the yeast, had not yet been "discovered". Rather, early brewers either relied on a natural fermentation (i.e., with wild yeast initiating the fermentation) or else used a portion of a previous batch to start the fermentation (i.e., backslopping).

The Reinheitsgebot is still in effect today in Germany, although it has been rewritten and is now more explicit. The current laws also have been modernized, such that hop extracts and filter aids are permitted. In addition, there is now a distinction made between lagers and ales, with exceptions regarding adjuncts and coloring agents allowed for the latter.

list of ingredients, the manufacture of a quality beer remains a challenging task. In part, this is because beer making consists of several different and distinct processes that are not always easy to control. In addition, some steps taken to improve one aspect of the process – for example, filtering the finished beer to enhance clarity and improve stability, may also remove desirable flavor and body constituents.

The actual brewing process involves not only the well-studied yeast fermentation, but also includes other biological, as well as chemical and physical reactions. It is, therefore, convenient to consider the beer manufacturing process as consisting of several distinct phases or steps.

The primary purpose of the first general series of steps is to transform non-fermentable starch into sugars that the yeast can ferment. The process, which is enzymatic in nature, involves several biochemical events that begin first with the conversion of cereal grain, usually barley, into malt. The malt is then used to make a mash, ultimately resulting in the formation of a nutrient-rich growth medium, called wort. Although other grains, such as sorghum, maize, and wheat can also be malted, barley is by far the most frequently malted cereal grain.

Next, in the fermentation phase, the wort sugars, amino acids, and other nutrients are used to support growth of yeasts that have been inoculated into the wort. Yeast growth, under the anaerobic conditions that are soon established, is accompanied by fermentation of sugars and formation of the main end products, ethanol and CO_2. Technically speaking, the fermentation step results in beer that could then be consumed. However, at this point, the beer contains yeast cells, insoluble protein complexes, and other materials that together result in a cloudy or hazy appearance. In addition, most of the carbon dioxide is lost during the fermentation. Beer at this stage is also microbiologically unstable and susceptible to spoilage. Therefore, additional measures are almost always taken to remove yeasts, other microorganisms, and any other substances that would otherwise affect product quality and shelf-life.

In this chapter, therefore, a final phase, consisting of important post-fermentation activities, also will be highlighted. These latter steps of the beer-making process, some might argue, are among the most important, since they have a profound effect on the appearance, flavor, and stability of the finished product. The general beer manufacturing process is outlined in Figure 10.3. As the reader will soon be aware, many beer-specific terms, such as mashing and pitching are used to describe particular steps in the brewing process. Some of these terms are defined in Box 10.3.

ENZYMATIC REACTIONS: MALTING AND MASHING

Other than water, the main ingredient of beer is malt. It is the malt that serves as the source of the enzymes necessary to convert starches into fermentable sugars. However, the manufacture of malt actually begins far from the brewing facility, in the malting houses that convert barley into brewing malt. Not only will this malt serve as the source of the amylases, proteinases, and other enzymes necessary for hydrolysis of large macromolecules, such as starch and protein, but for most beers, the malt also serves as the substrates for those enzymes. That is, the malt contains the starches and proteins that are hydrolyzed by the malt enzymes. In addition, malt is the primary determinant of color and body characteristics in beer, and it also influences flavor development. Considering the critical functions that malt contributes to the beer-making process, it is evident that malt quality has a profound influence on beer quality.

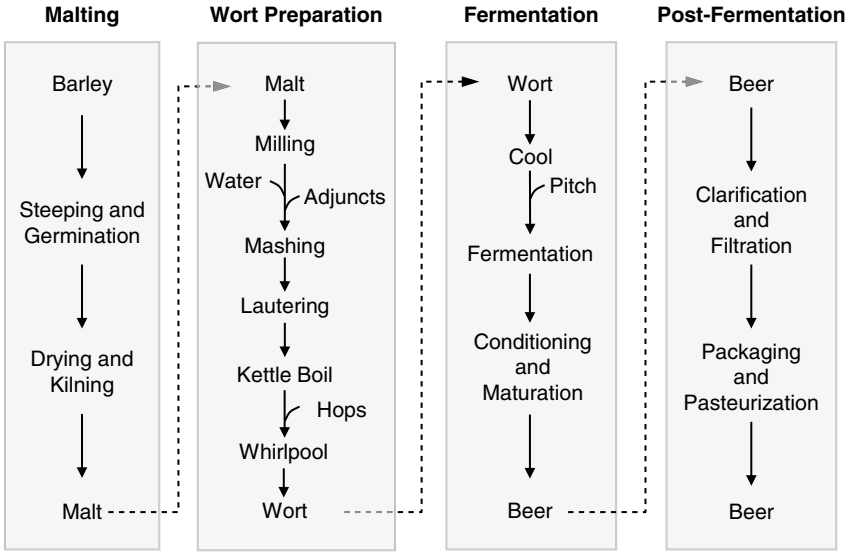


Figure 10.3 Manufacture of beer. The beer manufacture process consists of four distinct stages: malting, in which barley is converted to malt; wort preparation, where enzyme and substrate extraction and reactions occur (mashing) and a suitable growth medium is prepared; fermentation, where wort sugars are fermented to beer; and post-fermentation, in which the beer is made suitable for consumption.

Box 10.3 Beer terminology

The manufacture of beer, like that for wine making, bread making, and other food processing technologies, has evolved its own peculiar vocabulary to describe many of the manufacturing steps. Thus, the brewing jargon contains words and phrases unique to beer making and to brewers, but not so familiar to the casual reader. Below are some of the more common terms used in brewing science and technology.

Coppers. Vessels used for wort-boiling, called coppers because of the construction material used in their manufacture; even though these kettles are now made from stainless steel, they are still referred to as coppers.

Diastase, diastatic power. Refers to the overall starch hydrolyzing activity present in the malt.

Kiln. The oven used for drying and cooking malt.

Krausen. The carbon dioxide layer that forms at the top of the fermentation tank. The term "high krausen" refers to the period at which the krausen reaches its maximum, and krausening refers to the addition of this material (containing very active, log phase cells) to beer to promote a secondary fermentation.

Lagering. The act of storing or conditioning the beer at low temperature to promote maturation.

Lauter tun. A tank, containing a false bottom, used to promote clarification and separation of the spent grains from the wort.

Mash. The heated malt-water mixture, consisting of malt-derived enzymes and substrates.

Mash off/out. At the end of mashing, when the temperature is raised to about 72–77°C to inactivate enzymes.
Mash tun. Where the mashing step takes place; may contain a false bottom and be used for wort separation.
Oast house. The facility used for drying whole hops.
Pitch. The step when wort is inoculated with yeasts.
Racking. A step involving the transfer of fermented beer from one tank to another, usually for a secondary fermentation.
Trub. The precipitated material obtained from the wort after boiling; it is rich in protein and hop solids.

The general goal of the maltster is to convert barley to malt such that enzyme synthesis is maximized and enzyme activity is stable and well-preserved. The process starts with selection of barley. Although many different barley cultivars are used to produce brewers' malt, all are classified as either two-row or six-row, where the "row" refers to the number of rows of seeds on each spike. In North America, six-row barley is generally preferred, whereas in Europe and the UK, two-row barley is used. However, some six-row is also used in Europe and the UK and some two-row is used in the United States. Six-row barley contains more protein and less starch than two-row barley. The former also contains a higher level of starch-degrading enzymes, making it more suitable for beers containing adjuncts (discussed below). The harvested barley is then cleaned, graded, and sized. Unless the barley is to be used right away (which is usually not the case), it is dried from about 25% moisture to 10% to 12% so that it can be safely stored.

In the next step, the barley is allowed to germinate and develop the beginning of a root system. Barley is essentially a seed – if it is held under warm, moist conditions, it will begin to sprout or root, just as it would if it were growing in nature. For malting purposes, germination is done by steeping the barley in cool water at 10°C to 20°C for two to three days, or long enough to increase the moisture from 10% to 12% to about 45%. The steep water is usually changed every twelve hours, in large part to minimize the impact of potential spoilage organisms. During this step, the material is well aerated to promote the germination process. The moist barley is then removed from the steep water and incubated in trays or drums under cool, humid, and well-aerated conditions for two to eight days. The barley initially swells and then germinates, such that "rootlets" or sprouts appear. Germination is accompanied by the synthesis of myriad enzymes that the barley seeds would theoretically need for subsequent growth into new barley plants. The barley has reached its maximum enzyme activity when the sprout reaches a length of one-third the size of the grain.

Next, it is necessary to arrest further germination and to stabilize and preserve the enzymatic activity. This is done by a step-wise drying process, in which the germinated or "green" barley is air-dried incrementally within a temperature range of 45°C to 60°C. The purpose of this slow drying process is to remove water without inactivating enzymes. The dried malt, therefore, becomes a stable source of enzymes. Since moist heat is more detrimental to enzymes than dry heat, it is important to dry slowly at the start (when the grains are still wet), and only later, when the moisture is reduced, to dry at higher temperatures.

During the initial step or first stage of drying, the moisture drops from 45% at the start to about 15% to 18% at the end. In the second stage, the dried malt is further dried or "cured" or "kilned" at temperatures as high as 80°C, and the moisture decreases to less than 5%.

At this point the water activity of the malt is usually reduced to 0.3 or lower, so enzymes are well preserved and no microbial growth is expected to occur. After the kilning step, the malt contains mostly carbohydrate and protein (Table 10.1). Finally, the malt is packaged in bags and shipped to smaller brewers or placed in large trucks or train cars for delivery to large breweries.

Although the main purpose of the drying and kilning steps is to arrest germination and preserve enzyme activity, another important series of reactions also takes place. It is during kilning that non-enzymatic browning (i.e., the Maillard reaction) and associated heat-generated flavors and colors are formed. These products are readily apparent in the malt and especially later in the finished beers. Thus, dark beers (e.g., stouts, porters) use darker, more flavorful malt, whereas paler beers use lightly-colored, less roasted malt (Figure 10.4).

Table 10.1 Approximate composition of malt.

Component	% dry weight
Starch	58
Sucrose	4
Reducing Sugars	4
Hemicellulose	6
Cellulose	5
Lipid	2
Protein	12
Amino acids/peptides	1
Minerals	2
Other	6

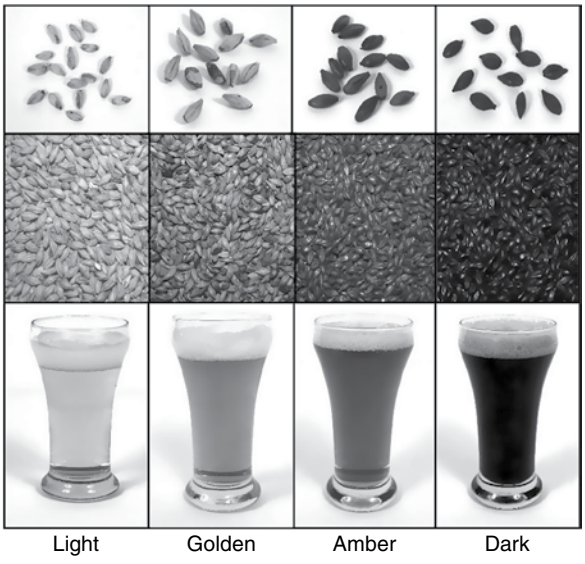


Figure 10.4 Types of malt used in brewing. Shown (from left to right) are four types of malt, ranging from light to dark. These malts are used to make beers having similar colors. Figure courtesy of Rich Chapin (Empyrean Ales, Lincoln, NE) and John Rupnow (University of Nebraska).

The flavors contributed by dark malts are often described as coffee- or chocolate-like, with toasty nutty notes. It would be a mistake, however, to conclude that darker beers, because of their stronger, more pronounced flavor and color, necessarily contain more ethanol than pale-colored beers. In fact, the darker the malt, the less amylolytic (or diastatic) activity is present. Ultimately, less sugar substrates are generated from starch, and less ethanol can theoretically be produced from the mashes (see below) made using dark malts. Indeed, the very dark, specialty malts that are used for stout-style beers have essentially no enzymatic activity. For this reason, stouts and other dark beers are made with a combination of mostly lighter base malts, to supply enzymatic activity, with just a small amount of highly-roasted malt, to provide color and flavor.

When malt arrives at the brewery, it is in an intact form, i.e., as whole malt. Although the malt contains starches, proteins, and the enzymes necessary for their degradation, these materials are not easily extracted from the intact malt kernel. Thus, to facilitate extraction, the malt must first be milled to break the kernel and expose the interior portion to the aqueous medium. Most breweries purchase whole or unground malt and do the milling themselves, according to their particular specifications. The kilned malt is most commonly milled in roller mills or hammer mills that can be set to deliver products ranging from a very fine to very coarse grind.

Prior to milling, the malt is surrounded by a tough husk, which separates from the grain during milling, but is generally only partially degraded. The fineness of the malt and the extent of husk degradation are extremely important and depend on the preference of the brewer. The finer the malt, the more easily will enzymes and other materials be extracted during the next step, the mashing phase. However, with finely milled malt, the insoluble residue remaining after mashing is difficult to separate, due to its small particle size. In contrast, the spent malt material will be more easily separated from the mixture if coarse malt, with minimum husk damage, is used. In other words, extraction of enzymes, starches, protein, nutrients, and flavors is greater with finely milled malts, but the filtration rates are slow. Conversely, good filtration rates but poor extraction occurs with coarsely milled malts. The brewer decides which is best.

Finally, the ground malt or "grist" is ready to be used in the step called mashing. The purpose of mashing is two-fold. First, the enzymes, starches, proteins, and other substances are extracted and solubilized in a hot aqueous solution. Second, the extracted enzymes, starches, and proteins react to form products that ultimately serve as substrates to support growth of yeasts during the fermentation phase. The precise steps involved in mashing in particular, the composition of the water, the temperature, pH, and the heating rate, are critical factors, subject to the preferences of the individual brewer.

Mashing begins when the malt is mixed with brewing water in a specialized tank called a "mash tun". About 20% to 30% malt is usually added. The water is usually pre-heated in the hot liquor tank. Water quality has a profound influence on the quality of the beer (it is, after all, the main ingredient). Thus, geographical considerations can be very important. Indeed, the reputations of many beers are based, in part, on the source, location, and properties of the water used to make the beer. For example, the famous Pilsener (also spelled Pilsner) beers were made from the very soft water in the Pilsen region of the Czech Republic. In contrast, the great English-style pale ales developed in Burton-on-Trent in England, where the relatively hard water is high in minerals and bicarbonate. Many beer manufacturers continue even today to make quality claims based on the water from which the beer is made (e.g., "made from Rocky Mountain spring water"). However, other brewers argue

Table 10.2 Components of brewing water and their function during mashing and brewing[1].

Component	Concentration (ppm)	Function
Calcium	10–250	When mashes are heated, insoluble calcium phosphate complexes are formed, resulting in a decrease in pH during mashing and an increase in activity of enzymes active at low pH (e.g., β-amylase). Calcium may increase thermal stability of α-amylase. Yeast flocculation also requires calcium.
Magnesium	5–100	Required for activity and stability of enzymes produced by yeasts. At high concentrations, magnesium may compete with calcium for phosphate, reducing calcium phosphate levels.
Bicarbonate	10–250	High bicarbonate concentrations increase the pH of the mash or wort. High pH decreases the activity and stability of amylases and proteinases.
Sulfate	5–500	Required for biosynthesis of sulfur-containing amino acids that are essential for yeasts. At high concentrations, sulfates may be reduced to sulfur dioxide and hydrogen sulfide, which confer undesirable aroma and flavor characteristics in beer.

[1] Adapted from Mosher and Trantham. 2017.

that by making adjustments for mineral content, pH, and ionicity, water variability is much less a factor in determining beer quality. One trend among craft brewers is to use reverse osmosis to purify the water and then add whatever minerals are suitable for their desired style.

Despite the chemical characteristics of the water preferred for particular beers, there are some general properties brewing water should have (Table 10.2). In general, brewing water should have medium hardness, containing about 100 ppm each of calcium and magnesium salts and 50 ppm or less of bicarbonate/carbonate. The pH should be between 5.2 and 5.8.

Returning to the mash tun, the malt–water mixture, or mash, is gradually heated from 20°C to 60°C to 65°C. Heating is done by infusion, decoction, or a combination of the two. Infusion heating is a simple step-wise process in which the mash is heated to an incremental temperature, held for a period of time, then the temperature is raised again, held, and so on until the final temperature is reached.

In decoction heating, a portion of the mash is separated and boiled in a separate kettle, then added back to the main mash, thereby raising the combined mash temperature. The main advantage of decoction mashing is an increase in starch extraction. Decoction mashing is used mainly for lager beers; in the United States a combination of infusion and decoction heating is common.

One might ask why the heating process during mashing is performed in a particular order, rather than simply heating the mash to some target temperature and then holding it there for a pre-determined time. Remember that the purpose of mashing is to extract and react. In reality, there are many enzymes in the malt, not just a single amylase or a single proteinase. Likewise, there are many sugar-containing polymers and starches with varying degree of branching, as well as heterogeneous proteins that will eventually serve as substrates. Pigments, flavors, and other substances are also extracted during mashing. The important

point is that each of the enzymatic reactions have optimum temperatures that are likely different from one another. By extracting the reactants in step-wise fashion, it is possible to coordinate the optimum or near-optimum temperature of a particular enzyme with the appearance of its substrate. For example, a particular α-amylase may hydrolyze its substrate at 45°C, but a different amylase (perhaps a β-amylase) may have a temperature optimum of 60°C. If the mash was heated directly to 60°C, then the α-amylase would not have had enough time at 45°C to perform its job.

Malt enzymology

The carbohydrate fraction of malt is mostly in the form of starch. Approximately one-fourth of malt starch is amylose, a linear polymer consisting of glucose, linked α-1, 4. The remaining starch, about three-fourths of the total, is amylopectin, which contains not only linear glucose, but also glucose in branched α-1,6 linkages. The main starch-degrading enzymes synthesized during malting are α-amylase and β-amylase. The former is an endoenzyme, acting primarily at intramolecular α-1,4 glucosidic bonds. Products formed by α-amylase are dextrins (short chain glucose-containing α-1,4 linear oligosaccharides) and limit dextrins (short chain glucose-containing α-1,4 and α-1,6 branched oligosaccharides). In contrast, β-amylase acts at the end or near the end of amylose and amylopectin chains. The main products are glucose, maltose, maltotriose, and small branched dextrins. Collectively, then, the products of starch hydrolysis by malt will consist primarily of fermentable glucose and maltose and non-fermentable dextrins and limit dextrins. Proteins are also hydrolyzed by malt proteases during mashing, yielding free amino acids and small peptides.

Adjuncts

The mash, as described so far, contains only the malt and brewing water. The malt, as stated above, supplies the enzymes and the enzyme substrates. Many of the European beers, as well as the micro-brewed beers produced in the United States, are made using 100% malt mashes. However, some of the most widely-consumed beers, certainly many of those produced by the large breweries, contain an additional source of starch-containing material in the form of corn, rice, or wheat. These materials are called adjuncts, and can account for as much as 60% of the total mash solids. Adjunct syrups, which contain sucrose, glucose, or hydrolyzed starch, are also commonly used. Adjuncts are allowed in most of the world, but their use is actually restricted in some countries, including Germany, where centuries-old beer purity laws are still enforced (see above). Recently, however some of these restrictions have been relaxed.

Adjuncts have several functions. First, they dilute the strongly flavored, dark-colored, "heavy" characteristics of the malt. Although these properties are preferred by some beer drinkers, most North American consumers favor the paler color and milder flavor associated with adjunct-containing beers. Second, adjuncts increase the carbohydrate content of the mash and provide the amylase enzymes with an additional source of substrate, and, ultimately, more fermentable sugar for the yeast. Third, they reduce the carbohydrate-to-protein ratio, such that fewer haze-forming proteins (discussed later) are present in the finished beer. Finally, adjuncts are less expensive than malt as a source of carbohydrate and, therefore, reduce the ingredient costs. In the United States, most of the adjuncts are derived from corn or rice. The two brands that dominate the US market, Budweiser and Miller, both incorporate adjuncts in their formulations.

It is important to recognize that adjuncts are not essential, since malt mashes contains 70% or more fermentable carbohydrate, which is more than enough to satisfy the energy and carbon requirement of the yeast. As noted above, adjuncts lower the protein/carbohydrate ratio, so if too much adjunct is added, relative to malt (e.g., >1:1 ratio), there may not be enough protein available for the yeast. Finally, if adjuncts are used, they are usually added to brewing water in a separate tank (e.g., cereal cooker). The mixture is brought to a boil to pre-gelatinize the starch and is then added to the mash tun.

Wort

Eventually, when the brewer has determined that sufficient saccharification has occurred in the mash tun, the temperature is raised to about 75°C. This effectively inactivates most enzymatic activity. The insoluble material in the mash liquid is then separated by one of several means (discussed below). This is an important step because the mash still contains grain solids and insoluble proteins, carbohydrates, and other materials. In addition, trapped within the mash solids is a reasonable amount of soluble materials, including fermentable sugars that would otherwise be discarded with the spent grains.

For some breweries, the mash tun can also provide its own means of filtration. These tanks contain a false bottom that allows most of the mash liquid to flow out of the tank and into a collection vessel, while at the same time retaining a portion of the mash containing the spent grains. The spent grains form a filter bed that enhance flow rate (depending on the fineness of the malt). This material can then be stirred and sparged with hot liquid to extract as much of the soluble material as possible. This liquid is added to that already removed from the mash tun. An alternative process involves pumping the mash into a separate tank called a lauter tun, which also contains a false bottom and sparging system and operates much like the mash tun, except it provides greater surface area and faster and more efficient filtration. Because the initial liquid material obtained from the lauter tun (or the mash tun) often still contains solids, it may be recycled back until a better filter bed is established and the expected clarity is achieved.

Another alternative means to manage the mash separation step is to use plate and frame type filtration systems. These systems consist of a series of connected vertical plates, housing filters of various composition and porosities. The mash liquid is pumped horizontally through the system. As for the mash and lauter tuns, the filtrate can be recycled and the entrapped material can be sparged to enhance extraction.

Ultimately, the liquid material or filtrate that is collected at the end of the mash separation step is called “wort”. Since it is the wort that will be the growth medium for the yeast and which will ultimately become beer, its composition is very important (Table 10.3). The main component of wort (other than water) is the carbohydrate fraction (90%). Most (75%) of the carbohydrates are in the form of small, fermentable sugars, including maltose, glucose, and maltotriose. If adjuncts were added, fructose and sucrose may also be present. The rest, about 25% of the total carbohydrate fraction, are longer, non-fermentable oligosaccharides that include dextrins (α-1,4 linked glucose molecules) and limit dextrins (α-1,4 and α-1,6 glucose molecules). A small amount of β-glucans (β-1,4 and β-1,6 linked glucose molecules), derived from the cell walls of the barley, may also be present.

In addition to the carbohydrate fraction, wort also contains 0.3% to 0.6% nitrogenous matter, including proteins, peptides, and free amino acids. Proportionally less protein, relative to the total solids, will be present if adjuncts are used. About half of the amino acids will be used to support yeast growth, and the other half will contribute to flavor and browning reactions.

Table 10.3 Approximate composition of mash, wort, and beer (g/100 ml)[1,2].

Component	Mash[3]	Wort	Beer
Water	70	88	88
Carbohydrates	15	10	4.0–6.0
Starch	13	<0.1	<0.1
Maltose	1	5	<0.1
Maltotriose	0.5	1	0.7
Dextrins	0.2	2	3.0
Sucrose	0.5	0.3	<0.1
Glucose	1	1	<0.1
Fructose	0.5	0.2	<0.1
Others	3	0.2	<0.1
Proteins + peptides	3	0.3	0.3
Amino acids	0.2	0.2	<0.1
Lipid	<0.1	<0.1	<0.1
Ethanol	<0.1	<0.1	4.5
pH	5.6	5.2	4.2
Specific gravity (°Plato)	<0.1	12	<0.1

[1] Adapted from multiple sources.
[2] Assumes an all-malt mash (no adjuncts).
[3] At start of mashing.

Various ions (e.g., ionic calcium, magnesium, and carbonates) are also present. The final pH of the wort is around 5.2.

The average molecular weight of the protein fraction is very important, because it determines the "palate fullness" or mouthfeel of the beer. Proteins (and the extent of protein hydrolysis) can also have an important effect on physical stability (and formation of cloudiness), color, and foam stability. In general, the greater is the protein hydrolysis, the less cloudy the beer. However, foam stability also is reduced. In contrast, the less hydrolyzed the protein, the more likely there will be cloudiness problems.

HOPS

In the final step prior to fermentation, the wort is pumped into a special heating tank called the brew kettle. It is here that the wort is boiled and other important reactions occur. One more essential beer ingredient, hops, are also added to the wort during the boil step. Note that exactly when hop addition occurs will depend on brewer preferences and the desired level of bitterness or aroma properties (see below).

Hops are botanically described as the flowers derived from the plant *Humulus lupulus* (in the family Cannabinaceae). Although they were not part of the "original" beer formula, they have been added to beer since the Middle Ages. Why hops came to be used in beer making is not known, but it seems likely that they were initially added as flavoring agents, and then later additional benefits were realized.

Clearly, hops provide two main sensory characteristics to beer – flavor and aroma. A large number of different hop cultivars or varieties are commercially available, and they are distinguished mainly on the basis of their aroma and flavor properties. By far, the

predominant flavor is bitterness, which is due primarily to the presence of alpha-acids. The main two alpha-acids are humulone and cohumulone; both are contained within the resin fraction. Another group of related compounds, the beta-acids, are also found in hops and also contribute some bitterness, but it is the alpha-acids that are more important for bitterness. In fact, brewers usually select hops based on their alpha-acid content (as a function of the whole hop weight), which can range from 5% (low bitterness) to as high as 14% (very bitter). While the intact alpha-acids do impart some bitterness, they actually serve as precursors for isomerization reactions that result in formation of so-called iso-alpha-acids. Among the iso-alpha-acids, isohumulone and isocohumulone are considered the primary bittering compounds.

The amount of bitterness ultimately contributed by the hops is expressed in terms of International Bitterness Units (IBU). One IBU is approximately equal to 1 mg of iso-alpha-acids per liter of beer. Most US lager beers have bitterness intensities of less than 15 IBU, compared to nearly 50 for some of the ales produced in the United Kingdom (hence, the name "bitters" for a typical British ale).

The other major component of hops is the essential oil fraction. This fraction, which is comprised of various terpenoids, esters, ketones, and other volatiles, is responsible for the aroma or bouquet properties of hops. Most of these substances are volatile and, if added at the beginning of the kettle boil step, would mostly be lost by the end of the step. Therefore, when high-oil hops are used (i.e., when the brewer desires a beer with a strong hoppy aroma), the hops are usually added near the end of the kettle boil step. In contrast, early hop addition is used for ales where more bitterness is desired.

In addition to providing flavor and aroma characteristics, hops also enhance preservation and increase shelf-life of beer. This is because the iso-alpha-acids formed during isomerization have considerable antimicrobial activity and inhibit lactic acid and other bacteria capable of causing beer spoilage (Box 10.4).

Hops are usually obtained from specialty suppliers in one of several forms, including liquid hop extracts, hop pellets, or as dried whole flowers or cones. Although the latter are still used, hop extracts and pellets have gained popularity due to their consistency, ease of use, and storage conveniences. Hop products can also be obtained as isomerized extracts or pellets, containing iso-alpha-acids. This allows the brewer to add the hops to the wort during kettle boil, as well as later stages, so that the desired hops flavor can more easily be achieved.

KETTLE BOIL

Wort mixtures are usually boiled for one hour, although some brewers will use shorter or longer boil times. The boiling step accomplishes many functions. First, it kills nearly all of the microorganisms remaining after mashing, making the wort, for all practical purposes, sterile. Second, boiling inactivates most of the enzymes still active after mashing or reduces their activity to barely detectable levels. Third, the boiling step enhances extraction of oils and resins from the hops and accelerates isomerization of hop acids. Fourth, proteins, tannins, and other materials that would ordinarily cause clarity and cloudiness problems precipitate during the boiling step. Subsequent removal of this precipitate (known as "hot break" or "hot trub") helps to prevent such defects. Fifth, wort boiling enhances color development by catalyzing formation of Maillard reaction products. Sixth, undesirable volatile components, such as sulfur-containing aroma compounds, are removed. Finally, during prolonged boiling, water evaporates and wort is concentrated. Because the mash or wort is diluted by sparge water, evaporation returns the density or specific gravity back to the desired level.

Box 10.4 Hop acids, antimicrobial activity, and mechanisms of resistance

It has long been known that beer is less prone to microbial spoilage than many other aqueous beverages. That is why beer has historically often been considered better preserved than even water (e.g., the Pilgrims on the Mayflower). It is now known that the preservation properties of beer are due to the presence of several components or constituents that inhibit a wide variety of microorganisms (Suzuki, 2011). In particular, ethanol concentrations above 3% and pH levels below 4.6 are inhibitory to many bacteria and yeasts, including those involved in spoilage. Carbon dioxide, low oxygen levels, and limiting nutrient concentrations also restrict growth of microorganisms. However, it's the hops that are considered one of the more potent antimicrobial constituents in beer, and are responsible, in large part, for beer's long shelf-life. In particular, hops inhibit many of the lactic acid bacteria that spoil beer via production of acids, diacetyl, and other products that confer flavor and aroma defects.

The antibacterial properties of hops are due primarily to the alpha-acid fraction. As shown in the model below (Figure 10.4.1A), the mechanism is actually similar to how other weak organic acid preservatives, such as benzoic and propionic acids, inhibit microorganisms in foods. Specifically, hop acids, like other weak acid preservatives, have ionophoric activity and are inhibitory due to their ability to decrease intracellular pH and to disrupt proton gradients. Given the low pH of beer (<5) and their low pKa (3.1), isomerized iso-alpha-acids exist in their undissociated or acid form (abbreviated as HA), and hence, are able to diffuse across the lipophilic cell membranes of spoilage lactic acid bacteria. Since the cell cytoplasm of these bacteria is near neutral, the iso-alpha-acids dissociate (according to the Henderson–Hasselbalch equation), forming the anion (A^-) and releasing a free proton (H^+). The latter then lowers the cytoplasmic pH. The cell may respond by activating ATP-dependent pumps that efflux the accumulated protons, but the continued proton cycling is expensive (energy-wise). Eventually, too many protons in the cytoplasm leads to dissipation of the pH gradient component of the proton motive force (PMF) and other ion gradients that the cell uses to drive various transport systems. Thus, energy depletion, cytoplasmic acidification, and reduced nutrient uptake all occur as a result of hop-derived alpha-acids and account for their inhibitory activity.

However, a second mechanism also may be involved, because it appears that the dissociated form (A^-) of the alpha-acids binds to cytoplasmic divalent cations, especially manganese (Mn^{2+}). The iso-alpha-acids-Mn^{2+} complex contributes to a change in the intracellular redox potential resulting in oxidative stress (Behr et al., 2007; Behr and Vogel, 2009; Behr and Vogel, 2010). Efflux of these complexes from the cell also results in low intracellular manganese levels and loss of a necessary nutrient that must then be re-transported, with the concurrent cost of energy.

Despite the effectiveness of hop alpha-acids as antimicrobial agents, some bacteria (mainly *Lactobacillus* or *Pediococcus*) have been isolated from spoiled beer that appear insensitive or resistant to hops. Several resistance mechanisms have been identified (shown in Figure 10.4.1B as dashed lines). In *Lactobacillus brevis*, a common spoilage organism, resistance is mediated, in part, by transport systems that operate as efflux pumps that are similar to multi-drug exporters. In general, they consist of

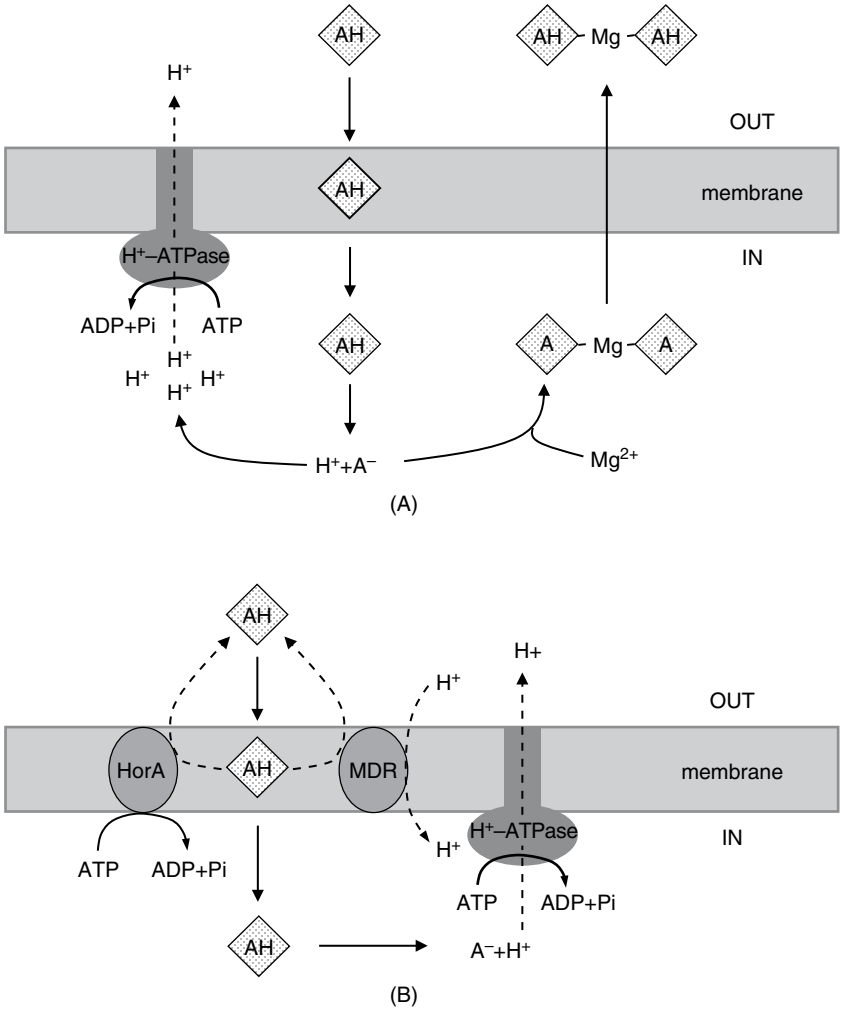


Figure 10.4.1 Inhibition of lactic acid bacteria by hops. In A, the means by which hop iso-alpha-acids inhibit sensitive organisms is shown; a model for hop resistance is shown in B. Adapted from Sakamoto et al., 2002 and Sakamoto and Konings, 2003.

membrane-integrated and other accessory proteins that use ATP or the PMF to drive efflux of iso-alpha-acids or iso-alpha-acids-Mn^{2+} complexes (Karabin et al., 2016). The HorA system, for example, is classified as an ATP-binding cassette (ABC) transport system that effluxes iso-alpha-acids via ATP hydrolysis. The HorC system also pumps out iso-alpha-acids, but uses the PMF as the driving force. A third system relies on increased activity of the proton translocating ATPase (H^+-ATPase) to pump out accumulated protons.

Another means by which hop resistance occurs is via expression of glutamic acid decarboxylase (GAD). Decarboxylation of glutamate by this enzyme results in consumption of an intracellular proton, as well as formation of gamma-aminobutyric acid

(GABA) that raises the extracellular pH (Schurr et al., 2013). It appears that the GAD system is induced and active in the presence of hops.

References

Behr, J. and R.F. Vogel. 2009. Mechanisms of hop inhibition: hop ionophores. J. Agric. Food Chem. 57:6074–6081.

Behr, J. and R.F. Vogel. 2010. Mechanisms of hop inhibition include the transmembrane redox reaction. Appl. Environ. Microbiol. 76:142–149.

Karabın, M., T. Hudcova, L. Jelınek, and P. Dostalek. 2016. Biologically active compounds from hops and prospects for their use. Comp. Rev. Food Sci. Food Saf. 15:542–567.

Sakamoto, K. and W.N. Konings. 2003. Beer spoilage bacteria and hop resistance. Int. J. Food Microbiol. 89:105–124.

Sakamoto, K., H.W. van Veen, H. Saito, H. Kobayashi, and W.N. Konings. 2002. Membrane-bound ATPase contributes to hop resistance of *Lactobacillus brevis*. Appl. Environ. Microbiol 68:5374–5378.

Schurr, B.C., J. Behr, and R.F. Vogel. 2013. Role of the GAD system in hop tolerance of Lactobacillus brevis. Eur. Food Res. Technol. 237:199–207.

Schurr, B.C., J. Behr, and R.F. Vogel. 2015. Detection of acid and hop shock induced responses in beer spoiling *Lactobacillus brevis* by MALDI-TOF MS. Food Microbiol. 46:501-506.

Suzuki, K. 2011. 125th anniversary review: microbiological instability of beer caused by spoilage bacteria. J. Inst. Brew. 117:131–155.

As noted above, a trub or precipitate forms during the kettle boil step. In addition, the wort may also contain insoluble hop debris, especially if whole hops had been used. The latter are removed via strainers or screen-type devices that may also allow for sparging and re-circulation. For hot trub removal, several separation systems can be used. The simplest method is sedimentation, but this is not a very efficient process. A much more efficient method is to centrifuge the hot wort using continuous centrifuges. Such units, however, are expensive and require frequent maintenance. Therefore, the most common means of separating out the hot trub is the whirlpool separator, which is typically a low-height, conical-shaped (cone pointing downward) tank whose geometry promotes whirlpool-type movement of the wort. The trub or precipitate collects in the center of the tank.

After the trub is removed, the wort is cooled rapidly in plate-type heat exchangers. During the cooling stage, when the wort reaches about 50°C, more precipitated material forms (known as the "cold break"), which can be separated by centrifugation or filtration. When the wort temperature is reduced to 12°C to 22°C (depending on yeast preferences and beer style), it is pumped into fermentation vats, where it is, at last, ready to be made into beer.

THE BEER FERMENTATION

All of the steps described above for making wort are performed for one main reason – to provide a suitable growth medium for the ethanol-producing yeast. Before yeast is added, however, the wort is first aerated or sparged with sterile air. Even though the beer fermentation eventually becomes an anaerobic process, creating an aerobic environment at the outset jump-starts the yeast and promotes a more rapid entry into a logarithmic growth phase. Importantly, oxygen also is necessary for biosynthesis of cell membrane lipids that are essential for growth of yeast in wort.

Fermentor design

The fermentation of the wort occurs in fermentor vessels of varying composition, size and configuration, and in either batch or continuous modes. Although most modern fermentors are now constructed of stainless steel, the traditional materials were wood, concrete, or copper. Size and shape depend on a number of considerations, but are mostly driven by whether the fermentation is top fermenting, as for ales, or bottom fermenting, as for lagers (see below).

In general, lager fermentation vessels are cylindric, with cone-shaped bottoms (cylindroconical). At the end of the fermentation, the yeasts flocculate and settle within the conical region and can be retrieved and then re-used. In contrast, traditional ale fermentors are uncovered, such that at the end of the fermentation when the yeasts flocculate and rise to the surface, they can easily be recovered and re-used. Although this design is intentionally open to the environment, the thick blanket of CO_2 foam that develops during ale fermentations provides some protection against the elements (e.g., oxygen in the air, airborne yeasts and bacteria, and other contaminants). Many modern ale fermentors are now enclosed or constructed with covers. These designs still provide access to yeast recovery. The nature of yeast flocculation and why lager yeasts settle and ale yeast rise are discussed below.

Currently, enclosed, pressurized, cylindroconical fermentors are used for lagers, as well as ales, with capacities of nearly 10^6 L. Enclosed fermentors obviously have the advantage of reducing exposure to air and airborne contaminants. Whereas open fermentors, whether round or rectangular, are necessarily more horizontal (i.e., width > height), enclosed fermentors are usually constructed in a vertical orientation, so less floor space is required. Regardless of shape, however, most modern fermentors are jacketed to provide efficient cooling.

Inoculation

After the wort is cooled and aerated, the yeast culture is, at last, added to the wort, in a step called pitching. Brewing yeast strains are dramatically different from baker's yeast and wine yeasts and are also dissimilar to the *Saccharomyces cerevisiae* strains used in laboratory research. Even between brewing strains, there are major physiological, biochemical, and genetic differences, starting with the type of beer being produced. Many of these differences are highly relevant to the beer fermentation (Table 10.4).

Two types of yeasts are used in brewing. Ale style beers are made using selected strains of *Saccharomyces cerevisiae*, otherwise known as the "ale" or "top-fermenting" yeast. Lager beers are fermented by *Saccharomyces pastorianus* (formerly called *Saccharomyces*

Table 10.4 Differences between ale and lager yeasts.

Ale ***(Saccharomyces cerevisiae)***	**Lager** ***(Saccharomyces pastorianus)***
Flocculated yeast rise to the top	Flocculated yeast settle to the bottom
Typical growth temperature, 18–25°C	Typical growth temperature, 8–15°C
Minimum growth temperature = 15°C	Minimum growth temperature = 7°C
Maximum growth temperature = 40°C	Maximum growth temperature = 34°C
Cannot metabolize melibiose	Able to metabolize melibiose
Slow assimilation of maltotriose	Efficient assimilation of maltotriose
Sporulating (at low frequency)	Non-sporulating

carlsbergensis), also known as the "lager" or "bottom-fermenting" yeast. Despite this terminology, growth of these yeasts in wort is not necessarily confined to the top or bottom regions of the fermentor. Rather, top-fermenting yeasts, as they grow in the wort, tend to form low density clumps or flocs that trap CO_2 and rise to the surface. In contrast, when lager yeasts flocculate, the flocs are heavier and they sediment or settle to the bottom. The ability of brewing yeast to flocculate and the point during the fermentation at which flocculation occurs are very important factors, as will be discussed later. It should also be noted that the distinction between top- and bottom-fermenting yeast has become somewhat blurry, as the use of enclosed cylindroconical fermentation vessels results in even ale yeasts dropping to the bottom of the fermentor (discussed below).

The preferred or optimum growth temperature is another major difference between ale and lager yeasts. Ales are normally fermented at fairly high temperatures, from 18°C to as high as 27°C, well within the range at which *S. cerevisiae* grows. It is no coincidence, therefore, that ales were historically commonly produced in warmer climates that are typical in the British Isles. Thus, many of the classic English-style beers, whether produced in the UK or in former British colonies, such as India, are ales. In contrast, lager style beers are fermented by yeast capable of growing at temperatures below 15°C. Not surprisingly, these beers evolved from Germany and other northern European areas, where the ambient climates were cooler. Interestingly, when German immigrants moved to the United States in the late 1800s and early 1900s, they brought with them German beer-making technology. The American beer industry subsequently became dominated by large breweries making lager beers (e.g., Schlitz, Anheuser-Busch, Coors, Miller, Stroh, Heileman, Pabst, and others).

Beer yeast cultures are commercially available, and many small-to-medium sized breweries use these cultures. However, most large breweries use their own proprietary or "house" cultures. Brewing strains can be bred, much like plants or seeds, to provide hybrids with specific traits. These cultures are maintained and propagated in the laboratory and, when needed, grown in volumes necessary for inoculation into the fermentation tanks. Some breweries may also use the yeast slurry left over from the previous fermentation to pitch the next batch. Only when the fermentation appears slow or sluggish or when quality attributes suffer will a new culture be prepared.

YEAST METABOLISM

The usual starting inoculum is intended to give an initial yeast population of about 5×10^6 cells per ml of wort. Depending on the activity of the yeast inoculum, a lag period of six to 18 hours may occur. Although no increase in cell number is observed during the lag phase, metabolic activity is well under way. The yeasts are synthesizing sugar and amino acid transport systems, as well as enzymes necessary for their metabolism. As noted earlier, the wort is sparged with oxygen prior to pitching, thus the wort medium is initially highly aerobic. This is an important step, as the oxygen-rich environment stimulates synthesis of membrane-associated sterols and unsaturated fatty acids. Sterols, and in particular, ergosterol, are essential for formation of yeast cell membranes; likewise, the unsaturated fatty acids provide membrane fluidity. Because of these requirements, yeasts will get off to a slow start and grow poorly in oxygen-deprived worts.

Following the lag phase, cells enter into the logarithmic growth phase, and a primary ethanolic fermentation period begins. Despite the aerobic conditions initially established by aerating the wort with oxygen, sugar metabolism by *Saccharomyces* does not occur by

aerobic respiration (i.e., the tricarboxylic acid or Krebs cycle). Rather, sugars will be fermented via the Embden-Meyerhof-Parnas (EMP) glycolytic pathway. Perhaps even more surprising is that Krebs cycle enzymes are not even expressed during growth of *Saccharomyces* in wort. This well-studied phenomenon, called the Crabtree effect, is due to catabolite repression by glucose. The repression of these genes under aerobic conditions occurs only for as long as the sugar concentration remains high. Thus, when the availability of glucose and other fermentable sugars is nearly diminished (i.e., < 0.1 g/L), metabolism in an aerobic environment shifts from fermentation to respiration. In the latter stages of beer fermentation, however, anaerobic conditions will prevail.

Catabolite repression of respiratory activity by glucose also influences other biochemical processes that occur during the fermentation. Although brewing strains of *S. cerevisiae* and *S. pastorianus* can use all of the major sugars normally present in wort (glucose, maltose, and maltotriose; fructose and sucrose if adjuncts are used), metabolism of these sugars does not occur concurrently. In fact, most worts contain more maltose and maltotriose than glucose, yet glucose is still metabolized first. This preferential use of glucose occurs because: (1) at least one of the glucose transporters is constitutively expressed; and (2) the gene coding for the α-glucoside (maltose and maltotriose) transporter (*AGT1*) is repressed by glucose. Only when glucose is fermented (after about one to two days), will expression of the α-glucoside transport gene occur.

Sucrose, in contrast, is hydrolyzed by an invertase located within the periplasmic space (i.e., between wall and membrane), yielding its component monosaccharides, glucose and fructose. The regulation of sugar metabolism becomes especially relevant during beer fermentations when high glucose adjuncts are used. Under these circumstances, repression of maltose and maltotriose metabolism may exist throughout the entire fermentation. The resulting beer will be poorly attenuated, meaning that fermentable sugars still remain.

Following transport, intracellular metabolism of wort sugars proceeds via the EMP pathway, yielding pyruvic acid and reduced NADH. Pyruvate is subsequently decarboxylated by pyruvate decarboxylase, releasing CO_2 and acetaldehyde. The latter is then reduced by NADH-dependent alcohol dehydrogenase, forming ethanol and re-oxidizing NAD. Because a small amount of the glucose carbon must be used to support cell growth, some of the pyruvate is diverted, via pyruvate dehydrogenase, to the Krebs cycle, where biosynthetic precursors are formed. Re-oxidation of NAD then requires an alternative source of electron acceptors, leading to the formation of glycerol, higher alcohols, and other minor end-products found in beer.

The logarithmic growth phase continues for two to three days for ales, and up to six or seven days for lagers (given the cooler incubation temperatures). At the end of the fermentation, all of the mono- and disaccharides, and most of the maltotriose, will have been consumed. Cell mass usually increases by less than two logs. Importantly, the fermentation is exothermic, meaning that heat is generated, so the fermentation tanks must be cooled and maintained. Either internal cooling coils or external jackets are used to maintain temperatures of 8°C to 15°C for lagers and 15°C to 22°C for ales.

For ale fermentations, yeast cells, as noted earlier, rise to the surface, along with the evolved CO_2. The yeast can then be skimmed off and re-used later. For lager fermentations, cells remain suspended, but there is enough CO_2 evolved during the early stages of the fermentation to form cauliflower-shaped clumps. This thick foam layer is called krausen, and as growth and CO_2 formation become more rapid, corresponding to the maximum growth rate of the cells, a period known as "high krausen" is reached. A portion of this cell-rich material can be collected and re-used to initiate a secondary fermentation, as described

below. In general, however, the CO_2 formed during the primary beer fermentation is lost to the atmosphere. Closing the vat toward the latter stages of fermentation could result in retention of CO_2 in the fermentation tank, although this is not a common practice.

Although it is possible to monitor the progress of the fermentation simply by observing CO_2 formation, the more common method is to measure the specific gravity. The more sugars (or solutes) are dissolved in an aqueous solution, the greater will be the density and the higher the specific gravity. Thus, as the fermentable sugars are consumed in wort, the specific gravity, expressed as ^{0}Plato, decreases. When the specific gravity is no longer decreasing, most of the fermentable sugars will have been depleted, and the fermentation is complete. The beer is considered to be fully attenuated. At this point, usually about seven days after the beginning of the primary fermentation period, the fermentation vessel is quickly cooled to 4°C or less.

Some brewers, prior to cooling and depending on the style of beer, may extend the fermentation period to promote a so-called "diacetyl rest". Diacetyl, as described previously, imparts buttery flavors in fermented foods and is considered a defect in beer (discussed in more detail later). Yeasts can produce diacetyl and other related vicinal diketones. Extending the fermentation period gives the yeast a chance to re-assimilate diacteyl and may reduce the time necessary during the post-fermentation conditioning phase, when diacetyl reduction would ordinarily occur.

Flocculation

The ability of yeast cells to agglomerate or adhere to one another in the form of clumps is called flocculation. As noted previously, when lager yeasts flocculate, the clumps have a density greater than that of the beer and settle to the bottom. Ale yeasts, in contrast, form clumps or flocs that entrap CO_2 bubbles and have a lower density, and, therefore, rise to the surface. The ability of yeast cells to clump or flocculate, and the time at which flocculation occurs, are very important properties in beer manufacture. In most cases, flocculation should occur at the end of the fermentation, when all of the monosaccharides (glucose and fructose), disaccharides (sucrose and maltose), and trisaccharides (maltotriose) have been fermented. The yeast will have done its job, fermentable sugars are depleted, and the beer is considered to be fully attenuated.

If, however, flocculation occurs prematurely, before the end of the fermentation, fermentable sugars will remain in the beer -a situation brewers refer to as a hanging or stuck fermentation. These residual sugars can affect maturation and flavor. In particular, these unattenuated beers have a lower than normal ethanol concentration and are relatively sweet which, depending on the intent of the brewer, may or may not be desirable. Of course, the presence of sugars in the beer also provides growth substrates for other organisms.

In contrast, if the yeast cells fail to flocculate, and instead remain in the beer, other problems are likely to occur. Suspended yeasts are difficult to remove, causing cloudiness problems in the beer. These yeasts may later autolyze in the beer, releasing enzymes that contribute to yeasty flavor defects. Although the conditions that induce flocculation are not completely known, several factors are relevant. In particular, the onset of flocculation appears to be triggered by entry into stationery phase, low pH, low temperature, low sugar concentration, and high ethanol concentrations. Hops and calcium (see below) also promote flocculation activity.

Despite the importance of flocculation in the brewing process, the physical-chemical basis of this property is not fully understood. Ale yeasts, in general, have been reported to be

more hydrophobic than lager yeasts, due to differences in cell surface charges, but it is not clear that this property affects flocculation. Rather, it now appears that flocculation occurs as a result of lectin-like domains, contained within cell surface proteins. In the presence of ionic calcium, these lectins bind to mannans (i.e., mannose-containing chains) located on the surface of adjacent cells. Thus a floc will consist of a network of lectin-associated cell surface proteins attached to cell-surface mannan ligands. Flocculation is also a heritable property, meaning it has a genetic basis. Several genes have been identified in *S. cerevisiae* that code for proteins involved in flocculation, making it possible to select for strains expressing this trait (Box 10.5).

Box 10.5 Flocculation – a case of beer yeasts sticking together

Why flocculate? The ability to flocculate, consistently and at the right time during the fermentation, is one of the most important traits of a good brewing yeast. If flocculation occurs too early or too late, beer flavor, clarity, and overall quality are bound to suffer. Yeast floccuation is now thought to be mediated via a lectin-like mechanism in which the N-terminal region of cell wall-associated proteins (called floculins) recognize and bind, in a calcium-dependent manner, to mannose residues (or trimannoside oligosaccharides) located on the surface of other cells (Figure 10.5.1; Soares, 2011; Vidgren and Londesborough, 2011). Although both flocculating and non-flocculating cells contain this mannan receptor, only flocculating cells produce the binding protein. Importantly, flocculation is influenced by several physiological and environmental factors, including growth phase, ethanol and sugar concentrations, oxygen, pH, and calcium ion availability (Soares, 2010; Table 10.5.1). These observations suggest that flocculation depends not only on physical interactions, but is also subject to genetic regulation.

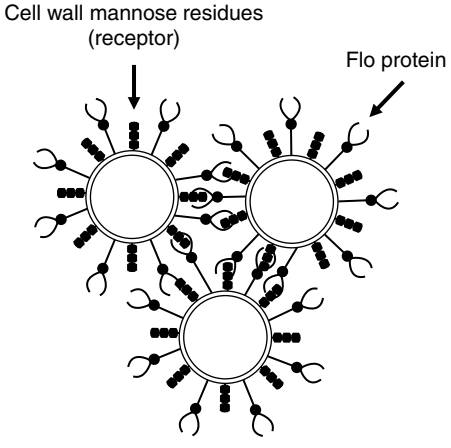


Figure 10.5.1 Model for yeast flocculation. Flocculating cells produce cell wall-attached lectin-like Flo proteins that recognize and bind to mannose residues on the surface of receptor cells. The receptors are present on most cells. Thus, it is the expression of the lectin-like proteins that is responsible for the flocculation phenotype. Adapted from Soares, 2011, with permission.

Table 10.5.1 Factors affecting flocculation.

Factor	Effect
Fermentable sugars	Inhibitory
Nitrogen, other nutrients	Little effect
Temperature	Strain-dependent, with broad range
pH	Optimal flocculation at pH 3.5–5.8
Oxygen	No direct effect
Ethanol	Strain-dependent, affects cell surface
Cell age	Old cells flocculate more than young cells
Inoculum handling	Ambient temperature increases flocculation

FLO genetics Eight genes currently have been identified in *Saccharomyces cerevisiae* that encode for proteins involved in adhesion. These include *FLO1*, *FLO5*, *FLO9*, *FLO10*, *FLO11, FLONL, FLONS*, and *Lg-FLO1* (Vidgren and Londesborough, 2011). Several of these genes, *FLO5, FLO9*, *FLONL,* and *FLONS* have high sequence homology to *FLO1*. However, several different adhesion phenotypes exist, including those based on cell-to-cell adhesion (i.e. flocculation), as well as adherence to other surfaces.

The expression of these genes is complex and unstable, and appears to be controlled by both genetic and epigenetic mechanisms (Verstrepen and Fink, 2009). Importantly, as noted above, the beer environment can have a profound influence on flocculation. Several flocculation phenotypes have been described for brewing strains of *S. cerevisiae* (Soares et al., 2010). For example, for cells having a *FLO1* phenotype, flocculation is inhibited by mannose. The NewFlo phenotype is inhibited by mannose, glucose, maltose, and sucrose. This phenotype is also more sensitive to low pH and cations than other cells. In contrast, the MI phenotype is mannose insensitive. Finally, flocculation can also be ethanol- and growth phase-dependent. While flocculation by most *FLO1* yeasts is constitutive, flocculation by NewFlo cells occurs during stationary phase (when ethanol is high). Thus, most lab strains have the *FLO1* phenotype, and most brewing strains have the NewFlo phenotype (Javadekar et al., 2000).

Inducing flocculation Brewing yeasts ordinarily do not flocculate in the presence of glucose and other fermentable sugars. Furthermore, when fermentable sugars are added to flocculant cells, the flocs will disperse and subsequent flocculation is lost (Soares et al., 2004). It also appears that, under experimental conditions, the rate of fermentation-dependent loss of flocculation correlates with growth rate and sugar use. That is, the faster the cells grow, the faster the flocs come apart. Despite these findings, however, it is not clear at a molecular level how the cells sense sugar availability and then respond by inducing or repressing flocculation. Nonetheless, it does appear that flocculation requires *de novo* synthesis of proteins that are induced as a result of the combined effects of nutrient shortage (of either fermentable sugars or nitrogen) and the presence of ethanol (Sampermans et al., 2005).

Flocculation stability The inherent instability of the flocculation property can be very frustrating for brewers. If flocculation occurs too early, the beer is not fully attenuated and the beer is considered "stuck". The residual sugars remain, leading to sweet flavors and availability of growth substrates for microorganisms. In addition, the ethanol content is too low. In contrast, yeasts that do not flocculate are perhaps even more of a problem. The loss of the flocculation phenotype is more common after successive pitching and re-use. Although these observations might suggest there is a genetic basis, the phenomenon appears to depend on the strain, as some strains remain flocculent for many generations (Soarres, 2010).

Opportunities Efforts to modify or improve flocculation properties of brewing yeast are being pursued actively as part of current strain improvement programs. Although the well-studied lab strain (*S. cerevisiae* S288C), is amenable to gene expression and mutation analysis (van Mulders et al., 2009), brewing strains are another matter. In part, this is because in contrast to lab strains, brewing strains are polyploidal and not easily manipulated. Perhaps more importantly, genetically modified strains are not popular among many beer consumers. Nonetheless, recent studies indicate that it is now possible to improve the flocculation properties of industrial brewing yeast strains without genetic engineering. In one study, chemically-mutagenized strains of *Saccharomyces pastorianus* were passed for 200 generations though high osmotic strength media (Ekberg et al., 2013). Selected strains were assessed in mini-wort fermentations and two fast-growing strains were also found to be strong flocculators. More recently, a novel adaptive evolution approach was used to select for strong flocculating brewer's yeast (Conjaerts and Willaert, 2017). They first constructed continuous mini-fermentors that were autoclavable using 3D printer technology. They were also built in a tower format such that the selective pressure was based on gravity. Thus, during continuous culturing in these fermentors, only cells that formed aggregates were retained, and non-aggregating cells passed through the vessels. Although the fermentations had been started with a low flocculating strain, after 2 weeks, the cells that were recovered had a multi-cellular floc appearance. The authors noted that the phenotype was likely independent of flocculin interactions, but rather due to the mother–daughter cell separation phenomenon.

References

Conjaerts, A. and R.G. Willaert. 2017. Gravity-driven adaptive evolution of an industrial brewer's yeast strain towards a snowflake phenotype in a 3d-printed mini tower fermentor. Fermentation 3:4.

Ekberg, J., J. Rautio, L. Mattinen, V. Vidgren, J. Londesborough, and B.R. Gibson. 2013. Adaptive evolution of the lager brewing yeast *Saccharomyces pastorianus* for improved growth under hyperosmotic conditions and its influence on fermentation performance. FEMS Yeast Res. 13:335–349.

Javadekar, V.S., H. Sivaraman, S.R. Sainkar, and M.I. Khan. 2000. A mannose-binding protein from the cell surface of flocculent *Saccharomyces cerevisiae* (NCIM 3528): its role in flocculation. Yeast. 16:99–110.

Sampermans, S., J. Mortier, and E.V. Soares. 2005. Flocculation onset in *Saccharomyces cerevisiae*: the role of nutrients. J. Appl. Microbiol. 98:525–531.

Soares, E.V. 2011. Flocculation in *Saccharomyces cerevisiae*: a review. J. Appl. Microbiol. 110:1–18.
van Mulders, S.E., E. Christianen, S.M.G. Saerens, L. Daenen, P.J. Verbelen, R. Willaert, K.J. Verstrepen, and F.R. Delvaux. 2009. Phenotypic diversity of Flo protein family-mediated adhesion in *Saccharomyces cerevisiae*. FEMS Yeast Res.9:178–190.
Verstrepen, K.J. and G.R. Fink. 2009. Genetic and epigenetic mechanisms underlying cell-surface variability in protozoa and fungi. Annu. Rev. Genet. 43:1–24.
Verstrepen, K.J., G. Derdelinckx, H. Verachtert, and F.R. Delvaux. 2003. Yeast flocculation: what brewers should know. App. Microbiol. Biotechnol. 61:197–205.
Vidgren, V. and J. Londesborough. 2011. 125th Anniversary review: Yeast flocculation and sedimentation in brewing, J. Inst. Brew. 117:475–487.

POST-FERMENTATION STEPS

As noted earlier, beer manufacture, at first glance, would appear to be a rather simple exercise: barley (and sometimes other grains) is converted to malt, which is converted to wort, which is then fermented by yeast. When the beer is removed from the tank, it will be somewhat cloudy, especially in the absence of any clarification steps. The problem is that at this point the beer still contains suspended yeasts and other microorganisms, as well as other insoluble and non-dissolved materials that give it an undesirable cloudy and hazy appearance. In addition, this so-called "green" or immature beer may contain chemical constituents that impart off-flavors. Finally, little or none of the carbon dioxide produced during the primary fermentation is retained in the beer, leaving the product without one of its key components. Thus, the challenge for the brewer is to promote acceptable flavor development and maturation, to remove remaining organisms and the undesirable cloud- and haze-forming materials, and to introduce carbonation into the beer. Indeed, it can be argued that the post-fermentation steps are as important as any of the preceding activities. This is true for large volume, high capacity brewers as well as small craft breweries.

Flocculation should occur as the fermentation ends, or more precisely, when the fermentable sugars are depleted and the beer is fully attenuated. Once the yeasts have flocculated, they are usually collected promptly and separated from the beer. It is important to recognize, as noted above, that even though most of the yeast cells are removed by the sedimentation or skimming step, the beer still contains suspended yeast cells. In addition, this green beer may contain undesirable flavor compounds, including sulfur dioxide and diacetyl, as well as proteins and tannins that can potentially form haze complexes. Therefore, it is essential that the beer be "conditioned" to enhance sedimentation of the remaining yeasts and haze-forming proteins, promote dissipation of off-flavors, and produce beer with a mature or finished flavor. Since yeast growth also occurs during the conditioning period, a secondary fermentation may take place, resulting in formation of CO_2. Depending on the specific conditions, enough CO_2 may be produced to naturally carbonate the beer. So-called cask-conditioned beers (see below) are carbonated in this manner.

Conditioning

Conditioning (or maturation) can be performed by one of several methods. In traditional ale manufacture, for example, the beer is pumped from the fermentor into wooden casks ranging in size from 20 L to more than 200 L. Stainless steel casks have replaced many of the wood

casks, although the latter are still available. A source of fermentable carbohydrates, in the form of fresh beer (i.e., shortly after pitching) is added to induce the secondary fermentation. Additional hops and fining agents (see below) may also be added. The casks are held at 12°C to 18°C for up to seven days. Following this maturation period, this cask-conditioned or draught beer is ready for consumption, without any additional clarification steps. Because the yeast sediment is still present, this beer has a relatively short shelf-life (from 2–3 weeks).

For traditional lager beers, conditioning or lagering occurs in tanks held at lower temperatures for a longer time. Typically, temperatures as low as 0°C, for as long as three months, can be used. A special variation of the lager method involves adding a portion of wort obtained at high krausen (i.e., cells in their most active growth phase) to the green beer. Since this material also contains wort sugars, this step, called krausening, essentially serves as a source of both fast-growing cells, as well as fermentable substrates. Since the vats are closed to the atmosphere, the CO_2 that evolves is trapped, and the beer becomes naturally carbonated.

Traditional conditioning of lagers and ales is time-consuming and expensive, and therefore, used mainly for premium beers that can bear higher production costs. Most modern breweries have adopted faster methods, referred to as accelerated lagering or brewery-conditioning. In these systems, the fully attenuated beer is pumped into storage tanks at 0°C to 2°C and held for one week or less. For some beers, small wood chips (less than 3 cm × 30 cm) may be layered on the bottom of the tank to promote maturation (e.g., "beechwood aging"), as well as serve a clarification function (see below). During the fermentation and later during conditioning, many volatile flavor compounds are formed that have a major impact on the overall quality of the beer (Table 10.5).

Clarification

Whether the beer has been conditioned via casking or lagering, suspended yeast cells will still be present. Because consumers have come to expect beers to be haze- or cloud-free, with excellent clarity, clarification and filtrations steps are almost always performed. The exception is for darker beers where the natural appearance of the beer is sufficient. Clarification can occur via physical separation methods, such as centrifugation, or by the addition of fining agents.

Fining agents act by promoting aggregation or flocculation of yeast cells, and include wood chips, gelatin, and isinglass. The latter, which is derived from swim bladders of fish

Table 10.5 Important volatile flavor compounds in beer.

Esters	Alcohols	Acids	Carbonyls	Sulfur compounds
isoamyl acetate	propanol	acetic acid	diacetyl	hydrogen sulfide
2-phenylethyl acetate	2-methylpropanol	caproic acid	2,3-pentenedione	sulfite
ethyl acetate	2-methylbutanol	caprylic acid	acetaldehyde	methanethiol
ethyl caproate	3-methylbutanol	caprylic acid		dimethyl sulfide
ethyl caprylate	isoamyl alcohol	lactic acid		
	amyl alcohol			
	isobutyl alcohol			
	2-phenylethylethanol			

Adapted from Blanco et al., 2016; Dufour et al., 2003 and Hough et al., 1971.

(really), is more commonly used for ales, especially in the UK, whereas gelatin is frequently used in the United States for lager-style beers. The suspended cells and other insoluble debris then settle to the bottom of the tank or cask. The clarified, or so-called "bright" beer that remains is not cell-free. Therefore, the beer may subsequently be filtered to remove residual yeast or bacterial cells that still remain.

Several filtration configurations exist, including plate and frame systems, leaf filters, and cartridge filters. In general, the filters or filter sheets do not exclude cells on their own, as the pores within these filters are not so small that they directly restrict passage of cells. Yeast and bacterial cells have approximate diameters of about 10 μm and 2 μm, respectively, and typical cellulose filters have much larger pore sizes. To remove cells by filtration, therefore, filtration aids are often used.

Filtration aids are inert, insoluble materials, such as cellulose or diatomaceous earth, that act to form a filter bed that traps cells without causing the filter to become plugged or fouled with a layer of cells. Cell counts of less than 10 per ml can be achieved in the filtered beer. Other filter aids, such as silica gel and polyvinylpolypyrrolidone (PVPP), can also be used, but their main function is to remove haze-forming proteins and polyphenolic materials, rather than cells (see below).

For large breweries, the use of filtration aids such as diatomaceous earth can impose a substantial environmental burden (see Sustainability later in this chapter). Filtration agents that can be recycled are now being used in some breweries. Alternatively, cross-flow filtration systems that effectively clarify beer without the need of filtration aids are also being used.

Despite the effectiveness of these bulk filtration systems, however, there are now many breweries that filter the beer through membrane filters with pore sizes less than 0.45 μm. Since bacteria and yeasts cannot pass through these filters, these beers can potentially become cell-free. Many of the large breweries now produce filter-sterilized beer. Because filtration is done at low temperature, this process is also referred to a cold-pasteurized beer (which also has the marketing advantage of distinguishing this process from heat-pasteurized beer).

If a sterile (or "polish") filtration step is to be performed, the beer must first be pre-filtered (as described above), to remove cells and other material that would otherwise foul the membranes. Although most brewers recognize that filtration is necessary to produce beer with the expected clarity and stability properties, they also realize that filtration may remove flavor, color, body characteristics, and other desirable components. Thus, filtration systems must be carefully designed to achieve the desired appearance without sacrificing flavor and body.

Process aids

Various approved additives are frequently used during the post-fermentation steps. For example, the filtration materials described above would qualify as a process aid. Other process aids include agents to improve flavor, color, appearance, and stability. One important additive is proteolytic enzymes, which are used as "chill-proofing" agents. These enzymes hydrolyze proteins that would otherwise precipitate and form complexes with tannins and other polyphenolic compounds at low temperatures and give a cloudy or hazy appearance when the beer is chilled. Hydrolysis of these proteins prevents this "haze" or cloudiness.

Several commercially-available types of enzymes are used. Papain, obtained from papaya, is commonly used, as are fungal- and bacterial-derived proteinases. The main requirement is that the enzyme be rather specific for haze-forming proteins (and not the foam-forming proteins). It may also be necessary that the enzyme be inactivated by a subsequent heating step, so residual activity in the finished beer is absent.

Other non-enzymatic, chill-proofing agents are also used, including tannic acid, bentonite, silica gels, and PVPP. Again, the use of these agents is permitted in some countries, and prohibited in others. In the United States, regulations regarding permitted additives and process aids are described in the US Code of Federal Regulations, Title 21, Part 173. Other general regulations on beer manufacturing are contained in Title 27, Part 25. Currently, in the US, they do not need to be declared on the label, although many microbreweries promote the absence of these agents in their beer.

Carbonation

Of all the post-fermentation steps, perhaps none is as important as carbonation. Carbonation provides sensual appeal by enhancing mouthfeel, flavor, and body. Indeed, other than color, the foam (or head) is one of the first attributes observed in a glass of beer and it has considerable value as a sensory trait (Box 10.6). In addition, the CO_2 preserves the beer by reducing pH and the oxidation-reduction potential (Eh), such that various aerobic, acid-sensitive spoilage organisms are inhibited. Carbonation of beer can occur naturally, via a secondary fermentation, or mechanically, by directly adding CO_2 after the conditioning and filtration steps. Although "flat" beer is generally undesirable, depending of the style, some beers are intentionally produced with less CO_2.

As described earlier, beer can be conditioned via several procedures. In the case of traditional ales, conditioning is commonly done in casks, whereas traditional lagers are conditioned, or lagered, in enclosed tanks. Some specialty beers (and those made by home-brewers) are conditioned directly in bottles. In all of these processes, the beers become naturally carbonated due to a secondary fermentation that yields CO_2 (as well as additional ethanol).

Box 10.6 Heady stuff – a lot of science goes into beer foam

Appearance as a sensory trait The visual appearance of beer has long been recognized as one of the most important quality attributes of beer (Bamforth, 2000). Significant appearance properties include clarity and brilliance, color, and foam. Although clarity and color are generally a function of brewing and post-brewing steps, formation of foam generally does not occur until the beer is poured and a "head" is formed. Nonetheless, foam properties are influenced by ingredients as well as processing steps that occur upstream from the actual formation of foam.

Foam appeal Despite its simplicity (after all, it's mainly CO_2), the foam layer is appealing to consumers for several reasons (Evans and Bamforth, 2009). For one, because foam formation occurs at the liquid-air interface, it provides an efficient mechanism for delivering aroma molecules to olfactory sensory receptors. Secondly, foam contributes important tactile effects in the mouth and even lips. Finally, there is an important visual quality to beer foam. The latter attribute is very subjective, however, as too little or too much foam can also be perceived as a negative quality (Donadini et al., 2011; Evans and Bamforth, 2009). Ultimately, foam formation, amount, stability, lacing (how it sticks to the glass), creaminess, and color are critical properties, but remain a challenge for brewers to control.

Factors affecting foam The importance of beer foam has attracted considerable research attention (Kosin et al., 2010). In particular, research is focused on identifying and manipulating the biological, physical, and chemical factors that affect foam formation, stability, and quality (Stewart, 2016). Research has shown, surprisingly perhaps, that many of the manufacturing steps and nearly every ingredient used in beer making contributes to the formation and stability of foam. This includes the phenotype of the yeast, the type of barley, composition of the malt and hops, and salts and ions present in the water. For draught beer, the manner in which the beer is moved to the tap and dispensed can also have a profound effect on foaming properties. Even glassware cleanliness can affect foam.

Therefore, achieving the desired amount of foam requires balancing these factors appropriately (Van Nierop et al., 2004). According to these authors, creating optimum foam characteristics depends on a combination of foam "favorable" and "unfavorable" factors. The former include hop acids, proteins, metal ions, and gas level. Among the foam negative factors are lipids, basic amino acids, ethanol, yeast protease activity, and excessive malt modification.

Foam proteins More recently, a range of mostly hydrophobic proteins have been studied for their importance in foam formation and stability. These include protein Z (and its isoforms Z4 and Z7), lipid transfer protein, and an α-amylase inhibitor (Iimure et al., 2012). These proteins originate from the barley and are therefore amenable to breeding strategies (Iimure et al., 2011). However, based on mutation analyses, the same authors suggested that proteins Z4 and Z7 may not contribute to foaming properties any more than other proteins (Iimure et al., 2012).

In addition to barley proteins, researchers have shown that yeast cells may influence foam. Specifically, a gene from the lager yeast, *Saccharomyces pastorianus*, was identified that appeared to contribute to foam stability (Blasco et al., 2012). This gene, called *CFG1* (for *C*arlsbergensis *f*oaming *g*ene), encodes for a cell wall protein that is homologous to previously characterized *Saccharomyces cerevisiae* cell wall mannoproteins, Awa1p and Fpg1p (Shimoi et al., 2002; Blasco et al., 2011).

Foam physics Finally, beer foam, whether for personal or scientific reasons, has also intrigued physical chemists. Accordingly, they view foam in terms of bubble formation and fragmentation, gas-liquid diffusive mass transfer reactions, and the dynamics of plumes and vortex rings (Mantič-Lugo et al., 2015). Sophisticated techniques are also employed to study foam behavior (Bamforth, 2011; Solórzano et al., 2013).

A somewhat simpler model, nicely summarized by Evans and Bamforth (2009), considers foam as a series of overlapping events. Initially, bubbles are formed following nucleation, then a portion of the liquid is drained from the bubbles to form a "dry" foam. Eventually the bubbles begin to collapse, while at nearly the same time, new bubbles are also recruited to the foam, an event called creaming. In contrast, the rupture of bubble films at the surface can lead to coalescence and formation of large, unappealing bubbles. Similarly, when large bubbles displace small bubbles (disproportionation), foam quality is also negatively affected.

References

Bamforth, C.W. 2000. Perceptions of beer foam. J. Inst. Brew. 106:229–238.

Bamforth, C.W. 2011. 125th Anniversary Review: The non-biological instability of beer. J. Inst. Brew. 117:488–497.

Blasco, L., P. Veiga-Crespo, A. Sánchez-Pérez, and T.G. Villa. 2012. Cloning and characterization of the beer foaming gene *CFG1* from *Saccharomyces pastorianus*. J. Agric. Food Chem. 60:10796−10807.

Blasco, L., P. Veiga-Crespo, and T.G. Villa. 2011. *FPG1*, a gene involved in foam formation in *Saccharomyces cerevisiae*. Yeast 28:437−451.

Donadini, G., M.D. Fumi, and M.D. de Faveri. 2011. How foam appearance influences the Italian consumer's beer perception and preference. J. Inst. Brew. 117:523–533.

Evans, D.E. and C.W. Bamforth. 2009. Beer foam: achieving a suitable head. Handbook of Alcoholic Beverages: Beer, A Quality Perspective. C.W. Bamforth, I. Russell and G.G. Stewart, eds. Elsevier, Burlington, MA, pp. 1–60.

Iimure, T., T. Kimura, S. Araki, M. Kihara, M. Sato, S. Yamada, T. Shigyou, and K. Sato. 2012. Mutation analysis of barley malt protein Z4 and protein Z7 on beer foam stability. J. Agric. Food Chem. 60:1548−1554.

Iimure, T., M. Kihara, S. Ichikawa, K. Ito, K. Takeda, K. Sato. 2011. Development of DNA markers associated with beer foam stability for barley breeding. Theor. Appl. Genet. 122:199–210.

Kosin, P., J. Savel, D.E. Evans, and A. Broz. 2010. Relationship between matrix foaming potential, beer composition and foam stability. J. Am. Soc. Brew. Chem. 68:63–69.

Mantič-Lugo, V., A. Cayron, P.T. Brun, and F. Gallaire. 2015. Beer tapping: dynamics of bubbles after impact. J. Physics. Conference Series 656, IOP Publishing.

Shimoi, I., K. Sakamoto, M. Okuda, R. Atthi, K. Iwashita, and K. Ito. 2002. The AWA1 gene is required for the foam-forming phenotype and cell surface hydrophobicity of sake yeast. Appl. Environ. Microbiol. 68:2018−2025.

Solórzano, E., S. Pardo-Alonso, J.A. de Saja, M.A. Rodríguez-Pérez. 2013. Study of aqueous foams evolution by means of X-ray radioscopy. Colloids Surf. A: Physicochem. Eng. Aspects. 438:159–166.

Stewart, G.G. 2016. Beer shelf life and stability. In: P. Subramaniam, ed. The Stability and Shelf Life of Food. Woodhead Publishing, Cambridge, pp. 293–309.

Van Nierop, S.N.E., D.E. Evans, B.C. Axcell, I.C. Cantrell, and M. Rautenbach, M. 2004. Impact of different wort boiling temperatures on the beer foam stabilizing properties of lipid transfer protein 1. J. Agric. Food Chem. 52:3120−3129.

Several requirements are necessary to induce a successful secondary fermentation. First, there must be sufficient fermentable sugar present in the beer to serve as substrate for the yeast. The cask, tank, or bottle must be able to withstand the pressure that accumulates as a result of CO_2 gas formation. Finally, either naturally present yeasts must remain in the beer, even after flocculation, or more yeast must be added. For lagers, in particular, the krausening technique can be used to initiate a secondary fermentation. Recall that the krausen is wort obtained during the most active phase of the fermentation and that it contains sugars as well as log phase yeasts. The CO_2 bubbles that form by krausening are considered by some brewers to be "finer" compared to those produced by other procedures. However, this technique is now only infrequently used.

The amount of CO_2 that forms during a secondary fermentation and is retained in the beer depends on several factors. The amount of available sugar present or added to the beer directly influences CO_2 production, but rarely is the sugar a limiting factor. Rather, the correct amount of carbonation is more a function of the counter-pressure and temperature in

the tank. Obviously, the more back pressure applied, the greater the dissolved CO_2 in the beer. Likewise, as the temperature decreases, solubility of CO_2 increases. Proper adjustment of temperature and back pressure, therefore, is critical to achieving the desired level of carbonation in the finished beer.

Typically, ales have less carbonation than lagers. In the United States, for example, lager-style beers contain about 2.6 volumes of CO_2 per volume of beer. Thus, if the dissolved CO_2 was completely dissipated and collected, it would have a volume 2.6 times that of the "flat" beer. Traditional ales, in contrast, contain about half as much CO_2 as that contained in lagers.

It should be noted that beers that have undergone a secondary fermentation still need to be clarified or filtered (or both) to remove cells and haze-forming material, but without losing any of the accumulated CO_2. Traditional cask-conditioned beer can be treated with fining agents to enhance sedimentation, leaving the sediment in the bottom of the cask (where it remains even during dispensing). In contrast, lagers or tank-conditioned beer must be processed under pressure to retain the CO_2.

In the absence of a secondary fermentation, mechanical carbonation provides a convenient and more controllable means for introducing CO_2 into the beer. Mechanical carbonation is now widely used in the beer industry. There are essentially two sources of CO_2. As noted earlier, the CO_2 that evolves during the primary fermentation can be collected, cleaned and purified, and then added back to the beer. This process is not inexpensive, however, and is not widely practiced. Therefore, the more common means of carbonation is simply to pump CO_2 from pressurized tanks directly into the beer. As for when a secondary fermentation occurs, the CO_2 back pressure and temperature dictate the necessary volume of CO_2 pumped into the beer. In some cases, CO_2 is even added to beer that has undergone a secondary fermentation to achieve the desired level of carbonation.

PACKAGING AND PASTEURIZATION

Following carbonation, the beer is ready to be packaged. Perhaps the simplest form of packaging is to fill the beer directly into kegs. Kegs are constructed of aluminum or stainless steel and vary in size between 20 L and 60 L. Kegs not only mimic the traditional cask-style beers (in terms of perceived quality), but provide a convenient means of delivering non-bottled or canned product to the consumer. Thus, kegs are widely used by bars and restaurants for serving draught (otherwise known as draft) beer.

Beer destined for kegging is processed like other beers, in that it is clarified and filtered. Kegged beer is often filter-sterilized, although some is heat-pasteurized (see below). Therefore, its shelf-life is usually considerably longer than traditional casked beer (about three months versus one month). Kegged beer that is neither filter- or heat-pasteurized has a shelf-life similar to that of casked beer.

Importantly, since kegs are re-usable containers, breweries must pay particular attention to the keg cleaning and sanitizing operation before the kegs are re-filled. The interior of the keg, including the valve housing assembly where microorganisms may collect, is typically steam-sterilized. And although it is outside the control of the brewer, the dispensing system used to pump beer from the keg to the glass is also a source of potential spoilage organisms.

The most common package for beer in North America is the bottle or can. Bottles are usually constructed of glass, although plastic (e.g., polyethylene terephthalate or PET)

bottles have become popular. Virtually all cans are made from aluminum. Most beer that is to be bottled or canned is pasteurized, either before or following packaging.

If the beer is pasteurized prior to filling, two options exist. The beer can be filter-sterilized, as described above, or it can be flash-pasteurized via a plate-type, regenerative heat exchanger (similar to that used for pasteurization of milk). In fact, the time-temperature conditions for heat pasteurization of beer, 71°C to 75°C for 15 to 30 seconds, are very near that used for milk. The intent is different, however, since for milk the target organisms include potential pathogens, whereas for beer, pasteurization is performed to inactivate potential spoilage organisms. Ultimately, beer pasteurization conditions achieve a very high level of microbial killing (>5 logs), such that the product is stable at room temperature for at least six months.

Whether the beer is filter-sterilized or flash-pasteurized, the packaging steps must be performed under rather stringent aseptic conditions to prevent post-processing contamination. Bottles (whether glass or plastic) and filling and capping (i.e., crowning) equipment must be free of microorganisms, and surrounding areas protected such that microorganisms are excluded. Sterile N_2 or CO_2 can be used to flush the environment, and sterile water used to ensure that the relevant equipment, especially the areas around the fillers, remain free of microbial contaminants. The beer filling and packaging operation is essentially no different from that used for milk, juice, or other fluid products.

One other important factor must be controlled during the filling and packaging step consideration – namely the exclusion of oxygen. As will be described later, oxygen can cause highly objectionable flavor and aroma defects in beer.

The alternative to pasteurizing beer prior to filling is to heat-pasteurize after the beer has been filled into cans or bottles. In fact, much of the beer consumed in the United States is processed via tunnel pasteurization systems in which filled and sealed bottles or cans are heated by hot water. Tunnel pasteurizers operate in a continuous mode, with the temperature being gradually raised to as high as 62°C and held for up to 20 minutes. The collective effect of longer heating at lower temperature has the same kinetic effect on the destruction of microorganisms in beer as flash-pasteurization.

Pasteurizing the beer after it is already in the sealed package has the obvious advantage of preventing post-pasteurization contamination. However, the longer exposure to heat may promote undesirable flavor changes in the beer, including formation of cooked and oxidized flavors. Of course, some beer manufacturers eschew any type of heat treatment and instead rely on filtration systems for preservation of beer. However, even filtration is not benign, as flavor and other beer constituents may be removed during the filtering process. Thus, the challenge for modern brewers is to produce not only a beer with desirable flavor, body, and appearance characteristics, but also one that meets the preservation requirements necessary to operate successfully in a highly competitive market. In particular, it is important, for both large and small breweries to produce shelf-stable beer. This allows retailers to position beer cases, six-packs, and other packages in the aisles or shelves at ambient temperatures.

BEER DEFECTS

Despite the low pH, high ethanol content, and hop antimicrobials ordinarily present in beer, microorganisms are responsible for many (but certainly not all) of the defects that occur in beer. Chemical and physical defects are also common and can cause significant problems for brewers. However, preventing or minimizing entry and growth of microbial contaminants

throughout the beer-making process is absolutely essential for consistent manufacture of high quality beer. This is no simple matter, because fungi, wild yeasts, and bacteria are naturally present as part of the normal microbiota of the raw ingredients, the brewery environment, and the brewing equipment. Moreover, even if a heat or filtration step is included at the end of the beer-making process, the damage may have already been done.

Microbiological problems can occur as early as the malting step. Although the water activity of stored barley is too low to support growth of fungi, yeasts, and bacteria, all of these organisms can grow as soon as the barley is steeped, with increases of about 1 to 3 logs. Kilning eventually inactivates some microorganisms; however, the finished malt may still contain as many as 10^6 bacteria, 10^3 fungi, and 10^4 yeast per gram. The majority of bacteria found in malt are comprised of various species of lactic acid bacteria. Several of these may cause several serious defects in the finished beer. However, even fungi that are present at relatively low levels can cause problems.

Among the fungi that are especially of concern are strains of *Fusarium*. This genus contains species that are plant pathogens and can infect barley in the field. Some strains also produce the toxin deoxynivalenol, which can then appear in beer. Aside from its possible toxicity to humans, the presence of deoxynivalenol-producing strains of *Fusarium* in barley also appears to be correlated with the "gushing" defect in packaged beer. Gushing is characterized by excessive gassing or over-foaming when non-agitated packaged beer is opened. One of the main contributors, if not the cause, of the gushing defect are hydrophobins, a class of small surface active proteins. Hydrophobins induce gushing by serving as nucleation sites at which carbon dioxide microbubbles are formed. As the diameter of the bubbles increases, the bubbles eventually reach a critical size and begin to foam or gush. Importantly, the hydrophobins that are responsible for this phenomenon are produced in barley by *Fusarium* and other filamentous fungi. Therefore, one of the best ways to prevent this problem is to avoid the use of contaminated barley.

Even though yeast are required for the beer fermentation, wild yeasts can cause several different types of product defects. Wild yeasts include species of *Zygosaccharomyces*, *Kluyveromyces*, and other *Saccharomyces*. If present in the wort, they may compete with brewing yeast during the fermentation, and, in some cases, reach high levels. Since these yeasts typically do not flocculate as do brewing yeasts, they remain in the beer, creating cloudiness and filtration problems. Some of these yeasts also produce killer toxins that can potentially inhibit the brewing yeast culture and dominate the fermentation.

Other yeasts are more important due to the post-fermentation problems they cause. In particular, *Saccharomyces cerevisiae* var. *diastaticus* and other so-called diastatic yeasts, can hydrolyze and ferment dextrins, producing phenolic off-flavors in the beer, often characterized as medicinal. The hydrolysis of dextrins also makes the beer "thinner". Finally, a group of aerobic yeasts, including members of the genera *Pichia*, *Debaryomyces*, and *Brettanomyces*, are capable of causing post-fermentation defects. These organisms produce acetic acid, volatile esters and alcohols, and other oxidized compounds, and can also cause turbidity problems.

Although high mashing temperatures (along with low pH) restrict growth of most bacteria, some heat-tolerant bacteria can survive and grow during the mashing step. Far more serious, however, are those bacteria that contaminate the wort after the kettle boil step and grow in the beer during the fermentation and post-fermentation steps. Lactic acid bacteria represent the most bothersome organisms in the brewing operation and are the cause of the most common defects. Of particular concern are species of *Lactobacillus*, including *Lactobacillus brevis*, *Lactobacillus casei*, *Lactobacillus plantarum*, and *Lactobacillus delbrueckii*.

During growth in wort, these bacteria ferment wort sugars and produce several undesirable end-products, especially lactic acid. Some lactic acid bacteria, *L. brevis* in particular, are heterofermentative, meaning that they produce acetic acid, CO_2, and ethanol, in addition to lactic acid. There are also lactobacilli that produce 3-hydroxypropionaldehyde, which serves as a precursor for synthesis of acrolein, a compound that imparts a bitter flavor. Importantly, several species of *Lactobacillus*, as well as other lactic acid bacteria most notably *Pediococcus acidilactici*, *Pediococcus inopinatus*, and *Pediococcus damnosus* are responsible for perhaps the most objectionable defect in beer, namely, the production of diacetyl.

As noted previously, diacetyl is a four-carbon molecule that imparts a buttery flavor and aroma that, while pleasant in cultured dairy products, is highly offensive in beer. It can be produced as a metabolic side product by the yeast during the primary fermentation, but most is re-assimilated during the maturation period. Among the lactic acid bacteria, pediococci (and *P. damnosus*, in particular) produce appreciably more diacetyl than other species and are perhaps the most serious microbial contaminants that affect beer quality. They are capable of growth over a wide range of temperatures, including those used during both the ale and lager fermentations. These bacteria are also tolerant of low pH, ethanol, and, depending on strain, hop alpha-acids.

The diacetyl defect caused by these bacteria is sometime referred to as "sarcina sickness" due to the original classification of pediococci as belonging to the genus, *Sarcina*. It has a low flavor threshold and is detectable at concentrations as low as 0.2 ppm (or 0.00002%). In addition to flavor defects, some pediococci and lactobacilli are also capable of producing extracellular polysaccharides that cause a ropiness defect. This ropy or "slime" material increases beer viscosity and gives the beer an undesirable mouthfeel.

Although the lactic acid bacteria are arguably the most serious spoilage organisms in beer, Gram-negative bacteria also can contaminate beer and cause spoilage problems. In fact, the organisms identified by Pasteur in his studies on beer spoilage were probably species of the genus *Acetobacter*, Gram-negative bacteria that oxidize ethanol to acetic acid. Strains of *Acetobacter* are used in the manufacture of vinegar, thus, they give beer a highly objectionable vinegary flavor. It is no wonder that the French beer industry had enlisted the technical advice of Pasteur to solve this problem. *Acetobacter* and the related genera, *Gluconoacetobacter* and *Gluconobacter*, consist of aerobic rods that grow well in the presence of high ethanol concentrations and are resistant to hop alpha-acids. Because these bacteria grow poorly, if at all, under the anaerobic conditions established during the beer fermentation, they are more likely to be a problem only when the beer is exposed to oxygen, such as during storage of beer in casks.

Other Gram-negative bacteria may also occasionally contaminate and spoil beer. Included are *Zymomonas*, ethanol-tolerant, facultative, or obligate anaerobes associated with ale spoilage, and *Citrobacter, Klebsiella,* and other bacteria belonging to the family *Enterobacteriaceae*. The former are prolific ethanol producers that also make lesser amounts of lactic and acetic acid, acetaldehyde, and glycerol. They may also produce sulfur-containing compounds, such as dimethyl sulfide and dimethyl disulfide. As spoilage organisms, they cause a fruity flavor defect that occurs most frequently in casked beer. Finally, anaerobic Gram-negative rods, including species from the genera *Pectinatus*, *Megasphaera*, and *Zymophilus* are capable of producing organic acids, H_2S, and turbidity in packaged beer. They are easily killed by heat, thus they probably gain entrance into beer during post-pasteurization packaging.

Beer spoilage does not occur only by microorganisms; a number of chemical and physical defects are also common in beer. One of the more frequent defects (perhaps less so in recent years) is referred to as "skunky" beer flavor or light-struck flavor. It is caused by

sunlight-induced photooxidation. This defect, which can range from slightly objectionable to nauseating, is most noticeable in beer bottled in clear glass. The most likely mechanism by which this defect occurs is via ultraviolet or sunlight-induced cleavage of side chains on hop-derived iso-alpha-acids. These reactive side chains then react with sulfur-containing compounds to form products with a skunky aroma. One thiol in particular, 3-methyl-2-butene-1-thiol or MBT, has a low flavor threshold (just a few ppb) and is characteristic of this defect. Given the potential for beer to become skunky, how is it that there are still many popular beers packaged in clear glass? In general, these beers are chemically "skunk-proofed" to prevent the light-induced reactions described above. Specifically, hop extracts (obtained via either liquid or supercritical CO_2 extraction) are used that are processed such that the isomerized iso-alpha-acids are converted to a reduced form. Depending on the extent of the reduction (i.e., how many hydrogen atoms are incorporated), dihydro-, tetrahydro-, or hexahydro-iso-alpha-acids are formed. Importantly, these modified iso-alpha-acids are very light-stable and non-reactive.

Another flavor defect is referred to as stale or oxidized. Stale beer is variously described as having cardboard-like, rotten apple, cooked, or toffee-like flavors. It is invariably caused by autooxidation reactions, not unlike the staling reactions that occur in other foods. Staling requires exposure to oxygen and is generally time-dependent, such that it increases during storage. Therefore, it is often the main factor that determines shelf-life of beer. The best way to control staling is simply to keep oxygen out of the finished beer.

Among the physical defects in beer, perhaps the most common is haze, which, as discussed above, occurs during cooling, and is caused primarily by proteins that form complexes with polyphenols and carbohydrates. Treatment with proteinases can reduce this problem. In addition, β-glucan-containing polysaccharides can also cause haze, gel, and filtration problems, as well as increasing viscosity. Bacterial β-glucanases can be added to the mash or wort to degrade these materials. Foam instability problems are also common and result in loss of foaming or head. Adding proteins, hop components, and gums increases viscosity and surface tension, and can therefore stabilize foaming.

SUSTAINABILITY IN THE BREWING INDUSTRY

Although sustainability affects all segments of the fermented foods industry, the issue is particularly important in the brewing industry for several reasons. First, beer manufacturing uses nearly a half-billion tons of grains each year in the US alone. Although some components of that grain are fermented, much of the grain is left behind. These spent grains represent a considerable disposal problem. Currently, this material is used in one of several ways. It can be further processed and used as a specialty food ingredient (e.g., as a fiber supplement). However this market is way too small to make much of a dent in the supply of spent grains. It is more common for breweries (small and large) to contract with local farmers, who use the spent grains as livestock feed. Spent grains can also be used as fertilizer. The grains can be dried to extend shelf-life and reduce shipping cost, but drying requires energy and is expensive. Still, both wet and dry grains are used. Another waste product that poses problems for the industry is the spent filter material used as filter aids. Alternative filtration systems that do not require filter aids or that use materials that can be recycled are now available.

Finally, brewing uses large volumes of water. As much as 5 L to 10 L of water are used for every liter of beer produced. Much of this water contains biological material, and its disposal into municipal water supplies may be restricted and expensive. Therefore, efficient water handling systems, such as anaerobic digesters that treat and/or recycle this water are critical.

RECENT DEVELOPMENTS IN THE BEER INDUSTRY

The beer industry is one of the most competitive segments of the food and beverage industry. This competition has led to new technologies, new innovations, and new products. At the same time, there has been remarkable growth in the microbrewing industry, and a return to traditional or craft brewing practices. While the major breweries have had only modest growth or even flat sales in recent years, sales of microbrewed beer have been increasing by about 10%. It is now possible to find nearly every type of beer at the local pub or retail outlet (Box 10.7). Thus, the beer industry, from the smallest to the largest brewer, continues to develop new and innovative products and processes, many of which involve biotechnology and bioprocessing.

Box 10.7 Beer from A to Z

There are two general types of beer, ales and lagers. However, this simple distinction is not really adequate to describe the many varieties that are produced throughout the world. In reality, there are so many different versions of these two styles, that to refer to a particular beer simply as an ale or a lager provides little useful information about the actual nature of that beer. In addition, beers made in a similar manner, but from different geographical regions, often have quite different characteristics, and are classified accordingly.

Given that many brewers adhere to the spirit of the Reinheitsgebot and rely on traditional brewing ingredients, it can be challenging for beer manufacturers to distinguish their beer from another. That is in part why selection of specific ingredients is so important. Hops and malt, in particular, have a major impact on the flavor, aroma, and color properties of the beer. Thus, apart from their status as ale or lager, beers are often classified based on their malt and hop type and content. For example, the Saaz hops used in Belgian pale ales and Czech Pilsner impart a flowery and fruity flavor to beer. Listed below are some of the major categories or styles of beer and brief descriptions for each.

Ale. Top-fermented beer, produced throughout the world, but most commonly in the UK. Ales generally have more hop aroma and bitter flavor than lagers, and are usually less carbonated.

Altbier. German style ale, historically made in Duusseldorf. It is copper-colored, with a modest bitter and malty flavor. Altbiers are lagered (and served) at lower temperatures than typical ales.

Bitter. A typical British-style ale, with a strong bitter flavor.

Bock. A German lager with strong malt flavor and sweetness, with little hop bitterness and little or no hop aroma. The color ranges from light to dark brown, and these beers generally are full-bodied.

Brown ale. An ale originated from Newcastle upon Tyne (in northeastern England), that is characterized by its full body, dark color, malty and generally sweet flavor, and low to moderate hop aroma and bitterness.

Cream ale. An ale made in the United States; it has a mild sweet flavor.

Dunkel. The classic Munich lager; characterized by its dark brown color, sweet and nutty malt flavor, and full body. Bitterness and hop aroma are usually mild.

Hefeweizen. A German wheat beer, characterized by a more than 50% wheat malt in the mash. Most versions are unfiltered and have a cloudy appearance, due to the presence of yeast.

Lager. Bottom-fermented beer that originated in Bavaria, probably in the fifteenth century. Lagers generally are less hoppy and are more carbonated than ales, although exceptions exist. In the United States, lagers are amber or golden in color, with medium body and flavor.

Lambic. A specialty beer that originated in Belgium that is noted for several unique characteristics. First, it contains unmalted wheat as a major ingredient (as much as 30% or more). Second, aged hops, having decreased iso-alpha-acids, are used, minimizing the antimicrobial activity and bitter flavor. Third, growth of a range of wild microorganisms, including lactic acid bacteria, Enterobacteriaceae, and various yeasts, with commensurate acid development by these organisms is encouraged. Finally, the fermentation is entirely natural or spontaneous – no yeast is added, and it may take place over a period of several months to a year or more. Lambic beer is sour and thin-bodied.

Malt liquor. An American-style, pale colored lager, with low hop bitterness or aroma, thin body, and little flavor, but with a higher-than-normal alcohol content (>4.5%).

Pale ale. Typical British ale, with a medium body and malty flavor and light bronze color. Pale ales are moderately dry and bitter, and have a hoppy aroma. Carbonation is generally light and ethanol content high. Adjuncts are often added. India Pale Ale is similar in most respects to conventional pale ale, except it contains more hops, which promotes shelf-life (hence, this style was developed for export from England to India).

Pilsner. A classic lager that originated in Pilzen, a city in the Bohemia region of what is now the Czech Republic. Pilsners are among the most popular lagers in Germany (Bohemia was once part of the Austrian Empire and has a heavy German influence), as well as other regions in Europe. Pilsner (or Pils) has a strong malt flavor and hop aroma, medium body and sweetness, and a golden color. German Pilsners generally are lighter in color and body, and are less sweet compared to Czech Pilsner.

Porter. A British ale that has characteristics of both stouts and pale ales. Porters have light-to-medium body and malty flavor, are well-bittered, and have dark color. North American versions often contain adjuncts.

Steam beer. Introduced (and trademarked) by the Anchor Brewery Co. in San Francisco in the late 1800s. Steam beers have both ale and lager properties, in that they are bottom fermented, but at a high ale-like temperature. They are fermented in wide, shallow tanks.

Stout. A type of ale that is noted for its very dark color (due to heavily roasted malt), rich malt and hop flavor, and moderate to high bitterness. Stouts have a creamy head, and vary from sweet to dry. The Irish stout, Guinness, is the most well-known example. Oatmeal stouts are made with oatmeal which enhances flavor and body.

Weissbier. Translated as "white beer", these beers contain wheat (malted or un-malted, depending on style) in addition to barley. Wheat beers can be cloudy (or not) and fruity (or not).

Zwickelbier. A German beer that is unfiltered and therefore rather cloudy.

Low-calorie beer

One of the most successful and influential products developed by the beer industry was low-calorie beer. Introduced in the 1970s, this "new" type of beer had a dramatic impact, not only in the beer industry, but throughout the food industry, as "light" (or "lite") became one of the most widely used descriptors for reduced calorie foods. In fact, low-calorie beers had been around for at least ten to twenty years prior to the introduction of Miller Lite in 1975, but these products were not very popular. More recently, these low-calorie beers have been re-branded as "low carbohydrate" beers to appeal to consumers on carbohydrate-restrictive diets.

Undoubtedly, whether labeled as low-calorie or low-carbohydrate or simple "light", highly successful advertising and marketing campaigns have led to what is now a major part of the beer industry. Currently (December, 2013), low-calorie beers have about 50% of the total US beer market, and three of the top four selling brands of all beer are in this category (Bud Light, Coors Light, and Miller Lite). When first introduced for beer, "light" had no official meaning. The widespread use of this term, however, eventually led the FDA to define light foods as those that are significantly reduced in fat, calories, or sodium. As applied to beer, "light" means that there must be one-third fewer calories.

The most obvious way to reduce the caloric content of beer would be to simply dilute regular beer with water. A typical American-style lager beer ordinarily contains about 150 calories in a 340 ml (12 ounce) serving, so adding 25% water would result in a beer with only 112 calories. Doing so, however, would obviously not only reduce calories, but would also reduce flavor, color, body, and the ethanol content. Instead, to make a "light" beer with fewer calories, but with a minimal loss of flavor and body characteristics, manufacturers had to consider beer composition and the components that contributed calories.

It's time for some beer math. In general, assume a typical US beer contains (on a weight basis) about 4% ethanol and 1.3% protein. Thus, in 340 ml of beer (i.e., a 12 ounce bottle), the caloric contribution of those components is about 95 from ethanol ($0.04 \times 340 \times 7$) + 18 from protein ($0.013 \times 340 \times 4$) = 113 calories. What is the source of the additional 37 calories (i.e., 150 minus 113)?

As discussed earlier in this chapter, the beer fermentation is considered complete or fully attenuated when the fermentable carbohydrates have been depleted. However, that is not to say that there are not carbohydrates in beer. In fact, wort contains a mixture of fermentable sugars (i.e., glucose and maltose), as well as more complex, non-fermentable sugars, in particular, dextrins and limit dextrins. The latter are a result of the incomplete hydrolysis of starch during the mashing step. Whereas the simple sugars are readily fermented, the complex carbohydrate fraction is not. Importantly, however, these carbohydrates are caloric (at 4 calories per gram), meaning they can be hydrolyzed to glucose during digestion, adsorbed into the blood stream, and either used as energy or stored as fat. Thus, one way to reduce calories is by removing these non-fermentable but caloric carbohydrates from the beer.

The strategy that was adopted, and which is still used today, was made possible by the availability of specific glucoamylases that can hydrolyze the caloric but non-fermentable carbohydrates. The free sugars that form are then fermented during the primary fermentation step. The enzymes are commercially available and are relatively inexpensive. Most are derived from *Aspergillus niger* and other fungi. They are added to the wort during mashing, and work in concert with the endogenous enzymes from the malt. Ideally, the fungal enzymes should be temperature labile so there is no residual activity after the kettle boil step.

Because more fermentable carbohydrate in the wort necessarily results in more ethanol in the beer (and at 7 calories per gram, would defeat the purpose, as well as add to the alcohol

excise tax), the extra ethanol is ordinarily removed. The carbohydrate content of these beers is reduced from 9–12 g per serving to less than 3 g, resulting in a calorie reduction of 25 or more. Thus, a typical light beer contains 100 to 120 calories. In the past decade, even lower calorie beers, containing fewer carbohydrates than conventional light beers, have been introduced. One popular "low-carb" brand contains only 2.6 g of carbohydrate and 95 calories per 340 ml serving.

An alternate strategy to the use of exogenous enzymes is based on the partial replacement of malt or starch adjuncts with adjuncts consisting of simple sugars. For example, if sucrose, fructose, or glucose syrups were used as adjuncts, at the expense of malt, the dextrin fractions would also be reduced. Both of these approaches, however, provide thin-bodied beers, since it is the dextrins that contribute mouthfeel and body properties to beer.

Several other approaches for reducing the dextrin (and caloric) content of beer have been considered, and are now being studied. Malt preparations with greater amylolytic activity can be used so hydrolysis of starch is more complete. Alternatively, brewers' yeast strains with greater amylolytic activity can be isolated via conventional yeast breeding programs or developed by molecular techniques. The goal is to increase activity of amyloglucosidase, which breaks down α-1,4 and α-1,6 glucosidic linkages in dextrin.

Finally, there is a perception that low-calorie or low-carbohydrate beers also have a much lower alcohol concentration than normal beer. In fact, the alcohol content for most of these beers is somewhat lower, generally by 0.5% to 1.0% (or about 20% less). For example, three of the most popular brands of light beer in the United States each contain less than 3.5% alcohol (on a weight basis), compared to 3.8% to 4.5% for their conventional counterparts. For other brands, the differences are less than 0.5%

Lambic beer

Many of the supposedly "new" or specialty styles of beer are not new at all and have been produced for 100s of years. This is especially true for lambic beers, a style that originated in the region around central Belgium. Indeed, authentic lambic beers are produced in and just to the west of Brussels. Lambic beers are dramatically different from other beers for several important reasons. First, they rely entirely on a wild fermentation – there is no pitching or starter culture involved. Second, the fermentation yields ethanol, but also lactic and other acids and flavors. Thus, these beers are known for their sour character. Third, lambic beers are often aged, as well as flavored with fruit. Collectively, these properties make lambic beers unique, which perhaps has contributed to their new-found popularity. They have certainly captured the attention of microbiologists (Box 10.8).

Gluten-free beer

Throughout the entire food industry, the demand for gluten-free products has increased dramatically. Beer is no exception. However, since the main ingredient in beer is malt, derived from gluten-containing barley, making beer gluten-free is a challenging task. Nonetheless, several strategies – ranging from alternative sources of malt to no grain at all – have been developed (Box 10.9). Adding proteinases capable of hydrolyzing the allergenic epitopes in barley gluten has become another viable approach. While these beers may not quite have the same functional or sensory properties as conventional beer, they do provide consumers with gluten-free or gluten-reduced choices.

Box 10.8 Everyone's wild about Lambic beer

Introduction

The popularity of traditionally-produced, craft style beers has had considerable implications for the beer industry. In particular, these beers provide a way for breweries to distinguish their products from the competition, whether by adding unique combinations of hops or using specialty malts and adjuncts. Another style, long popular in parts of Europe, has emerged just in the past ten years as a major category in the craft beer market. These beers, called lambics, are produced and have sensory properties unlike any other beer. The most apparent difference is their sour flavor – for this reason lambic style beers are often referred to as "sours".

Lambic beers originated in a region of Belgium very near the capital city of Brussels. The location is noted not just for historical reasons, but also because the traditional lambic fermentation has geographic significance. Specifically, the microorganisms responsible for the lambic fermentation (at least originally) just so happen to reside in this region. As will be described below, lambic beers can be produced outside of Belgium and indeed are now widely manufactured around the world.

Making lambic beer

It might be fair to say that there are more differences than there are similarities between how lambic and other beers are produced. For starters, the ingredients are different. The malt or grain bill is generally comprised of a mix of 70% malted barley and 30% unmalted wheat (Roels et al. 2016). This will yield different enzymes and carbohydrate substrates later during mashing and when the product is fermented. Another significant difference is the hops component, as lambics are made with aged, dried hops that have a lower iso-alpha-acid content than regular hops. This results in less bitterness in the wort, although anti-microbial activity is retained.

General overview of the lambic fermentation

Undoubtedly, what makes lambic beers unique is the manner in which fermentation occurs. Whereas nearly every other beer is started by a pitching step, the lambic fermentation occurs naturally or "spontaneously", without adding culture. Thus, the organisms that initiate the fermentation originate mainly from the air, although equipment and the brewing environment likely harbor these organism. They find their way into the wort as it cools overnight in the colder winter months, and it is for this reason that traditional lambics are seasonal.

Once "inoculated", the wort is then transferred to casks – unlike other beers that ferment in stainless steel fermenters, lambic beers ferment entirely in casks. In addition, the fermentation occurs as a microbial succession, such that the one group of organisms creates an environment favorable to another group of organisms that then becomes established. Some members of the previous group may be diminished in number or even be displaced entirely. Yeasts and bacteria from several phyla or taxa contribute. Lambic beers can be aged for one year or longer, so ample time exists for several succession events. Despite the length of the fermentation and the considerable

microbial diversity and seemingly chaotic nature of the lambic fermentation, it is still remarkably predictable and consistent. Lambic beers are now produced throughout Belgium, and indeed have become popular in the US, where they are known as American coolship ales, so-named after the specialized vessel used to cool the wort (Spitaels et al., 2015).

The lambic fermentation, in detail

Until relatively recently, identifying the specific members that comprise the lambic microbial community and measuring their abundances over time had been a difficult task. Culture methods alone were simply not sufficient. The use of 16s RNA and other molecular approaches has made it possible to characterize the organisms involved in the lambic fermentation at a high level of resolution.

As noted above, the lambic fermentation occurs as a succession, with four overlapping, but distinct periods (Figure 10.8.1; Bokulich et al., 2012; Spitaels et al., 2015). The initial phase is dominated by members of the family *Enterobacteriaceae* that includes *Enterobacter*, *E. coli*, and *Klebsiella.* Several genera of yeast also are present. The next phase commences after about one month with the emergence of more typical brewing *Saccharomyces*. This is the primary phase when the ethanolic fermentation occurs. Interestingly, both ale and lager yeast (i.e., *Saccharomyces cerevisiae* and *Saccharomyces pastorianus*) are ordinarily present (Roels et. al. 2016; Spitaels et al. 2015).

The third phase, starting at about month six and lasting from two to four months, is when the beer becomes acidified by ethanol-tolerant lactic acid bacteria. The main genera are *Pediococcus* and *Lactobacillus*; both produce enough lactic acid to induce a prominent sour flavor. Finally, the high acidic, high ethanol conditions provide an environment suitable for only few organisms, in particular, the yeast *Brettanomyces bruxellensis* (or its spore-forming version*, Dekkera bruxellensis*). While this organism has a long reputation as a spoilage organism in ethanolic fermentations, especially in wine, it is a key contributor to the lambic fermentation (Steensels et al., 2015). Specifically, this organism produces a range of phenolics, pyradines, esters, and acids, collectively referred to as "Brett flavor" (Crauwels et al., 2015). *Brettanomyces* are also important for maturation of the beer as they ferment carbohydrates left behind from the primary fermentation, a phase called super-attenuation. Acetic acid-producing bacteria from the genera *Gluconobacter* and *Acetobacter* may also be present during maturation, as well as at earlier phases of the fermentation (Spitaels et al., 2015).

The final product is described as sour or wine-like, with little carbonation. These beers are often blended with younger, less attenuated beers, such that when bottled, a secondary fermentation can occur. These so-called gueuze beers are the more common form of commercial lambics. Various fruits can also be added to the base beer; hence the many different styles, such as kreik (cherry) or framboise (raspberry).

The appeal of lambic beer

For most brewers, lactic acid bacteria, lactobacilli and pediococci in particular, are a major problem due to their ability to cause sourness in beer (Suzuki, 2011). Likewise, wild yeasts can also produce acids and other flavor defects in conventional beers (Shimotsu et al., 2015). As noted throughout this text, it is sometimes a fine line

between a successfully-fermented product and one that is considered as spoiled. In part, the difference is based on metabolite concentration, but also on the consumers' expectations (Wedral et al., 2010). Perhaps, the fact that lambics and related beers are made via a spontaneous fermentation, are matured in barrels, and have dramatically different flavor characteristic compared to other beers adds to their appeal.

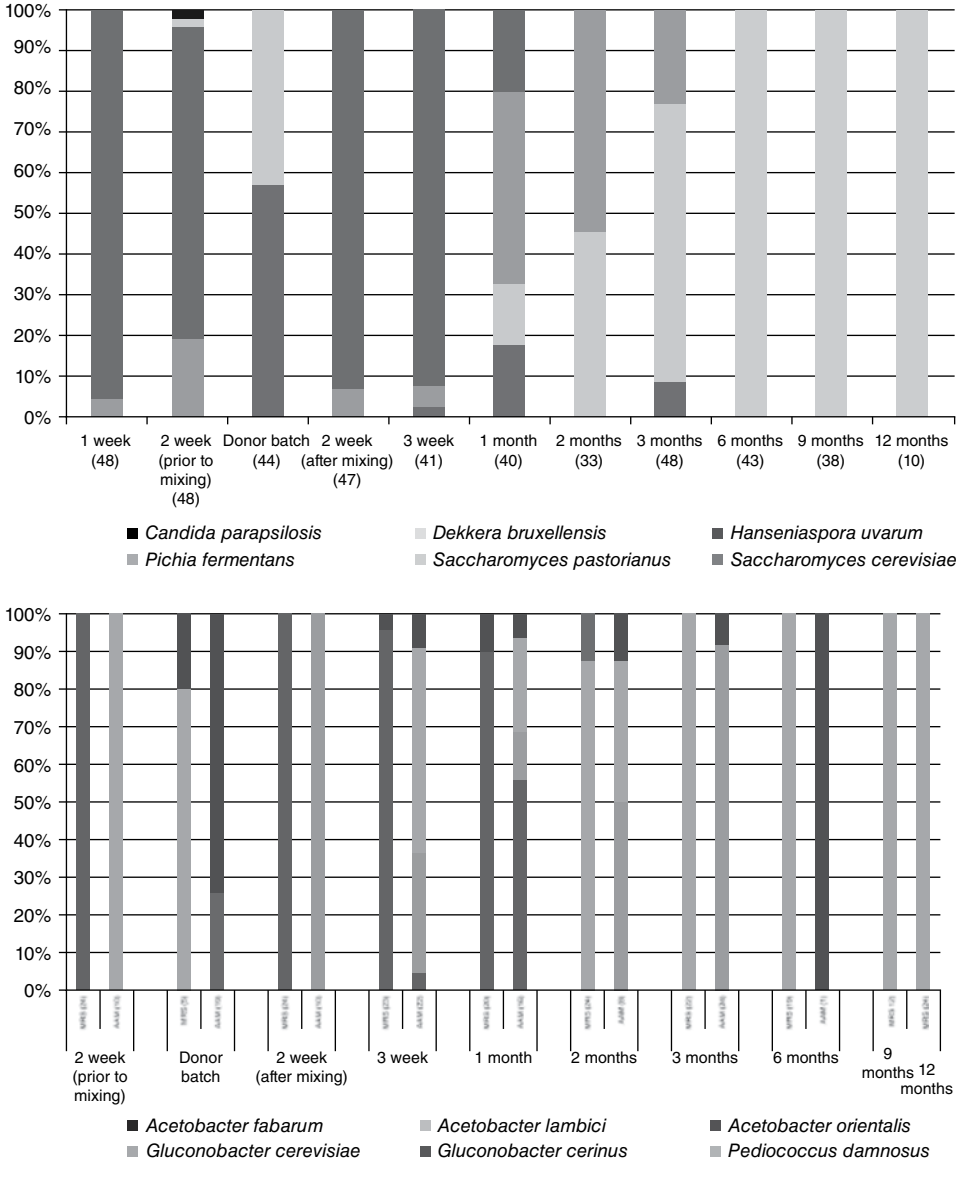


Figure 10.8.1 Identification of yeasts (upper panel) and bacteria (lower panel) isolated from lambic beer, from 3 weeks to 24 months, aged in casks. The number of isolates are indicated in the parentheses. From Spitaels et al., 2015, with permission.

It was not that long ago that one brewing researcher asked the question, "Can Lambic Survive?" (De Keersmaecker, 1996). The answer, based on considerable research interest as well as industrial production, is that lambic beers are not only surviving, they are thriving.

References

Bokulich, N.A., C.W. Bamforth, and D.A. Mills. 2012. Brewhouse-resident microbiota are responsible for multi-stage fermentation of American coolship ale. PLoS One, 7:e35507.

Bokulich, N.A., J. Bergsveinson, B. Ziola, and D.A. Mills. 2015. Mapping microbial ecosystems and spoilage-gene flow in breweries highlights patterns of contamination and resistance. Elife 4:1–21.

Crauwels, S., J. Steensels, G. Aerts, K.A. Willems, K.J. Verstrepen, and B. Lievens. 2015. *Brettanomyces bruxellensis*, essential contributor in spontaneous beer fermentations providing novel opportunities for the brewing industry. Brewing Sci. 68:110–121.

De Keersmaecker, J. 1996. The mystery of lambic beer. Sci. Am., 275:74–81.

Roels, S., F. Van Nieuwerburg, A. Landschoot, L. De Vuyst, and P. Vandamme. 2016. Microbial diversity and metabolite composition of Belgian red-brown. Int. J. Food Microbiol. 221:1–11.

Shimotsu, S., S. Asano, K. Iijima, K. Suzuki, H. Yamagishi, and M. Aizawa. 2015. Investigation of beer-spoilage ability of *Dekkera/Brettanomyces* yeasts and development of multiplex PCR method for beer-spoilage yeasts. J. Inst. Brewing 121:177–180.

Spitaels, F., A. Wieme, M. Janssens, M. Aerts, A. Laschot, L. De Vuyst, and P. Vandamme. 2015. The microbial diversity of an industrially produced lambic beer shares members of a traditionally produced one and reveals a core microbiota for lambic beer fermentation. Food Microbiol. 49:23–32.

Steensels, J., L. Daenen, P. Malcorps, G. Derderlinckx, H. Verachtert, and K.J. Verstrepen. 2015. *Brettanomyces* yeasts – From spoilage organisms to valuable contributors to industrial fermentations. Int. J. Food Microbiol. 206:24–38.

Suzuki, K. 2011. 125th Anniversary Review: microbiological instability of beer caused by spoilage bacteria. J. Inst. Brew. 117:131–155.

Wedral, D., R. Shewfelt, and J. Frank, 2010. The challenge of *Brettanomyces* in wine. LWT – Food Sci. Technol. 43:1474–1479.

Box 10.9 Gluten-free beer

Gluten basics As described previously (Chapter 9), gluten is an important constituent of wheat and several other grains. One simple definition considers gluten to be "the rubbery mass that remains when wheat dough is washed to remove starch granules and water-soluble constituents" (Wieser, 2007). Technically, gluten is formed when flour is hydrated and mixed. Although most students learn that wheat gluten is comprised of two main wheat storage proteins, gliadin and glutenin, its composition is much more complex. Indeed, gluten consists of a range of more than 100 proteins. Thus, while gliadins and glutenins are the most common group of gluten proteins in flour, secalin is the main constituent of rye gluten, and hordein is abundant in barley (Knorr et al., 2016). Related wheat species (sometimes referred to as ancient grains), like spelt and kamut also contain gluten proteins. Some oat cultivars also contain gluten.

Why barley for beer? It is no accident that beer making has long relied on malt derived from barley. This is in part because barley has high α- and β-amylase activity and plenty of starch, and also because the barely hulls provide a suitable bed for lautering the mash (Rubio-Flores and Serna-Saldivar, 2016). In addition, barley contains structural components that protect the embryo during grain handling and malting, resulting in more uniform germination (Burger and LaBerge, 1985). In addition, steeped barley grains are firmer than other cereals, making them less prone to structural damage during processing.

Why not barley for beer? Until recently, barley had no real disadvantage as a source of malt. In the 1950s, researchers discovered that gluten exacerbated celiac disease, a small intestine inflammatory disorder. Individuals with this disease who consume gluten may suffer from nutrient malabsorption, diarrhea, constipation, and iron-deficiency anemia. The only treatment for this disease was to exclude gluten-containing foods from the diet. In addition, proteins in wheat (not necessarily gluten proteins) may also induce an allergic reaction. Although celiac disease and wheat allergy are relatively uncommon (about 1% prevalence) and the etiologies are very different, even individuals who have no clinical or immunological evidence of either disease have adopted a gluten-free diet (Mansueto et al., 2014). So many people claimed to have improved intestinal function by following such diets, that the term "non-celiac gluten sensitivity" is used to describe this phenotype (Lebwohl et al., 2105). For these consumers, this means no beer.

Making gluten-free beer Making beer sans barley malt is not only possible, but given the potential market, gluten-free beers are now a growth category for the beer industry. As described below, several processing technologies and biological approaches now exist:

1. **Use other grains as a source of malt.** In contrast to barley and wheat, cereal grains such as corn, rice, and sorghum do not contain allergenic forms of gluten. However, while these grains are rich sources of starch, they are not easily converted to malt. Sorghum beers are the best studied, and germination conditions for making sorghum malt have been described (Owuama, 1999). However, identifying the most suitable cultivars, establishing extraction and mashing conditions, and optimizing filtration remain challenging (Taylor et al., 2013). Similar challenges exist for rice and corn. While many of the other brewing steps are not that different from conventional beers, gluten-free beers made from alternative grains often require addition of adjuncts and exogenous enzymes.
2. **Use barley malt that is enzymatically hydrolyzed to remove gluten.** Many of the gluten-derived, immune-reactive peptides in barley that are responsible for inducing the celiac cascade have been identified. These peptides are proline-rich and are resistant to digestion by gastric enzymes. Since beer manufacture includes several steps at which proteins are precipitated or removed, it cannot be assumed that all of these peptides actually end up in the beer (Hager et al., 2014). In part, the presence/absence of celiac-active peptides depends on the analytical method used

for detection. Nonetheless, one widely-used approach is to add exogenous enzymes (usually derived from microorganisms) that specifically hydrolyze the prolyl-peptide bonds that ordinarily resist digestion. Again, the efficacy of this approach depends on the detection method (Hager et al., 2014).

3. **Use non-grain raw materials.** One of the simplest approaches is to make beer without adding any grain- or cereal-derived protein. Instead, the wort is assembled from non-malt sources of fermentable sugars and amino acids, including sugar syrups and yeast extracts, respectively. Hops are added, as for conventional beer, and the wort is pitched with suitable yeasts. These beers are pale and coloring agents would ordinarily be added.
4. **New and novel approaches.** As noted above, exogenous microbial-derived enzymes can be added to beer to degrade barley gluten. However, it is also possible to optimize germination procedures such that the barley malt has high prolyl-peptidase activity (Knorr et al., 2016). Beer produced using this malt extract were gluten-free, and, importantly, also complied with German regulatory requirements.

Finally, plant geneticists have begun to consider the gluten problem at the source, by breeding low gluten barley. In one recent report, researchers targeted the genes encoding the hordein proteins (Tanner et al., 20916). They reported the successful development of an ultra-low gluten barley variety that contained less than 5 ppm hordein, which is even less than that considered to be gluten-free (20 ppm) by the FDA and EFSA.

"Gluten-free" has been one of the most successful categories in the entire food and beverage industry. The demand for such products continues to grow, with market projections ranging from 6–9% per annum between 2015 and 2023. So important is this segment to the brewing industry that some breweries are devoting entire manufacturing plants to gluten-free beers.

References

Burger, W.C. and D.E. LaBerge. 1985. Malting and brewing quality. In: Rasmussen, D.C., ed. Barley. American Society of Agronomy Monograph No. 26, ASA, CSSA, SSSA, pp. 367–401.

Hager, A.-S., J.P. Taylor, D.M. Waters, and E.K. Arendt. 2014. Gluten free beer – a review. Trends Food Sci. Technol. 36:44–54.

Knorr, V., H. Wieser, and P. Koehler. 2016. Production of gluten-free beer by peptidase treatment. Eur. Food Res. Technol. 242:1129–1140.

Lebwohl, B., J.F. Ludvigsson, and P.H.R. Green. 2015. Celiac disease and non-celiac gluten sensitivity. B.M.J. 351.

Mansueto, P., A. Seidita, A. D'Alcamo, and A. Carroccio. 2014. Non-celiac gluten sensitivity: literature review. J. Am. Coll. Nutr. 33:39–54.

Owuama, C.I. 1999. Brewing beer with sorghum. J. Inst. Brew. 105:23–34.

Rubio-Flores, M. and S.O. Serna-Saldivar. 2016. Technological and engineering trends for production of gluten-free beers. Food Eng. Rev. 8:468–482.

Tanner, G.J., M.J. Blundell, M.L. Colgrave, and C.A. Howitt. 2015. Creation of the first ultra-low gluten barley (*Hordeum vulgare* L.) for coeliac and gluten-intolerant populations. Plant Biotechnol. J. 14:1139–1150.

Taylor, J.R.N., B.C. Dlamini, and J. Kruger. 2013. 125th anniversary review: the science of the tropical cereals sorghum, maize and rice in relation to lager beer brewing. J. Inst. Brew. 119:1–14.

Wieser, H. 2007. Chemistry of gluten proteins. Food Microbiol. 24:115–119.

Non-alcoholic beer

Low- or non-alcoholic beers were first produced in the United States more than eighty years ago (during Prohibition), and have been available ever since. However, the relatively low demand for these products did not drive the industry to devote very much research effort into new technologies. Due to a marked increase in the consumer demand for low- or non-alcohol products, the technology for making these beers has improved dramatically in the last decade. The quality of these beers, not surprisingly, has also improved (although there are still many detractors who would argue otherwise). These products are now widely available in North America, as well as world-wide.

Non-alcoholic beers must contain less than 0.5% ethanol. Historically, several different processes have been used to produce non-alcoholic beer. The earliest methods generally involved removing the ethanol from normal beer by evaporation or distillation. Although these methods are still used, various filtration configurations, in particular, dialysis and reverse osmosis (RO), are now more widely used. Beer quality is arguably much better by the latter processes because they operate at low temperatures (<10°C) and the heat-generated reactions that affect flavor do not occur.

In RO systems, the beer is pumped or circulated at high pressure (more than 5000 Pascals) through small pore membranes with average molecular weight cut-offs of less than 0.01 μm. The ethanol molecules pass through the membrane as part of the permeate, along with a portion of the water. The retentate contains nearly all of the beer solids and most of the water, but little or none of the ethanol. Water can either be added back to its normal level after RO, or the beer can be diluted beforehand to account for the water lost from the retentate. Dialysis operates in a similar manner, except that the driving force is simply the concentration difference across the membrane. Despite the less intrusive nature of these processes, however, flavor compounds may still be lost.

Another entirely different way to make these beers relies not on ethanol removal, but rather on modifying the fermentation so that ethanol is not made as an end product. The fermentation can simply be abbreviated, such that yeast growth is stopped or curtailed before much ethanol has been produced. Alternatively, the fermentation can be conducted at a low temperature (<5°C) that restricts yeast growth and ethanol formation. However, these methods may result in a beer that is too sweet and microbiologically unstable, due to high levels of residual sugars. Modifying the wort composition by removing fermentable sugars prior to fermentation is another way to limit ethanol production. Finally, metabolic engineering of the yeast, such that the ethanol pathway is blocked, may be an ideal way to reduce the ethanol content in beer, but without leaving behind fermentable sugars (Box 10.10).

Wheat beer

Wheat beers have long been consumed in Europe, and in the past two decades they have become popular among US consumers. They are made using wheat malt combined with barley malt in ratios varying from 1:3 to as high as 2:1. These beers are generally thinner and more sour than barley malt beers, and are usually high in phenolic compounds. They also often have a somewhat cloudy appearance. However, their unique flavor, due in part, to vinyl guaiacol generated from ferulic acid, is particularly appreciated by some consumers.

Box 10.10 Metabolic engineering approaches in the manufacture of non-alcoholic beer

Two general processing approaches have been used to make non-alcoholic beer. One involves removing or separating ethanol from beer by physical means (e.g., distillation, evaporation, and reverse osmosis). The other approach has been to curtail the fermentation such that little or no ethanol is produced. Both of these approaches have their pitfalls, as already described.

The availability of molecular tools has now made it possible to consider biological strategies to produce non-alcoholic beer. Although the selection of brewers' yeasts has been based, for hundreds of years, on their ability to ferment wort sugars to ethanol, recent studies have shown that it is possible to redirect metabolism away from ethanol production and toward synthesis of other end products.

During the beer fermentation, several end products, other than ethanol, are ordinarily produced. Glycerol, in particular, can reach concentrations of more than 2 g per liter. Lesser but still relevant amounts of acetaldehyde, 2,3-butanediol, and acetoin are also formed. These products are derived from the glycolytic intermediates, dihdyroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (G-3-P), at the expense of ethanol (Figure 10.10.1).

Glycerol, for example, is formed from G-3-P via glyceraldehyde-3-phosphate dehydrogenase (GPD) and glycerol-3-phosphatase (GPP). By diverting even more of the glucose (or maltose) carbon to these alternative pathways, less ethanol would theoretically be produced. In the approach adopted by Nevoigt et al., 2002, the *GDP1* gene encoding for GPD was cloned and over-expressed in an industrial lager yeast strain. The transformants produced more than four times the amount of GPD, compared to the parent strain, and more than five times more glycerol. Importantly, the ethanol concentration in a typical brewing wort during a simulated beer fermentation was reduced by 18%, from 37 g/L to 30 g/L. This reduction, however, was less than half that achieved previously when GPD was over-expressed in a laboratory strain of *Saccharomyces cerevisiae* (Nevoigt and Stahl, 1996).

There were also large increases in the concentrations of acetaldehyde and diacetyl during the primary fermentation, and although these levels decreased during a subsequent secondary fermentation, they were still high enough to affect the flavor in a negative way. Thus, more metabolic fine-tuning of the competing pathways will be necessary to engineer a yeast capable of producing non-alcohol beers.

A completely different strategy for making non-alcoholic beer also was described by Navrátil et al. (2002). Their approach was based on the knowledge that: (1) non-alcoholic beer is sensitive to microbial spoilage; (2) low wort pH is inhibitory to contaminating microorganisms; and (3) addition of lactic acid or lactic acid bacteria to the wort stabilizes the beer. Because acidification of wort with lactic acid or lactic acid bacteria is either not allowed or is difficult to control, another way to promote acidification was needed.

Therefore, strains of *S. cerevisiae* defective in enzymes of the tricarboxylic acid (TCA) pathway and known to produce elevated concentrations of organic acids were used under simulated batch or continuous fermentation conditions (and using free or immobilized cells). In all cases, the beer pH was 3.25 or less when the mutant

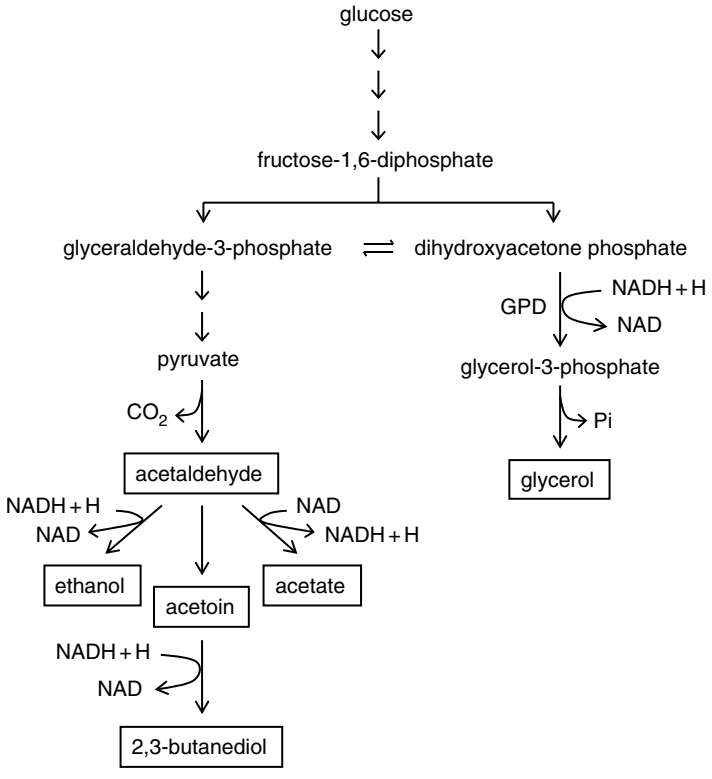


Figure 10.10.1 Formation of glycerol from glucose by *Saccharomyces cerevisiae*. The glycolytic reactions from glucose to pyruvate are not shown. Once acetaldehyde is formed, end-products other than ethanol can be formed (acetoin, acetate, and 2,3-butanediol). Over-expression of glycerol-3-phosphate dehydrogenase (GPD) results in glycerol production from dihydroxyacetone phosphate.

cells were used (compared to pH 4.1 to 4.2 for the control strain). Although the mutations were not located within ethanol production genes, the test strains produced very low amounts of ethanol (<0.31% for free cells and <0.24 for immobilized cells). The latter result presumably was due to the inhibition of ethanol formation, specifically, pyruvate decarboxylase and alcohol dehydrogenase, at low pH. Although other end products, including diacetyl, were also produced, informal sensory analysis suggested that the beer compared favorably to conventionally-produced non-alcoholic beer.

References

Blanco, C.A., C. Andrés-Iglesias, and O. Montero. 2016. Low-alcohol beers: flavor compounds, defects, and improvement strategies. Crit. Rev. Food Sci. Nutr. 56:1379–1388.

Liguori, L., G. De Francesco, P. Russo, G. Perretti, D. Albanese, and M. Di Matteo. 2016. Quality attributes of low-alcohol top-fermented beers produced by membrane contactor. Food Bioprocess Technol. 9:191–200.

Navrátil, M., Z. Dömény, E. Šturdík, D. Šmogrovičová, and P. Gemeiner. 2002. Production of non-alcoholic beer using free and immobilized cells of *Saccharomyces cerevisiae* deficient in the tricarboxylic acid cycle. Biotechnol. Appl. Biochem. 35:133–140.
Szollosi, A., Q.D. Nguyen, A.G. Kovacs, A.-L. Fogarasi, S. Kun, and B. Hegyesne-Vecseri. 2016. Production of low or non-alcoholic beer in microbial fuel cell. Food Bioprod. Process. 98:196–200.
Ivanov, K., I. Petelkov, V. Shopska, R. Denkova, V. Gochev, and G. Kostov. 2016. Investigation of mashing regimes for low-alcohol beer production. J. Inst. Brew. 122:508–516.
Yeo, H.Q. and S.-Q. Liu. 2014. An overview of selected specialty beers: developments, challenges and prospects. Int. J. Food Sci. Technol. 49: 1607–1618.

Ice and dry beers

Two other types of beer that gained a following in the 1990s, but whose popularity has since faded, are ice beers and dry beers. Although made by different processes, both ice beer and dry beer contain more ethanol than conventionally-processed beers and are thought to impart a smoother flavored beer with less after-taste. Ice beers are manufactured according to the freeze concentration principle, namely by cooling the beer to temperatures as low as –4°C, ice crystals will form, which can then be removed. The actual technology for the manufacture of these beers is not new – a German version called Eisbock has been made for many years. The resulting beer contains less water and more ethanol, and is generally sweeter, with good body and color. Dry beers, in contrast, are less sweet (hence the term "dry"), and are made by using less malt and more readily fermentable adjuncts. These beers are rather similar to low-calorie beers, in that they have less flavor, color, and body compared to conventionally-made products. They are also produced in a similar manner, in that enzymes are used to hydrolyze dextrins. This results in a more complete fermentation, less residual sugars, and more ethanol.

High-gravity fermentations

Another technology that has captured interest among brewers, especially large, high-volume producers, involves developing a wort with a specific gravity considerably higher than that used ordinarily. The concentrated wort, once made, can be diluted before or, more commonly, after fermentation. The net effect is an increase in brewing capacity and product throughput without having to incur addition capital expenses for equipment (e.g., mash tanks, lauter tanks, and fermentors). Moreover, the production of high-gravity worts reduces energy, labor, and effluent costs. Although high-gravity fermentations place extra demands on the yeast (e.g., the osmotic pressure and ethanol concentrations are higher), the quality of the beer is considered to be comparable to conventionally brewed beers.

BIOTECHNOLOGY AND THE BREWING INDUSTRY

For the first 5000 years that humans made and consumed beer, little was known about the actual scientific principles involved in its manufacture. Beer making was an art, practiced by craftsmen. Only in the last 150 years have biochemists and microbiologists identified the relevant organisms and metabolic pathways involved in the beer fermentation. Since the

early 2000s, genomes from more than 200 brewing strains of *Saccharomyces* have been sequenced, with many of the genes now having an assigned function.

This sequence information is now being used to understand yeast physiology, especially as it relates to brewing (Box 10.11). However, despite this knowledge, and the thousands of years of "practice", the brewing process is still far from perfect, and producing consistent, high-quality beer is still a challenge, even for large, highly sophisticated brewers. In addition, economic pressures, quality concerns, and perhaps most importantly, new market demands, have led the industry to consider new ways to improve the beer-making process. Advances in molecular genetics and biotechnology have made it possible to address these challenges via development of new brewing strains with novel traits and tailored to perform in a specific manner.

Box 10.11 Beer-omics

Of all the microorganisms described in this text and that are used in food and beverage fermentations, *Saccharomyces cerevisiae* is arguably the most important, both in terms of the economic value of the products, as well on a biomass basis. It should not be surprising therefore, that *Saccharomyces cerevisiae* was among the first microorganisms whose genome was sequenced (Goffeau et al., 1996). Although the sequenced strain was a laboratory strain, as opposed to the industrial strains used for brewing, genome sequences for both ale and lager strains were subsequently published (Borneman et al., 2011; Libkind et al., 2011). In one recent study, the genomes from more than 100 ale strains were described (Gallone et al., 2016). In addition to these genomics studies, transcriptomics, proteomics, and metabolomics are now being used widely to address questions related to yeast biology, evolution, and of course, their beer-making properties.

One of the first discoveries that emerged from genomic research on brewing yeasts was that while most laboratory strains have a haploid genome, industrial strains are polyploidal, meaning they contain multiple genomes. Indeed, the genome of the lager yeast, *Saccharomyces pastorianus*, is actually a hybrid consisting of one entire genome that evolved from an ale strain of *S. cerevisiae* and another whose ancestral progenitor was unknown (Casaregola et al., 2001). The latter was eventually identified as *Saccharomyces eubayanus* (Libkind et al., 2011). It is interesting to speculate when, where, and how these strains managed, literally, to come together (Gibson and Liti, 2015). Perhaps, prevailing brewing conditions provided an environment that selected for hybrids that could tolerate low temperature and that retained high fermentative capacity. It is also possible the hybridization event did not occur just once – the diversity of lager strains around many geographical areas suggest multiple independent events. Indeed, it now appears that variations in the wild *S. eubayanus* genomes accounts for most of the genetic variation in the *S. pastorianus* hybrids (Peris et al., 2016).

Beer transcriptomics

While genome information can predict the genes that are present in the chromosome(s) of an organism, it is often more informative to identify genes that are actually transcribed during the beer fermentation. The transcriptome can be determined using oligonucleotide microarrays (Gibson et al., 2008; Minato et al., 2009) or more directly

by mRNA sequencing. Such analyses can reveal how gene expression is influenced by environmental conditions, growth phase, or other temporal factors. For example, in the case of beer strains of *S. cerevisiae*, one might wish to identify genes expressed during growth on maltose or that are induced or repressed when ethanol or oxygen are present or absent. In fact, it is now possible to monitor gene expression throughout the entire beer fermentation process, generating an expression profile of all the transcripts produced at any given time during the course of the fermentation. Outcomes of such studies have revealed, for example, that formation of esters (compound that contribute to flavor and aroma) were correlated with transcription of specific genes (Krogerus et al., 2016).

Beer proteomics

The yeast transcriptome, as described above, provides a means of identifying the mRNA transcripts that are produced at a given time during growth. In contrast, the proteome represents the complete set of actual expressed gene products (i.e., the proteins) that are synthesized during growth. Proteome maps of industrial lager and other yeast strains during growth in synthetic medium revealed that about 1200 polypeptides are produced, although many appear to be duplications (Joubert et al., 2000). There is also a high degree of similarity between the proteins produced by lager strains and lab strains, adding further evidence that lager strains are hybrids and contain a genome derived from *S. cerevisiae*. However, as many as thirty-two other proteins are made by lager yeasts that do not appear in the *S. cerevisiae* proteome (Joubert et al., 2001). Analysis of these non-*S. cerevisiae* proteins by peptide mass fingerprinting and mass spectroscopy techniques revealed that many are involved in maltose metabolism, glycolytic pathways, and production of ethanol. Another study showed that the non-*S. cerevisiae* proteins expressed by lager yeasts were similar to those produced by the hybrid species, *Saccharomyces bayanus.*

Proteomics approaches were also used to investigate induction of protein synthesis by lager yeasts during the lag and early log phase of growth (Brejning et al., 2005). Interestingly, the induced protein expression pattern differed from the gene expression patterns (at least for the genes investigated), indicating that post-transcriptional regulation was involved. Among the early expressed proteins were those involved in amino acid and protein synthesis, glycerol metabolism, glycolysis, and ergosterol biosynthesis. Moreover, the expression profile for cells grown in minimal medium was consistent with those grown under brewing conditions.

Finally, yeast autolysis is an important phenomenon that affects beer quality, usually for the worse. A recent proteomics-based analysis revealed that expression of autolysis-response proteins depended on the energy status of the cells as well as amino acid and carbohydrate metabolic processes (Xu et al., 2014). Ultimately, these authors suggest such that this approach may lead to strategies for controlling autolysis during beer-making.

Beer metabolomics

It would be understatement to suggest that wide variations exist in the chemical composition of different beers. After all, differences in composition account, to a large

extent, for the many different types of beer that are produced around the world. Still, determining on a quantitative basis what the actual chemical composition is for a given beer can provide considerable information regarding the brewing process, yeast metabolism, and overall beer quality. This chemical profile, or the metabolome, which can be determined by a variety of spectrophotometric and other analytical methods combined with principal component analysis or qualitative multivariate statistics, has been shown to distinguish between ales and lagers, as well as between different types of manufactured beers (Duarte et al., 2002; Duarte et al., 2004).

More recently, identification of complex sugars present in beer, as well as sites of hydrolysis was performed using nuclear magnetic resonance (NMR) (Petersen et al., 2014). Similarly, NMR was used to assess the metabolites formed and consumed during the entire pre- and post-fermentation process, i.e., from kettle boil through secondary fermentation (Spevacek et al., 2016). These researchers were able to identify more than 70 metabolites, and they also showed that hopping practices influenced formation of key metabolites, especially those containing adenine.

Finally, mass spectroscopy has been used to identify a range of peptides and small molecules, including those that correlated with particular flavors and therefore could be considered as flavor markers (Vivian et al., 2016). Even the raw materials can be analyzed, providing a metabolome that could be predictive for the final product (Gonçalves et al., 2014).

References

Borneman, A.R., B.A. Desany, D. Riches, J.P. Affourtit, A.H. Forgan, I.S. Pretorius, M. Egholm, and P.J. Chambers. 2011. Whole-genome comparison reveals novel genetic elements that characterize the genome of industrial strains of *Saccharomyces cerevisiae*. PLoS Genet. 7(2), p.e1001287.

Brejning, J., N. Arneborg, and L. Jespersen. 2005. Identification of genes and proteins induced during the lag and early exponential phase of growth of lager brewing yeasts. J. Appl. Microbiol. 98:261–271.

Caesar, R., J. Palmfeldt, J.S. Gustafsson, E. Pettersson, S.H. Hashemi, and A. Blomberg. 2007. Comparative proteomics of industrial lager yeast reveals differential expression of the cerevisiae and non-cerevisiae parts of their genomes. Proteomics 7:4135–4147.

Casaregola, S., H.-V. Nguyen, G. Lapathitis, A. Kotyk, and C. Gaillardin. 2001. Analysis of the constitution of the beer yeast genome by PCR, sequencing and subtelomeric sequence hybridization. Int. J. Syst. Evol. Microbiol. 51:1607–1618.

Duarte, I., A. Barros, P.S. Belton, R. Righelato, M. Spraul, E. Humpfer, and A.M. Gil. 2002. High-resolution nuclear magnetic resonance spectroscopy and multivariate analysis for the characterization of beer. J. Agric. Food Chem. 50:2475–2481.

Duarte, I.F., A. Barros, C. Almeida, M. Spraul, and A.M. Gil. 2004. Multivariate analysis of NMR and FTIR data as a potential tool for the quality control of beer. J. Agric. Food Chem. 52:1031–1038.

Gallone, B., J. Steensels, T. Prahl, L. Soriaga, V. Saels, B. Herrera-Malaver, A. Merlevede, M. Roncoroni, K. Voordeckers, L. Miraglia, C. Teiling, B. Steffy, M. Taylor, A. Schwartz, T. Richardson, C. White, G. Baele, S. Maere, and K. Verstrepen. 2016. Domestication and divergence of *Saccharomyces cerevisiae* beer yeasts. Cell 166:1397–1410.

Gibson, B., and G. Liti. 2015. *Saccharomyces pastorianus*: genomic insights inspiring innovation for industry. Yeast 32:17–27.

Gibson, B.R., C.A. Boulton, W.G. Box, N.S. Graham, S.J. Lawrence, R.S. Linforth, and K.A. Smart. 2008. Carbohydrate utilization and the lager yeast transcriptome during brewery fermentation. Yeast 25:549–562.

Goffeau, A., B.G. Barrell, H. Bussey, R.W. Davis, B. Dujon, H. Feldmann, F. Galibert, J.D. Hoheisel, C. Jacq, M. Johnston, E.J. Louis, H.W. Mewes, Y. Murakami, P. Philippsen, H. Tettelin, and S.G. Oliver. 1996. Life with 6000 genes. Science 274:546–567.

Gonçalves, J.L., J.A. Figueira, F.P. Rodrigues, L.P. Ornelas, R.N. Branco, C.L. Silva, and J.S. Câmara. 2014. A powerful methodological approach combining headspace solid phase microextraction, mass spectrometry and multivariate analysis for profiling the volatile metabolomic pattern of beer starting raw materials. Food Chem. 160:266–280.

Joubert, R., P. Brignon, C. Lehmann, C. Monribot, F. Gendre, and H. Boucherie. 2000. Two-dimensional gel analysis of the proteome of lager brewing yeast. Yeast 16:511–522.

Joubert, R., J.-M. Strub, S. Zugmeyer, D. Kobi, N. Carte, A.V. Dorsselaer, H. Boucherie, and L. Jaquet-Gutfreund. 2001. Identification by mass spectrometry of two-dimensional gel electrophoresis-separated proteins extracted from lager brewing yeast. Electrophoresis 22:2969–2982.

Krogerus, K., M. Arvas, M. De Chiara, F. Magalhães, L. Mattinen, M. Oja, V. Vidgren, J. Yue, G. Liti, and B. Gibson. 2016. Ploidy influences the functional attributes of de novo lager yeast hybrids. Appl. Microbiol. Biotechnol. 100:7203–7222.

Libkind, D., C.T. Hittinger, E. Valerio, C. Goncalves, J. Dover, M. Johnston, P. Goncalves, and J.P. Sampaio. 2011. Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast. Proc. Natl. Acad. Sci. USA 108:14539–14544.

Minato, T., S. Yoshida, T. Ishiguro, E. Shimada, S. Mizutani, O. Kobayashi, and H. Yoshimoto. 2009. Expression profiling of the bottom fermenting yeast *Saccharomyces pastorianus* orthologous genes using oligonucleotide microarrays. Yeast 26:147–165.

Peris, D., Q. Langdon, R. Moriarty, K. Sylvester, M. Bontrager, G. Charron, J. Leducq, C. Landry, D. Libkind, and C. Hittinger. 2016. Complex ancestries of lager-brewing hybrids were shaped by standing variation in the wild yeast *Saccharomyces eubayanus*. PLoS Genet 12(7):e1006155.

Petersen, B.O., M. Nilsson, M. Bøjstrup, O. Hindsgaul, and S. Meier. 2014. 1H NMR spectroscopy for profiling complex carbohydrate mixtures in non-fractionated beer. Food Chem. 150:65–72.

Spevacek, A.R., K.H. Benson, C.W. Bamforth, and C. M. Slupsky. 2016. Beer metabolomics: molecular details of the brewing process and the differential effects of late and dry hopping on yeast purine metabolism. J. Inst. Brew. 122:21–28.

Vivian, A.F., C.T. Aoyagui, D. de Oliveira, and R.R. Catharino. 2016. Mass spectrometry for the characterization of brewing process. Food Res. Int. 89:281–288.

Xu, W., J. Wang and Q. Li. 2014. Comparative proteome and transcriptome analysis of lager brewer's yeast in the autolysis process. FEMS Yeast Res. 14:1273–1285.

Strain improvement strategies

Since the beginning of beer making, all the way to the present, brewing strains have been used continuously, being passed down from batch to batch. These strains are highly adapted to wort and beer and are not very amenable to classical strain improvement strategies. That is, trying to select strains, either spontaneously or following mutagenesis, with improved fermentative, flavor-producing, or other relevant properties, is not an easy proposition. In addition, whereas laboratory strains are usually diploid and capable of sporulating and forming haploid spores, brewing strains are polyploid, containing multiple alleles for many genes. Brewing strains also sporulate poorly. Thus, even mating and hybridization techniques, the standard means for modifying yeast in the laboratory, are often unsuccessful in brewing strains. Moreover, changes in one trait often have pleiotropic effects and lead to undesirable changes in other performance characteristics.

Although strain improvement programs still rely on these classical approaches, the ability to target specific genes or traits is now possible using recombinant DNA and genetic engineering techniques. However, such approaches must be carefully considered, due to the public perception (often supported by regulatory authorities) that genetically modified organisms pose risks to the consumer or the environment. Rather, it seems that commercial applications can still be realized, but will require the use of more benign techniques (i.e., no foreign DNA used, no antibiotic resistance markers) for strain construction. Of course, in the absence of any genetic manipulation, another strategy relies simply on blending different strains to achieve the desired fermentation outcomes.

Examples of modified brewing strains

In the brewing industry, several traits have attracted the most research attention (Table 10.6). As noted earlier, brewing strains of *S. cerevisiae* generally do not hydrolyze dextrins and limit dextrins during the beer fermentation. These sugars remain in the beer, and although they may have a positive influence on body and mouthfeel characteristics, they also contribute calories. Reducing these dextrins provides the basis for making low-calorie or light beers. Most brewers use commercially available enzymes, usually fungal glucoamylases that are added to the wort during mashing. Another approach is to use yeast strains that express glucoamylase and that degrade these dextrins during the fermentation. Such strains have been isolated (e.g., *S. cerevisiae* var. *diastaticus*), but, unfortunately, when used for brewing, the beer quality is poor, due, in part, to the production of phenolic off-flavors. Even hybrid strains, obtained by mating brewing yeasts with diastatic yeasts, may still produce off-flavors.

Another problem that occurs in beer production is due to the β-glucans that are derived from the cell walls of barley malt. Consisting of β-1-4 and β-1-6 linked glucose polymers, these materials form gels and foul filters, and increase beer viscosity and haze formation. They can be digested by commercially available enzymes, but, like the glucoamylase example described above, brewing strains that express these enzymes directly would be very useful.

Table 10.6 Traits targeted for strain improvement.

Trait	Relevant gene[1]	Function
Dextrin utilization	*STA2*	Glucoamylase
Diacetyl utilization	*ALDC*	Acetolactate dehydrogenase
Glucan utilization	*EG1*	β-glucanase
Maltose utilization	*MAL, AGT1*	Maltose permease
H_2S reduction	*MET10*	Sulfite reductase
Dimethyl sulfide reduction	*MXR1*	Sulfoxide reductase
Enhanced flocculation	*FLO1*	Flocculation
SO_2 production	*MET3*	ATP sulfurylase
Ester production	*ATF1*	Alcohol acetyltransferase

[1] From Verstrepen et al., 2006; Stewart et al., 2013, and other sources.

BIBLIOGRAPHY

Bamforth, C.W. 2017. Progress in brewing science and beer production. Annu. Rev. Chem. Biomol. Eng. 8:161–176.

Bamforth, C.W. 2004. Beer: Health and Nutrition. Blackwell Science, Oxford.

Blanco, C.A., C. Andrés-Iglesias, and O. Montero. 2016. Low-alcohol beers: flavor compounds, defects, and improvement strategies. Crit. Rev. Food Sci. Nutr. 56: 1379–1388.

Goldammer, T. 1999. The Brewer's Handbook. KVP Publishers, Clifton, VA.

Hill, A., ed. 2015. Brewing Microbiology: Managing Microbes, Ensuring Quality and Valorising Waste. Woodhead Publishing, Cambridge.

Krogerus, K., F. Magalhães, V. Vidgren, and B. Gibson. 2017. Novel brewing yeast hybrids: creation and application. Appl. Microbiol. Biotechnol. 101:65–78.

McGovern, P.E. 2017. Ancient Brews: Rediscovered and Re-created. W.W. Norton and Co., New York.

Mosher, M. and K. Trantham. 2017. Brewing Science: A Multidisciplinary Approach. Springer Publishing, Switzerland.

Priest, F.G., and I. Campbell. 2003. Brewing Microbiology, 3rd edn. Kluwer Academic/Plenum Publishers. New York.

Rasmussen, S.C. 2014. The Quest for Aqua Vitae. Springer, New York.

Stewart, G.G. 2016. *Saccharomyces* species in the production of beer. Beverages 2:34.

Stewart, G.G., A.E. Hill, and I. Russell. 2013. 125th Anniversary Review: Developments in brewing and distilling yeast strains. J. Inst. Brew. 119:202–220.

Verstrepen, K.J., P.J. Chambers, and I.S. Pretorius. 2006. The development of superior yeast strains for the food and beverage industries: challenges, opportunities and potential benefits. In: Yeasts in Food and Beverages. Querol, A. and G.H. Fleet, eds. Springer, Berlin, Heidelberg, pp. 399–444.

Wendland, J., 2014. Lager yeast comes of age. Eukaryot. Cell 13:1256–1265.

11 Wine

> I do like to think about the life of wine, how it is a living thing. I like to think about the year the grapes were growing, how the sun was shining that summer or if it rained… what the weather was like. I think about all those people who tended and picked the grapes, and if it is an old wine, how many of them must be dead by now. I love how wine continues to evolve, how every time I open a bottle it's going to taste different than if I opened it on any other day. Because a bottle of wine is actually alive – it's constantly evolving and gaining complexity. That is until it peaks, like your '61 and it begins a steady, inevitable decline.
>
> From the film, Sideways, screenplay by Alexander Payne and Jim Taylor.

INTRODUCTION

By now the reader should have observed a consistent theme in this book. Namely, that all of the fermented foods so far described have involved conversions of simple and inexpensive raw food materials into finished fermented products having enhanced value. Fermentation, therefore, is one of oldest examples of "value-added" processing where the value of raw materials is increased by virtue of a processing step. Perhaps no other process or product illustrates this principle as much as the conversion of grapes into wine. Consider for example, the following: In October, 2014, a lot containing 114 bottles of a Burgundy (France) wine from the Romanee-Conti vineyard sold for $1.6 million (about $14,000 per bottle). Or this: A single bottle of Cheval Blanc from the Bordeaux region sold in 2010 for $300,000. Of course, it was an Imperial bottle, meaning it did contain 6 liters of wine! Not that all top end wines are French – a Syrah from the Sine Qua Non winery in California's Ventura Valley recently (2014) sold for $43,000. While it's true that cheese, sausage, beer, kimchi, and other fermented products certainly appreciate in value by the act of fermentation, when considering the value-added potential of wine, no other product is even in the neighborhood.

Although these thousand dollar wines are certainly the exception, high quality California and European wines still average nearly $30 (25 Euro) per bottle. Given these examples, one may reasonably assume that wine making must be a highly complicated process, involving sophisticated processes and technologies. Indeed, wine manufacture does rely on modern microbiology and biochemistry, as well as technological and engineering skills. However, traditional techniques and tried-and-true manufacturing practices are still important, and in

Microbiology and Technology of Fermented Foods, Second Edition. Robert W. Hutkins.

many cases, required to produce high quality products. Thus, wine making serves not only as an example of value-added processing, but also as an example of an ancient technology that has adopted twenty-first century science.

HISTORY

The history of wine is nearly as old as the history of human civilization. The earliest writings discovered on the walls of ancient caves and in buried artifacts contain images of wine and wine-making instruments. For thousands of years, even to the present day, wine has had great ritual significance in many of the world's major religions and cultures. Wine is mentioned more than 100 times in both the Hebrew and Christian bibles and many of the most well-known passages involve wine. According to Genesis 9, verse 20, Noah becomes perhaps the first winemaker, planting a vineyard immediately after the Flood waters receded. Reading one line further, Noah "drank of the wine" (resulting, it seems, in the first recorded hangover). Later, in the New Testament, Jesus performed the miracle of turning water into wine. Wine also was an important part of Greek and Roman mythology and is described in the writings of Homer and Hippocrates. Wine is also a considerable part of the world economy and commerce. Although wine is also important in Asian cultures, most are made from rice rather than grapes. The latter will be discussed in more detail in Chapter 14.

The cultivation of grapes for wine making (the science of viniculture) appears to have begun in the Zagros Mountains and Caucasus region of Asia (north of Iran, east of Turkey). Domestication of grapes dates back to 6000 BCE, and large-scale production, based on archaeological evidence, appears to have been established shortly thereafter (Box 11.1). A fermented wine-like beverage made from honey and fruit appears to have been produced in China around 7000 BCE, and rice-based wines, similar to modern day sake, were produced in Asia a few thousand years later. Wines were imported into Italy, France, and other Mediterranean countries by sea-faring traders sometime around 1000 BCE, and vines and viticulture techniques were likely introduced into those regions several centuries later. Wine making spread widely during the Roman Empire, and soon was established throughout Europe.

Vines and wines eventually reached the Americas in the post-Columbus era. In due course, European vines were planted and wine making technology was adopted. Wine making soon became an important activity in Canada, South America (especially Argentina and Chile), and the US (see below). Likewise, grape vines were introduced into South Africa in the seventeenth century and Australia at the turn of the eighteenth century, and shortly thereafter in New Zealand. All have become major producers of wine. As mentioned above, wine making in Asia was long based on rice as a starting material, and only in the past two decades have grape wines been produced on an industrial scale. However, even in that short time, China has already become one of the leading producers of wine.

Wine is not only one of the oldest of all fermented products, it also one of the first that became commercialized, mass-produced, and studied. In fact, many of the early chemists and microbiologist were concerned with wine making and wine science. Nearly 150 years ago, when the very existence of microorganisms was still being debated, Pasteur showed that not only did microorganisms exist, but that they were responsible for both production and spoilage of wine. Of course, wine preservation has been important since ancient days, when early Egyptian and Roman wine makers began using sulfur dioxide (in the form of burned sulfur fumes) as perhaps the first application of a true antimicrobial agent.

Box 11.1 The origin of wine yeast and its European adventure

Identifying the origins of yeasts (and other cultures) responsible for food and beverage fermentations is one of the most intriguing and fundamental challenges for fermentation scientists. How is it possible, for example, that the fermentation of beer, wine and bread all require yeasts with specific physiological and biochemical traits, yet all are made with *Saccharomyces cerevisiae*? Was there a progenitor *S. cerevisiae* strain that somehow evolved over thousands of years, resulting in specialized strains that were especially suited for these different fermented foods? If so, where did that progenitor strain arise and what were its properties? And how did these strains spread across the planet?

Molecular archaeology These are obviously questions that are difficult to answer directly. However, it is possible, using molecular techniques, to make inferences regarding the evolution of these important fermentative yeasts (Cavalieri et al., 2003). Indeed, molecular archaeologists have been analyzing artifacts from nearly every continent to identify and track the movement of these organisms. It is also possible to "back-track" the origins of wine yeast by analyzing the genomes of oak-associated isolates, as recently reported (Almeida et al., 2015).

As noted discussed, wine making appears to have developed in the Caucasus and Zagreb Mountain areas of the Near East (the "Fertile Crescent") more than 7000 years ago. Ancient clay jars (estimated date about 3150 BCE) from these regions once contained wine, according to chemical and instrumental analyses. Furthermore, DNA residues were found when organic residues (presumably the lees, comprised of dead yeast cells) from these artifacts were analyzed. In fact, the amount of DNA present in the residue was high enough (it could even be seen in a gel) to eliminate the possibility that it had come from a stray microbial contaminant. Given the low water activity, low relative humidity (essentially zero), and overall ideal conditions for storing a biological material, it was also not surprising that the DNA was in such good shape.

The DNA was subsequently extracted and used as a template for PCR. Primers were based on *S. cerevisiae* rDNA sequences, such that the spacer regions between the 18S and 28S rRNA genes would be amplified. Three PCR products (540, 580, and 840 base pairs) were initially sequenced. Based on a BLAST search, all of the sequences had homologies to existing GenBank sequences. The sequence of the 580 bp PCR product was similar to various fungi, but remarkably, there was strong homology (nearly 90% identity) between the 540 bp sequence and a similarly derived sequence obtained from a fungal clone isolated from the clothing of "The Iceman." The latter dates back to 3300 BCE. The investigators suggest that both of the ancient fungi had been "buried" along with the wine.

Perhaps the most interesting finding from this analysis, however, concerned the 840 bp PCR product. This fragment, as well as smaller pieces obtained using internal PCR primers, had very high sequence similarity with a 748 bp region from chromosome 12 (part of the 5.8S rDNA) from contemporary strains of *S. cerevisiae*, *Saccharomyces bayanus*, and *Saccharomyces paradoxus.* Across this entire region there were only four nucleotide mismatches and no deletions or insertions between *S. cerevisiae* and the ancient wine sequence. This level of similarity should not be so surprising, perhaps, since the DNA regions represented by the PCR products are known to be quite

stable. Thus, this stability could account, in part, for the lack of sequence alterations between the ancient and modern strains during the past 5000 years of yeast evolution. Importantly, these findings suggest the yeast used to carry out the wine fermentation in ancient days was *S. cerevisiae,* just as it is today.

To Europe and beyond Alas, although wine making may have originated in the Fertile Crescent region, it long ago relinquished its position as the epicenter of wine-making. Rather, it was primarily France, Italy, and Spain where wine-making eventually spread. Presumably, traders and merchants took to the sea, island hopping across the Mediterranean (McGovern et al., 2013), introducing wine along the way. Eventually, viticulture became well established, first in Italy, and then in France.

Again, evidence for the movement of wine to Europe was based on molecular and chemical analyses of amphora – pottery, metal, and glass vessels used to hold wine. Scientists detected tartaric acid and other grape-derived chemicals in these materials, collected from southern France, which were dated to about 500 BCE. The discovery of stone platforms that similarly contained these same residues suggested these devices were used to collect juice from pressed grapes. Interestingly, these researchers also identified various resins in these samples, and concluded they had been added for their medicinal role as well as for preservation.

References

Almeida, P, R. Barbose, P. Zalar, Y. Imanishi, K. Shimizu, B. Turchetti, et al. 2015. A population genomics insight into the Mediterranean origins of wine yeast domestication. Mol. Ecol. 24:5412–5427.

Cavalieri, D., P.E. McGovern, D.L. Hartl, R. Mortimer, and M. Polsinelli. 2003. Evidence for *S. cerevisiae* fermentation in ancient wine. J. Mol. Evol. 57:S226–S232.

McGovern, P.E., B.P. Luley, N. Rovira, A. Mirzoian, M.P. Callahan, K.E. Smith, G.R. Hall, T. Davidson, and J.M. Henkin. 2013. Beginning of viniculture in France. PNAS. 110:10147–10152.

McGovern, P.E. 2003. Ancient Wine: The Search for the Origins of Viniculture. Princeton University Press, Princeton, NJ.

PRODUCTION AND CONSUMPTION

Most wines are made in temperate climates, particularly those areas near oceans or seas. Nearly 60% of the wine produced in the world is made in the Mediterranean areas of Europe (Table 11.1). Italy, France, and Spain are the largest producers and are responsible for half of the nearly 27 billion liters of wine produced from around the world (2016 data). Not surprisingly, these countries also devote the most acreage to grape production. However, as noted above, several relatively new entrants into the global wine market have made a significant impact. In particular, Australia, China, South Africa, Chile, and Argentina, are now responsible for nearly 20% of worldwide wine production. Several of these countries produce far more wine than they consume and therefore compete with the traditional European export market. The United States is also one of the leading producers of wine (more than 2.3 billion liters per year), with most production coming from California (which alone accounts for about 85% of all US wine). In fact, California as a "region" would be the fourth largest

Table 11.1 Top 20 wine-producing countries in 2016 and their total consumption.

Country	Production (L × 10^6)	% of world total	Consumption (L × 10^6)	% of world total
Italy	5090	19	2040	8
France	4350	16	2700	11
Spain	3930	15	990	4
United States	2390	9	3180	13
Australia	1300	5	540	3
China	1140	4	1730	7
South Africa	1050	4	440	2
Chile	1010	4	220	1
Argentina	940	4	940	4
Germany	900	3	1950	8
Portugal	600	2	460	2
Russia	560	2	930	4
Romania New	330	1	380	2
Zealand	310	1	92	< 1
Greece	260	1	230	1
Austria	200	1	240	1
Hungary	190	1	190	1
Brazil	160	1	290	1
Bulgaria	120	1	100	< 1
Switzerland	100	< 1	280	1
Total	26,700	100	24,514	100

Source: The International Organization of Vine and Wine (http://www.oiv.int/en/).

producer of wine. Not surprising, according to 2015 wine industry statistics (www.wineinstitute.org), the economic impact of the California wine industry is estimated to be more than $100 billion.

On a total consumption basis, the top four wine-drinking countries are the US, France, Italy, and Germany (Table 11.1). In just the past 10 years China and Russia have emerged as major consumers of wine. On a per capita basis for 2016, consumers in Portugal, France, Italy, and Switzerland drink the most wine, more than 35 liters per person per year (Figure 11.1). This compares to the world per capita average of about 3.3 liters. In the United States, per capita consumption is about 10 liters. However, in contrast to many European countries where wine consumption has either been the same or decreased in recent years, wine consumption in the United States increased about 3% annually from 2000 to 2013. Nonetheless, US wine consumption has leveled off in the past few years. The average cost of a 0.75 L bottle of wine in the United States is now about $10.

WINE BASICS

In principle, the manufacture of wine shares several similarities to beer manufacture – both involve an alcoholic fermentation performed by yeasts, and both rely on high quality ingredients. However, the similarities, for the most part, end there. Wine is often

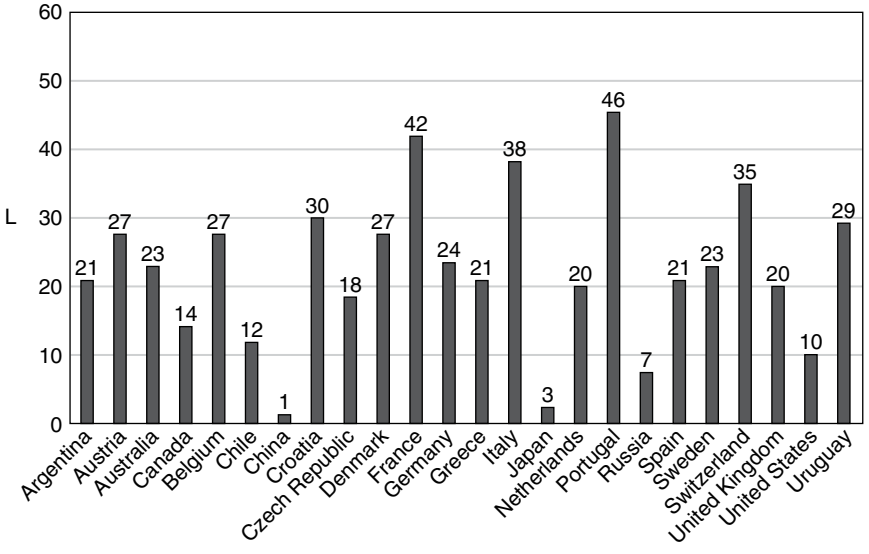


Figure 11.1 World-wide per wine consumption (L per person per year). Adapted from the Wine Institute 2001 Statistics (www.wineinstitute.org).

made by a natural fermentation, without the benefit of a starter culture. When cultures are used, the yeasts are selected on the basis of wine-specific traits. The fermentation substrates are different from those used in brewing, and they can vary from season to season. Although the main fermentation products are the same (i.e., ethanol and CO_2), other metabolic products are formed by wine organisms that affect product quality. Whereas beer is generally best consumed fresh, many wines improve during an aging period that can sometimes last for many years and has a profound influence on the finished product. Finally, although most wines, like most beers, start with relatively inexpensive raw materials, the quality of premium wines arguably depends, more so than any other fermented product, on the quality (some would say the very essence) of the raw material used in their manufacture.

Although about 99% of the wine produced throughout the world is made from grapes, juice from other fruits can also be made into wine. Berries, including raspberries, boysenberries, and strawberries, are common substrates. Many wines also are made from tree fruits, including apples and pears. The only requirement is that the fruit and the juice from that fruit must contain enough free sugars to support growth of the yeast and to yield a sufficient ethanol concentration (about 12% by volume).

Viticulture and grape science

The starting material for most wines, as noted above, is grapes. The main wine grape grown in temperate zones throughout the world is *Vitis vinifera*. Another grape, *Vitis labrusca,* grows well in northeastern regions in the United States and is frequently used for Concord varieties. This grape is also used in authentic Balsamic vinegar (Chapter 12). It is important to note that, despite the existence of only a few major grape species, there are many different grape cultivars grown throughout the world. For example, many of the "famous" grapes,

including Cabernet Sauvignon, Chardonnay, Gamay, Mission, Gewurztraminer, Grenache, and Sangiovese all refer to different varieties or cultivars of the *V. vinifera* grape. These grapes not only have different compositions, sugar contents, and pigmentation, they also grow better in different climates and soils and are used for different types of wine. This point cannot be over-emphasized, as it forms the basis of terroir – the principle that has long guided wine making (discussed below). Thus, most Bordeaux wines (those produced in the Bordeaux region of France) are made from the grapes that grow particularly well in that region, namely Cabernet Sauvignon and Merlot. Those same grapes, however, can be grown in California or anywhere else in the world, for that matter, and used to make Bordeaux-style or other types of wine.

Although it is common throughout Europe to name a wine after a region, in other countries, wines are ordinarily named after the grape variety from which the wine was produced. How a bottle of wine is actually labeled is itself an important issue. Labels not only must comply with local regulations, but they may also reveal useful information on the precise source of the raw materials, how the wine was made, and the expected quality attributes of the finished product (Box 11.2).

Assuming that it is possible to successfully grow Cabernet Sauvignon grapes, say, in central Minnesota, which is on the same general latitude as central France, could a bottle of wine similar to that of a Bordeaux be produced? Or to phrase the question another way, what distinguishes wine made from the same grape but from different geographical locations? Several answers are evident. First, different regions have different growing soils and climates. For example, in the famous grape-growing regions in Europe, different districts within the same region may yield grapes with markedly different composition and properties due to differences in soil composition, temperature, moisture, topology, and sunlight. Even different vineyards within the same district may yield different grapes. In addition, grapes cultivated in the same vineyard but harvested at different times within the same season, or from two different growing seasons, may be quite different and yield distinctly different wines. Climatic factors have such an important effect on grape quality and maturity that sophisticated computer-generated meteorological models have been developed to allow viticulturists to predict how grapes will grow and mature in a given geographical environment.

Vineyard management also affects grape maturation. Important factors include vine spacing and density, pruning and thinning, training and trellising, use of canopies, and application of pesticides. These vineyard practices, together with the combined effects of soil, climate, moisture, terrain, sun, and season have a profound influence on grape composition and quality. The collective contribution of all of these factors has led to the concept of "terroir," a term that translates from the French as "earth" or "land". However, the terroir concept extends even beyond the grape or the soil. Rather, it evokes an entire ethos or philosophy about the nature of the wine, so much so that winemakers claim that the terroir is a vital constituent of the wine and can be tasted and smelled (Box 11.3). The special wine region designations in Europe (e.g., "Appellation d'Origine Controle") and the US ("American Viticultural Areas") are a reflection of the terroir concept.

Certainly, it takes good grapes to make good wines. However, it is also true that wines made from premium grapes can sometimes be very ordinary, indicating that attention to proper wine-making techniques is also critical. The bottom line is that the basic raw material for wine, i.e., the grape, is prone to considerable variability, which may have profound effects on the physical, chemical, sensory, and other properties of the finished wine.

Box 11.2 Wine label nomenclature

Few, if any, food labels reveal or imply as much information on the source of the raw materials and when, where, or how the product was made, as do the labels on wine bottles. Indeed, wine label nomenclature can be so precise that informed and experienced consumers can even anticipate how a particular wine will taste even before the bottle is opened.

Appellation controlled labels This is especially true in the wine-producing regions in Europe. For example, in France, wineries are subject to strict regulatory authority by virtue of Appellation d'Origine Contrôlée (AOC) laws. As a result, French wine labels can be extremely informative, at least for those who understand how to "read" such labels. Moreover, European Union labeling laws prevent use of generic labels (i.e., naming a wine after a specific wine region, when the wine was produced from somewhere else). This labeling requirement is comparable to how other products are labeled. Thus, just as Parmesan cheese must have been made in Parma-Reggio Emilia and near-by regions of Italy, so too must a bottle of Champagne have been produced in the Champagne region of France. Also, because authentic Champagne (and Parmesan cheese, for that matter) is made using traditional methods (i.e., Méthode Champenoise), labeling a bottle of wine as Champagne necessarily tells the consumer not only where the product was produced, but also how that wine was made.

Situation in the US For the most part, few of these nomenclature rules exists in the United States, making comparisons between wines from different countries, based on labels, a challenging exercise. What does it mean, for example, for a California-made wine to be labeled as Burgundy or Champagne? In the past decade, however, these designations have become less common, and even many US sparkling wine producers no longer label their wine as Champagne.

Label details In general, there are three types of information on a wine label that provide an indication as to the nature or quality of that wine. Although different countries vary with respect to the specific details (see below), most labels indicate the type of grape (varietal), where the grapes were grown (the appellation), and the owner and/or bottler of the wine (proprietor).

Varietal refers simply to the cultivar (e.g., Chardonnay, Riesling, Cabernet Sauvignon) used to make the wine. For many French wines, the variety is not stated, but can be inferred by virtue of where it was made. In the United States (as well as most other countries), the varietal name can be used only when that variety comprises more than 75% of the grapes used in its manufacture.

Appellations are essentially geographical designations and refer to the location where the grapes were grown, such as Champagne, Chablis, Chianti, and Napa Valley. In France and other European countries, even small sub-regions and villages within the larger regions can be designated. In fact, protecting the geographical integrity of wine is one of the main reasons why labeling laws evolved. Although no law prohibits an American wine manufacturer from adopting a European geographical designation,

this so-called generic labeling is merely a marketing device and provides no real information. This practice is now much less of a problem than it was previously. To account for the geographical and varietal properties, most countries have adopted a form of the French AOC labeling regulations.

In France, most wines fall into one of three main categories: Appellation d'Origine Contrôlée, Vins de Pays, and Vin de Table. Wines bearing an AOC declaration are those that are (1) produced in one of the designated regions; (2) comprised of specific cultivars; and (3) made according to traditional manufacturing practices. In theory, wines bearing the AOC designation are of the highest quality.

Wines produced from grapes from different regions, outside the established AOC boundaries, are referred to as Vins de Pays (wine of the country). These wines are generally considered to be of somewhat lower quality, but in many cases, they are of similar quality as AOC wines. Finally, wines bearing the Vin de Table (or Vin Ordinaire) designation are of the lowest quality. The label makes no mention of the region or area where the wine was produced or of the grape variety. Other than declaring the wine as "vin blanc", "vin rouge", or "vin rosé", the label simply states the proprietor and the country.

In the United States, regulations regarding wine labeling are established by the Alcohol and Tobacco Tax and Trade Bureau (TTB), a division within the Department

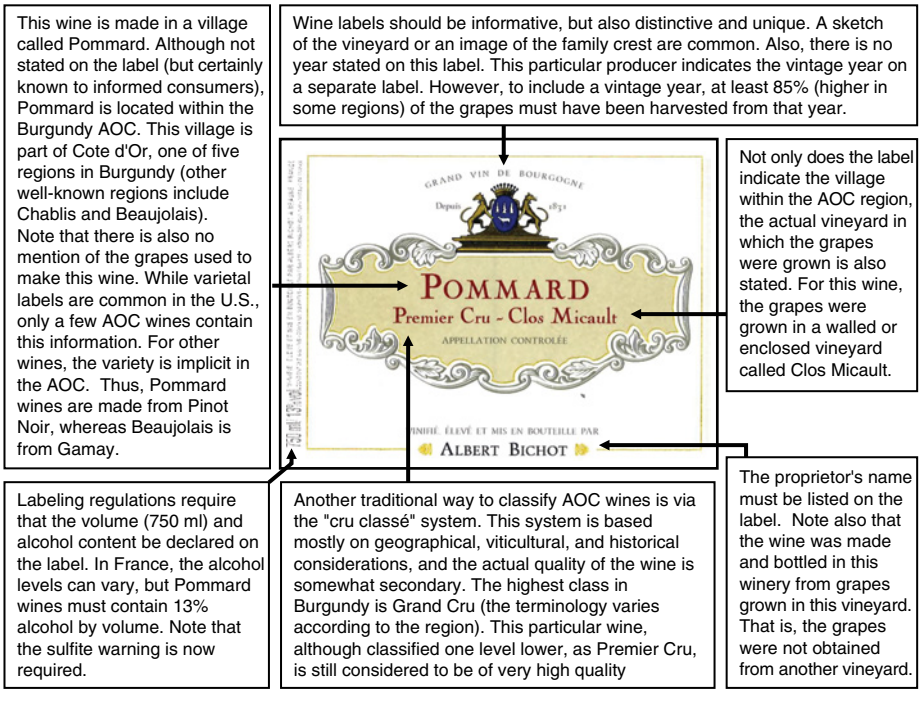


Figure 11.2.1 Understanding wine labels. The label above is from a French Burgundy made in the Cote d'Or region. The label on the next panel is from a bottle of Cabernet Sauvignon, made in the Napa Valley region of California. Labels were provided courtesy of the Albert Bichot winery and Far Niente winery.

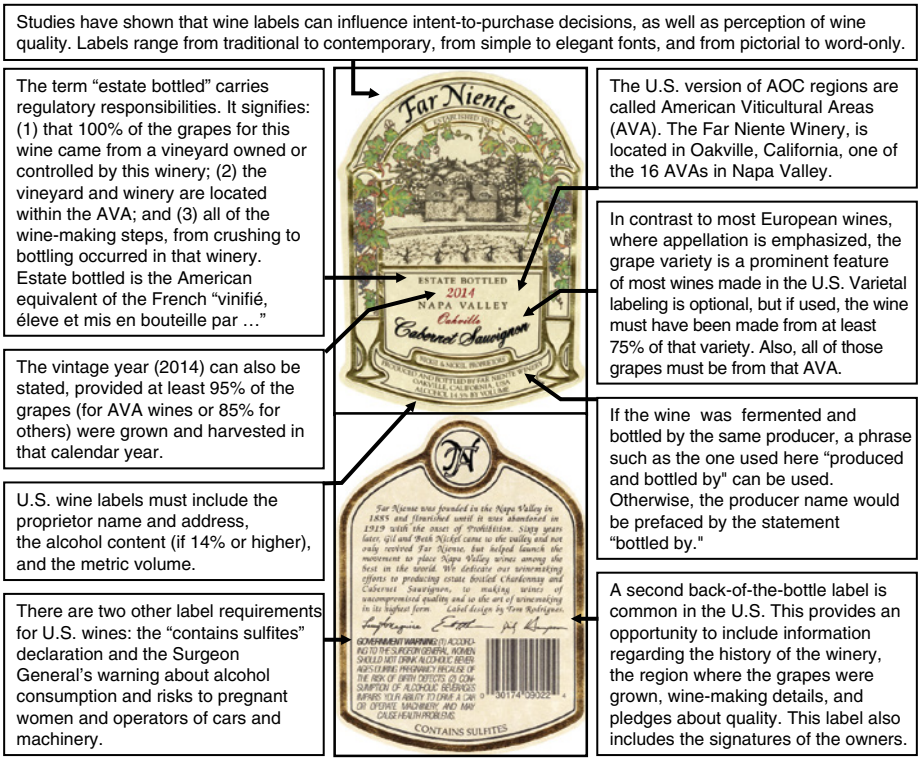


Figure 11.2.1 (*Continued*)

of the Treasury. In addition to a health warning on alcoholic beverages and a sulfite warning, there are a minimum of five other TTB requirements that must also be on the label: (1) the brand name or owner name; (2) the class or type; e.g., Table Wine, Sparkling Wine, Carbonated Wine, Citrus Wine, etc., (3) the alcohol content, by volume; (4) the bottler's name and address; and (5) the fluid volume, in metric measurements.

If the wine is made from grapes harvested and fermented during a particular year and from a particular region, a vintage year may be indicated on the label. Similarly, an appellation or geographical designation may be allowed, provided that more than 85% of the grapes were grown within the appellation boundaries. The boundaries for these appellations, called American Viticulture Areas (AVA), are defined by the TTB. More than half of the approved 239 AVA designations are in California (examples include Napa Valley, Sonoma Coast, and Carmel Valley), but 32 other states also have AVA regions.

Finally, although some consumers are intimidated by wine labels (especially when they are not written in English), the relevant information can be readily understood, provided one knows a few simple basics (Figure 11.2.1).

Box 11.3 Location, location, location – the importance of terroir in wine making

As noted throughout this book, fermented foods are very often associated with the specific locations at which they are manufactured. Parmesan, Roquefort, and Feta cheeses are well-known examples. Not only do they refer to a particular style of cheese, but the very use of the appellation (in Europe at least) requires that they be made in the designated regions in Italy, France, and Greece.

Real estate matters For all of the fermented foods described in this book, however, location or place is nowhere more intimately associated with product identity as it is for wine (Charters et al., 2017; Trubeck, 2008). There is even a word, if not a philosophy that describes this sense of place. Terroir, translated from the French as "earth" or land", refers, at its most simple level, to the ground or location in which wine grapes are grown. In France, the entire appellation control system (Appellations d'Origine Contrôlées or AOC) that forms the basis for how French wines are labeled is based on terroir. Thus, each of the so-called AOC regions has its own terroir (e.g., the Bordeaux terroir, the Burgundy terroir, and so on). Note that since 2008, AOCs are now referred to as Appellation d'Origine Protégée (or PDO in English).

In the broader sense, however, terroir theory holds that wine quality, apart from the actual manufacturing steps, is a function of a range of environmental and agronomic factors. Relevant factors include the nutrient composition of the soil in which the grapes were grown, the cultivar of the grapes, the topography of the vineyard, watering conditions, climatic conditions, exposure to sunlight, vine density, and training and pruning practices. Some wine makers might suggest that the specific microorganisms that are present on the grapes also contribute to terroir (Box 11.9).

On the surface, one might wonder why controversy regarding terroir exists. Is it not unreasonable to expect that wine quality might be influenced by where the grapes were grown? In fact, it is well established that sugar concentration, phenol content, other grape constituents and overall grape maturity are affected by climate, sunlight, and other growing conditions. Thus, on a general level, there is probably not much argument – environmental conditions have a profound effect on grape composition which ultimately affects wine quality.

Broader questions Why the issue of terroir raises concern among enologists, it seems, relates to two other issues. First, there is the view held by terroir advocates that the soil itself contributes to wine flavor by somehow translocating minerals and other soil molecules directly into the grapes. Thus, the wine has the "gout de terroir or "taste of the soil". This claim is disputed by many viticulturists, who would argue that grape vines and other plants adsorb water, minerals, nutrients, and other inorganic and organic molecules from the soil, but not flavor compounds per se.

A second issue is more philosophical in nature. Terroir, according to the popular wine writer, Jamie Goode, is considered by many to be an "ethos … a unifying theory encapsulating a certain approach to wine that encompasses the almost metaphysical circle of soil, nature, appellation, and human activity" (www.wineanorak.com).

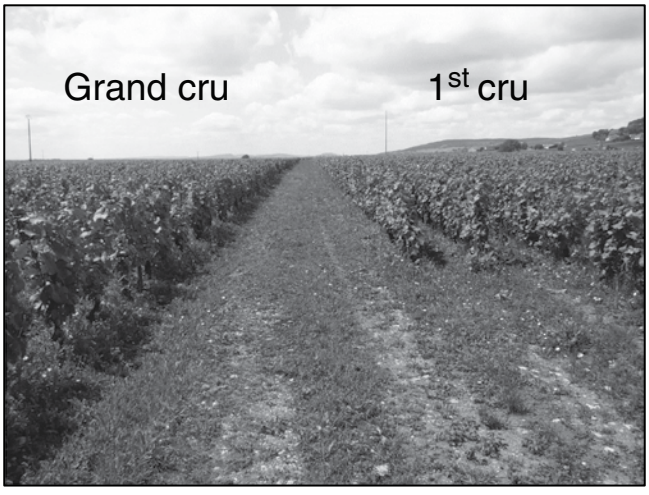


Figure 11.3.1 A vineyard in the Burgundy region. Photo courtesy of Albane de Vaux.

In other words, it is terroir, and not only the skills of the wine-maker, that is responsible for the overall sensory characteristics of a wine. Ultimately, the argument goes, terroir makes a particular wine unique.

By definition, therefore, it would not be possible to replicate a particular wine produced from grapes from one region with a wine made from the same cultivar, but grown even a few meters away (Figure 11.3.1). Further, and this is probably what really annoys "new world" wine makers, terroir implies or reinforces the perception that "old world" (and French, in particular) wines are superior to those produced elsewhere. Despite whether or not there actually is a scientific basis for terroir, there are clearly considerable market advantages for French wines bearing AOC designations (which are essentially synonymous with terroir). And even though wines produced in the Napa Valley of California or the Finger Lakes region of New York, or anywhere else for that matter, could also make terroir claims, there is little question that it is difficult to match the history and reputation of Bordeaux, Mosel Valley, Champagne, or any of the other terroir regions.

References

Charters, S., N. Spielmann, and B.J. Babin. 2017. The nature and value of terroir products. Eur. J. Mark. 51:748–771.

Trubeck, A.B. 2008. The taste of place: A cultural journey into terroir. Vol. 20, University of California Press.

Grape composition

The two major constituents of wine, water and ethanol, have no flavor, color, or aroma. Thus, it should not be surprising that the other grape components contribute so much to the organoleptic properties of wine. Some of these substances, however, can be problematic, causing

Table 11.2 Constituents of juice and red table wine (g/100 ml)[1].

Compound	Juice	Wine
Water	70–85	80–90
Carbohydrates	15–25	0.1–0.3
Glucose	8–12	0.05–0.1
Fructose	8–12	0.05–0.1
Other[2]	1–3	0.1–0.2
Organic acids[3]	0.5–2.0	0.3–0.6
Glycerol	0.0	0.7–1.0
Inorganic salts[4]	0.3–0.5	0.2–0.4
Nitrogenous	0.1–0.4	0.01–0.1
Phenolics	0.1–0.2	0.2–0.3
Ethanol	0.0	8–15
Other alcohols	0.0	0.01–0.04

[1] Adapted from Amerine et al., 1980, Boulton et al., 1996, and Waterhouse et al., 2016.
[2] Mainly sucrose and various pentoses.
[3] Mainly tartaric, malic, citric, lactic, and acetic.
[4] Mainly potassium, magnesium, and calcium.

a variety of defects. In addition, the composition of grapes changes during growth and maturation on the vine, such that the time of harvest can have a major influence on the chemical constituents of the grape as well as the wine. For example, the sugar concentration increases as grapes ripen on the vine, due to increased sugar biosynthesis, and to a lesser extent, to water evaporation and subsequent concentration of solutes. In contrast, acid concentrations decrease during maturation. Finally, in discussing the composition of wine, it is often more useful to consider the liquid juice just after the grapes have been crushed as the starting material, rather than the intact grape. As listed in Table 11.2, the juice, or "must," consists of several major constituents and many other minor components that are important, but which are present at relatively low concentrations.

Sugars

Other than water, which is 70% to 85% of the total juice volume, simple sugars represent the largest constituent of grapes or must. Depending on the maturation of the grapes at harvest, must usually contains equal concentrations of glucose and fructose, with the latter increasing somewhat in over-ripened grapes. Sucrose is usually present at very low concentrations (less than 1%), except for musts from *V. labrusca* grapes, which can contain as much as 10% sucrose. In general, most grape cultivars contain about 20% sugar (i.e., 10% glucose and 10% fructose), but the actual amount of total sugar varies, depending on maturity. Other sugars also may be present, but at very low concentrations, including the sugar alcohol, sorbitol, and the pentoses, arabinose, rhamnose, and xylose. It is common practice among wine makers to refer to the total sugar concentration in units of Brix or ^{0}Brix. The ^{0}Brix value is actually a measure of density or specific gravity and is easily and quickly determined using a hydrometer. Juice from mature grapes at 20% sugar is ordinarily about 21^{0}Brix to 24^{0}Brix.

As will be discussed later in more detail, glucose and fructose (and to a lesser extent, sucrose) serve as the major growth substrates for the fermenting yeasts. In fact, the main wine yeast, *Saccharomyces cerevisiae*, ferments only simple sugars, and all of the ethanol

that is produced is derived from fermentation of these sugars. In addition, a small amount of the sugar is converted to esters, aldehydes, higher alcohols, and other volatile organic compounds (formed also from metabolism of fatty acids and amino acids) that contribute important flavor and aroma characteristics. When nearly all of the sugar is fermented, and only residual (1–2 g/L or 0.1% to 0.2%) amounts remain, the wine is considered "dry". In contrast, when the residual sugar concentration at the end of fermentation is 10 g/L or higher, a "sweet" wine is produced. Very sweet wines, such as those consumed as dessert wines, can contain as much as 100 g/L to 200 g/L (10% to 20%). For these wines, the extra sugar may be part of the original must, mainly in botrytized or late-harvest wines and ice wines, described later. However, sugar can also be added, usually to make less expensive sweet wines. Finally, it should be noted that when the sugar concentration in the must is low, as might occur in grapes grown in cool climates, it is permissible, in some countries, to add sugar to the must, a process known as chaptalization.

Organic acids

Organic acids comprise the second most plentiful constituent in must. Although they are present at relatively modest concentrations, ranging from 1% to 2%, their effect on wine quality is extremely important. These acids are responsible for the low and well buffered pH of the must and the wine (usually between 3.0 and 3.5). That wine is so well preserved is due not only to the ethanol and low pH, but also to the presence of organic acids that have significant antimicrobial activities. At these pH values, pathogenic and other microorganisms are inhibited, including *Salmonella*, *Escherichia coli*, and *Clostridium* spp. The exceptions are aciduric and acidophilic organisms such as lactic acid bacteria, acetic acid-producing bacteria, and some fungi and yeast. In addition to antimicrobial effects, low pH also stabilizes the anthocyanins that give red wine its color, inhibits oxidation reactions, and contributes desirable flavor.

Wine chemists generally categorize wine acids into two general groups. Volatile acids are those that are volatilized or removed by steam treatment. Those that remain under these conditions are considered fixed acids. There are little, if any, volatile acids in must. During the ethanolic fermentation, small and usually inconsequential amounts (<0.5 g/L) of acetic acid and other short- and medium-chain fatty acids may be produced. However, if higher concentrations are produced, either by oxidation of ethanol by bacteria or as an end product of microbial metabolism, the wine will suffer serious defects and may be unsalable (as discussed later).

The main fixed acids, depending on the grape, condition, and maturity, are tartaric acid and malic acid. The ratio of these two acids can range widely but is usually about 1:3 (tartaric:malic). However, this ratio can be reversed in some grapes. These acids are important in wine for several reasons. Since malic acid contains two carboxylic acid groups, it contributes more protons in solutions, and makes the must more acidic. If the malic acid concentration is too low, as might occur in overly mature grapes grown in warm climates, the wine pH may be too high (e.g., when the pH is above 4.0). The wine will lack the desirable acid flavor and may be more prone to spoilage by bacteria. In contrast, although a minimum acidity is desirable in wine, excess acidity is also a defect and results in an inferior sour-tasting wine. Musts obtained from grapes grown in cool climates may contain high levels of malic acid, and are, therefore, problematic. A natural, biological method for deacidifying wine is commonly used for such musts (see below). Finally, it should be noted that other important acids may be present in wine, including fixed acids such as succinic acid and citric acid, as well as lactic acid, produced by yeast or bacteria during the wine fermentation.

Nitrogenous compounds

Grapes contain both inorganic and organic sources of nitrogen. Total nitrogen concentrations in grapes (or musts) range from about 0.1 g/L to 0.7 g/L. Free amino acids account for most of the nitrogen in must, with ammonium ions representing less than 0.1 g/L. Despite the relatively low concentration, the nitrogen content of most musts is generally adequate for rapid growth of yeasts. In fact, the primary role of nitrogen in wine appears to be as a nitrogen source for the yeasts, rather than affecting any of the organoleptic or other properties of the wine, per se. That being said, not all of the must nitrogen is utilized. Thus, the so-called yeast assimilable nitrogen (YAN) refers to that fraction consumed by yeast during wine fermentations.

In general, *S. cerevisiae* and other wine yeasts first consume free amino acids. However, they can also use ammonia directly as a source of nitrogen. Nonetheless, nitrogen deficiency can occasionally occur, and is one of the main causes for sluggish or "stuck" fermentations. Some wine makers, therefore, routinely add ammonium salts to the must, in the form of a yeast food (especially for those occasions when the grapes are deficient in nitrogen).

The main organic nitrogen-containing compounds are amino acids, amides, amines, and proteins. The free amino acids are not only synthesized into proteins (following transamination reactions), but several can also be used as an energy source. Among the proteins found in must, the most important are the enzymes. Some grape enzymes serve useful functions, whereas others can present problems. The pectinases and other hydrolases that enhance extraction of juice from the grapes during crushing are especially important. In contrast, phenol oxidases participate in the well-known enzymatic browning reaction. In the presence of oxygen, these enzymes form undesirable brown pigments that discolor the wine. This reaction, however, is effectively inhibited by addition of sulfur dioxide.

Polysaccharides

The main polysaccharide in grapes is pectin, a structural carbohydrate that provides structural integrity to the plant cell walls. The pectin concentration in the must can be as high as 5 g/L, which could potentially cause the wine to become cloudy. However, most of the pectin is either precipitated out during fermentation or is hydrolyzed to soluble sugars by exogenous microbial pectinases. The latter are commercially available and when added to must, they not only enhance maceration and pressing, but also improve wine clarity. Other polysaccharides, such as cellulose and hemicellulose, are not soluble in the juice and are removed along with the other non-soluble solids in the form of pomace.

Sulfur compounds

Several sulfur-containing substances are found or are formed in must that have a pronounced effect on the wine fermentation and wine quality. In particular, hydrogen sulfide (H_2S) and various organic forms of sulfur, especially the mercaptans which are derived from H_2S, impart highly offensive odors in the wine. They are produced in trace amounts by grape yeasts during fermentation.

The other major sulfur compound found in wine is sulfur dioxide (SO_2). Other related aqueous forms also exist as sulfite ions. These substances are produced naturally by yeast, and are invariably present in wine, albeit at concentrations usually less than 50 mg/L. However, sulfur dioxide and bisulfite salts are now commonly added to must due to their strong antimicrobial, antioxidant,

and anti-browning properties. It is important to recognize that these activities occur only when the SO_2 is in its free, un-bound form. When bound or fixed with other wine compounds, such as acetaldehyde, reducing sugars, or sugar acids, SO_2 activity is diminished. How SO_2 specifically functions in wine and its important role in wine making will be further discussed later.

Phenols, tannins, and pigments

Among the most important naturally occurring substances in grapes and musts are the phenolic and polyphenolic compounds. Some phenols can also be introduced into the wine following aging in wooden casks or via yeast and bacterial metabolism. These chemically diverse compounds contribute color, flavor, aroma, and mouth feel to the wine. They can also react with other grape components and can either improve or diminish wine quality. Finally, many of the phenolic compounds found in wine are thought to be responsible for the putative health benefits associated with wine consumption (Box 11.4).

Box 11.4 L'chaim! Healthful properties of wine

However one says it L'chaim! Salut! A votre sante! Cheers! – all are often said just prior to consuming wine, and all mean the same thing – to life and good health. But is wine simply a symbol for expression of this sentiment or might there be an actual scientific basis to support the healthful properties of wine? Let's examine some of the epidemiological, as well as mechanistic evidence collected in recent years.

Epidemiology

If drinking wine promotes health or well-being, one would expect an association between moderate wine consumption and longevity. Likewise, residents of countries where wine is regularly consumed would be expected to have longer life expectancies. Lower rates of heart disease and cancer might also be expected in those countries compared to where wine consumption is low. Indeed, that is exactly what such studies have shown – moderate wine drinking is associated with longevity and heart health (Figure 11.4.1).

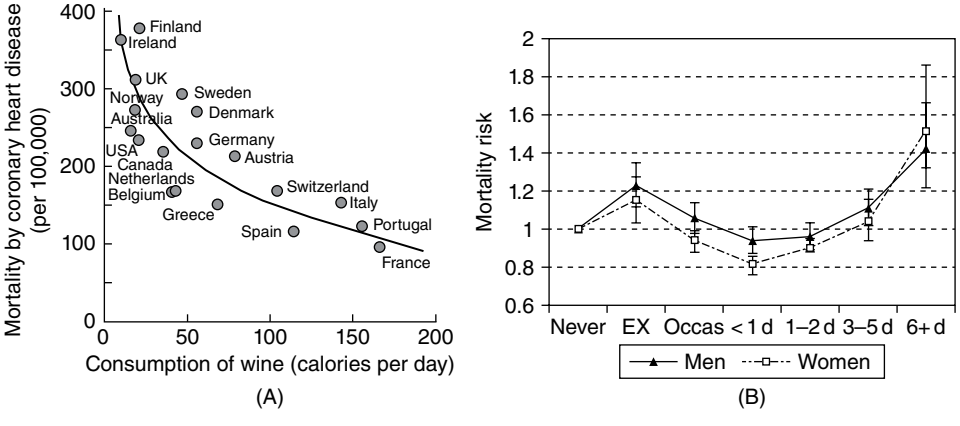


Figure 11.4.1 Correlation between wine consumption and mortality by coronary heart disease (A), adapted from Renaud and de Lorgeril, 1992; and total mortality among men and women as a function of alcohol drinking frequency (B), from Klatsky and Udaltsova, 2007, with permission.

Several recent studies have provided additional epidemiological evidence to support this hypothesis. In one large study (Gea et al., 2014), a cohort of nearly 20,000 Spanish adults was tracked for 20 years. One of the primary goals was to assess the relationship between drinking habit and mortality. They were particularly interested in individuals whose diet or lifestyle conformed to the so-called Mediterranean alcohol-drinking pattern (MADP). The MADP is characterized by moderate total alcohol intake, with a preference for red wine, consumed with food and spread out over the week. The results revealed higher risks of mortality among both abstainers, who either did not drink alcohol, and individuals who had a low MADP score. In other words, consuming alcohol in the traditional Mediterranean manner (i.e., red wine) lowers one's risk of mortality (Giacosa et al., 2016).

The association of wine, diet, and life style with longevity also forms the basis of the so-called "Blue Zone" concept (Buettner and Skemp, 2016; Pes et al., 2015). People who live in these Blue Zone regions live longer and have fewer diseases. Of the five Blue Zone locales, two are in the Mediterranean region, including Sardinia, Italy and Ikaria, Greece. Importantly, among the dietary features that comprise the Blue Zone lifestyle is moderate consumption of red wine.

It is relevant to note that epidemiological studies that show positive associations of wine consumption and reduced mortality do not always account for other factors that may influence mortality, such as exercise, diet, other health habits, and genetics. Also, it is important to distinguish between wine and other forms of alcoholic beverages that may show similar effects. Finally, it should be emphasized, as is evident from so-called J- or U-curves, that as wine or alcohol consumption increases above a particular level, mortality risk also increases.

Wine consumption and heart disease

High rates of coronary heart disease are generally associated with high saturated fat diets. Yet, in some countries, such as France and Italy, where saturated fat consumption is high, heart disease rates are significantly lower than in other countries where fat consumption is high. What is different about France and Italy is that they also have high rates of red wine consumption. These observations formed the basis of the so-called French Paradox – namely, that red wine protects people from heart disease (Renaud and de Lorgeril, 1992).

Several epidemiological and meta-analyses studies support this claim (Chiva-Blanch et al., 2013; Costanzo et al., 2011; Levantesi et al., 2013). For example, the latter study followed the dietary and drinking habits of 11,000 subjects who had suffered a myocardial infarction (i.e., heart attack). They concluded that moderate wine consumption reduced the risks of mortality and subsequent cardiovascular events. Red wine consumption was also associated with reduced risks of metabolic syndrome (Tresserra-Rimbau et al., 2015). In this study (nearly 4000 subjects), elderly moderate red wine drinkers at a high cardiovascular risk were compared to non-drinkers. Of the five metabolic criteria for metabolic syndrome, wine drinkers had lower risks for four, including abnormal waist circumference and high blood pressure.

Biochemical mechanisms

Given the strong epidemiological evidence showing an inverse relationship between coronary disease and wine consumption, what biochemical mechanisms would

account for this finding? Several possibilities have been suggested (Lippi et al., 2010). First, alcohol consumption, in general, is known to increase the high-density lipoprotein (HDL) cholesterol concentration in blood, a factor known to reduce heart disease risks. Alcohol may also increase fibrinolytic activity and decrease platelet aggregation, which similarly reduce plaque formation. Wine, however, contains specific components (other than those due to alcohol) that may also account for the reduction in cardiovascular disease. Among the compounds thought to be most important are polyphenols and flavonoids (Yamagata et al., 2015). These compounds have antioxidant activity and can scavenge superoxide and other reactive oxygen species. Thus, formation of oxidized low-density lipoprotein (LDL), which is known to be toxic to endothelial cells and impair vasorelaxation, are inhibited. Similarly, the antioxidant activity of wine flavonoids can prevent the reaction of superoxide and nitric oxide (NO). The latter has antithrombotic activity, prevents atherosclerosis, and inhibits platelet adhesion and aggregation, and, therefore, is necessary for normal endothelial function.

Other explanations for the role of wine in reducing coronary disease and endothelium-dependent constriction, in particular, have been described (Pes et al., 2006; Corder et al., 2006). These investigators reported that polyphenols in red wine, mainly oligomeric procyanidins (OPC) suppress endothelial synthesis of endothelin-1 (ET-1), a peptide that has vasoconstriction activity and is involved in development of atherosclerosis. These authors further reported that the OPC content and biological activity of red wine from Sardinia were more than double from other wines.

Effect of wine consumption on cancer

Based on epidemiological studies, consumption of alcohol, in the form of beer or spirits, generally increases cancer risk mortality, whereas moderate wine consumption appears to lower risks of some cancers (Chao, 2007). Several wine polyphenolic compounds, such as quercetin and catechin, have been shown to have inhibitory effects, *in vitro*, against tumor and cancer cells. Two other compounds found in red wine, resveratrol and acutissimin A, have recently attracted considerable attention as molecules that may have anti-cancer properties.

Resveratrol is a polyphenol that may contribute cardiological benefits (as do other wine phenols), in addition to its protective effects on carcinogenesis. Specifically, resveratrol is thought to interfere with the cell signaling cascades that result in the synthesis of NF-κB and AP-1 (Athar et al., 2009). The latter are transcription factors that activate expression of genes involved in promotion of cell cycle progression, inflammation, anti-apoptosis, and tumorigenesis.

Despite the mostly positive attention that the wine-health connection continues to receive in the popular press, as well as in the scientific literature (Ayala, 2011), it would be misleading not to note that there are many detractors. Some investigators have argued that dietary diversity, rather than wine, is responsible for the French Paradox, that statistical biases underestimate coronary deaths in France, and that the risk/benefit ratio does not support wine consumption as a means of promoting overall health (Chikritzhs et al., 2015; Stockwell et al., 2016).

References

Athar, M., J.H. Back, L. Kopelovich, L., D.R. Bickers, and A.L. Kim. 2009. Multiple molecular targets of resveratrol: anti-carcinogenic mechanisms. Arch. Biochem. Biophys. 486:95–102.

Ayala, F.J. 2011. Elixir of life: In vino veritas. PNAS 108:3457–3458.

Buettner, D., and S. Skemp. 2016. Blue Zones: lessons from the world's longest lived. Am. J. Lifestyle Med. 10:318–321.

Chao, C. 2007. Associations between beer, wine, and liquor consumption and lung cancer risk: A meta-analysis. Cancer Epidemiol. Biomarkers Prev. 16:2436–2447.

Chikritzhs, T., T. Stockwell, T. Naimi, S. Andreasson, F. Dangardt, and W. Liang. 2015. Has the leaning tower of presumed health benefits from "moderate" alcohol use finally collapsed? Addiction 110:726–727.

Chiva-Blanch, G., S. Arranz, R.M. Lamuela-Raventos, and R. Estruch. 2013. Effects of wine, alcohol and polyphenols on cardiovascular disease risk factors: evidences from human studies. Alcohol Alcohol. 48:270–277.

Corder, R., W. Mullen, N.Q. Khan, S.C. Marks, E.G. Wood, M.J. Carrier, and A. Crozier. 2006. Red wine procyanidins and vascular health. Nature 444:566.

Costanzo, S., A. Di Castelnuovo, M.B. Donati, L. Iacoviello, G. de Gaetano. 2011. Wine, beer or spirit drinking in relation to fatal and non-fatal cardiovascular events: a meta-analysis. Eur. J. Epidemiol. 26:833–850.

Gea, A., M. Bes-Rastrollo, E. Toledo, M. Garcia-Lopez, J.J. Beunza, R. Estruch, and M.A. Martinez-Gonzalez. 2014. Mediterranean alcohol-drinking pattern and mortality in the SUN (Seguimiento Universidad de Navarra) Project: A prospective cohort study. Br. J. Nutr. 111:1871–1880.

Giacosa, A.. R. Barale, L. Bavaresco, M.A. Faliva, V. Gerbi, C. La Vecchia, E. Negri, A. Opizzi, S. Perna, M. Pezzotti, and M. Rondanelli. 2016. Mediterranean way of drinking and longevity. Crit. Rev. Food Sci. Nutr. 56:635–640.

Klatsky, A.L. and N. Udaltsova. 2007. Alcohol drinking and total mortality risk. Ann. Epidemiol. 17:S63–S67.

Levantesi, G., R. Marfisi, D. Mozaffarian, M.G. Franzosi, A. Maggioni, G.L. Nicolosi, C. Schweiger, M. Silletta, L. Tavazzi, G. Tognoni, and R. Marchioli. 2013. Wine consumption and risk of cardiovascular events after myocardial infarction: results from the GISSI-Prevenzione trial. Int. J. Cardiol. 163:282–287.

Lippi, G., M. Franchini, E.J. Favaloro, and G. Targher. 2010. Moderate red wine consumption and cardiovascular disease risk: beyond the "French paradox". Semin. Thromb. Hemost. 36:59–70.

Pes, G.M., F. Tolu, M.P. Dore, G.P. Sechi, A. Errigo, A. Canelada, and M. Poulain. 2015. Male longevity in Sardinia, a review of historical sources supporting a causal link with dietary factors. Eur. J. Clin. Nutr. 69:411–418.

Renaud, S. and M. de Lorgeril. 1992. Wine, alcohol, platelets, and the French paradox for coronary heart disease. Lancet 339:1523–1526.

Stockwell, T., J. Zhao, S. Panwar, A. Roemer, T.S. Naimi, and T. Chikritzhs. 2016. Do "moderate" drinkers have reduced mortality risk? A systematic review and meta-analysis of alcohol consumption and all-cause mortality. J. Stud. Alcohol Drugs 77:185–198.

Tresserra-Rimbau, A., A. Medina-Remón, R.M. Lamuela-Raventós, M. Bulló, J. Salas-Salvadó, D. Corella, M. Fitó, A. Gea, E. Gómez-Gracia, J. Lapetra, F. Arós, M. Fiol, E. Ros, L. Serra-Majem, X. Pinto, M.A. Muñoz, and R. Estruch. 2015. Moderate red wine consumption is associated with a lower prevalence of the metabolic syndrome in the PREDIMED population. Brit J. Nutr. 113:S121–S130.

Yamagata, K., M. Tagami, and Y. Yamori. 2015. Dietary polyphenols regulate endothelial function and prevent cardiovascular disease. Nutrition 31:28–37.

The phenols important in wine are grouped according to their chemical structure (Figure 11.2). Phenols containing a single phenolic ring and functional groups at carbon 1 or 3 are referred to as non-flavonoids. In contrast, compounds containing two or more phenolic

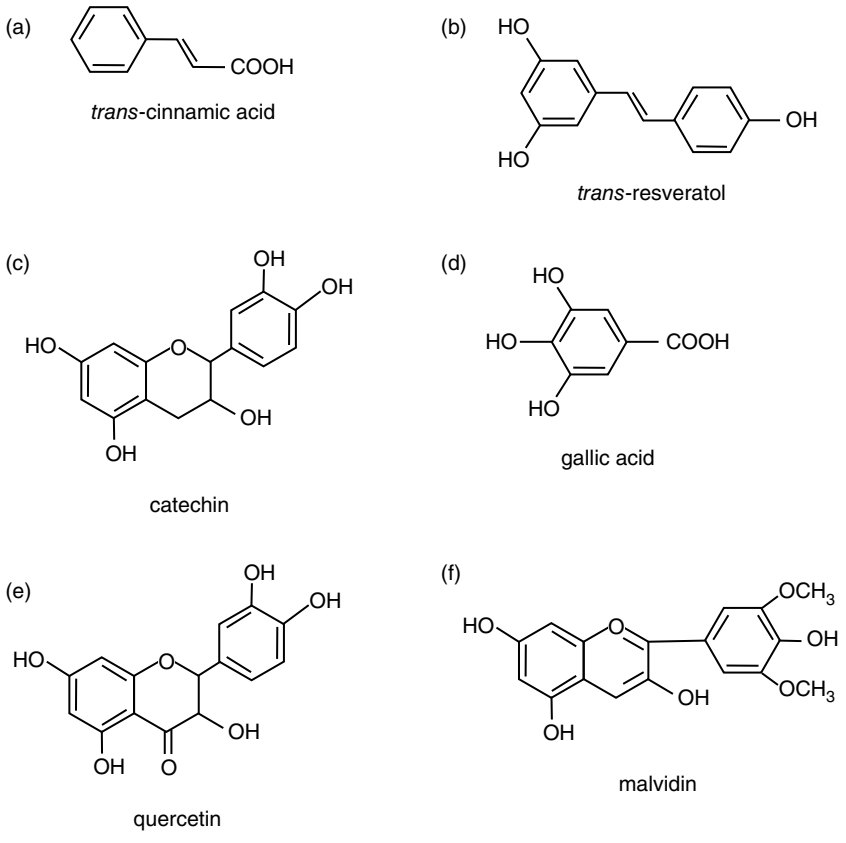


Figure 11.2 Representative phenolic compounds in wine. Examples include: a. cinnamic acid, a phenolic acid; b. resveratol, a derivative of cinnamic acid; c. catechin, a flavan-3-ol; d, gallic acid, a tannin; e. quercetin, a flavonol; and f. malvidin, an anthocyanin.

rings connected by pyran ring structures are called flavonoids. Both non-flavonoids and flavonoids can be polymerized to form another important class of phenolic compounds called tannins. Tannins consisting of flavonoid phenols are especially important in wine due to their bitter flavor and color stabilization properties.

Within the flavonoid group are several different types of compounds, including the flavonols, catechins, and anthocyanins. They are constituents of the grape skins and seeds, but are extracted into the juice shortly after the grapes have been crushed. In general, the longer the wine is in contact with the seeds and skins, the more of these substances will be extracted. High temperatures also enhance extraction. The extraction of these phenolic compounds from grape skins and seeds is one the distinguishing differences between red and white wines. Thus, in the manufacture of red wine, the seeds and skins are left in contact with the pressed juice for several days (i.e., during fermentation), whereas this material is removed almost immediately after crushing for white wine manufacture (see below). Thus, although both red wine and white wine contain phenolic compounds, the concentration in red wines is usually at least four times higher than that of white wine. Importantly, many of these phenolic compounds are responsible for pigmentation of grapes and, subsequently, the color of the wine. Anthocyanins, for example, have red or blue-red color and make red wines red.

Most of the anthocyanins are attached, via ester linkages, to one or two glucose molecules, which enhances their solubility and stability.

WINE MANUFACTURE PRINCIPLES

Making wine, as far as the actual steps are concerned, appears to be a rather simple and straightforward process (Figure 11.3). Grapes are harvested and crushed, the crushed material (i.e., juice or must) is fermented by yeasts and bacteria, the organisms and insoluble materials are removed, and the wine is aged and bottled. In reality, the process is far from easy, and each of these pre-fermentation, fermentation, and post-fermentation steps must be carefully executed if high-quality wine is to be consistently produced.

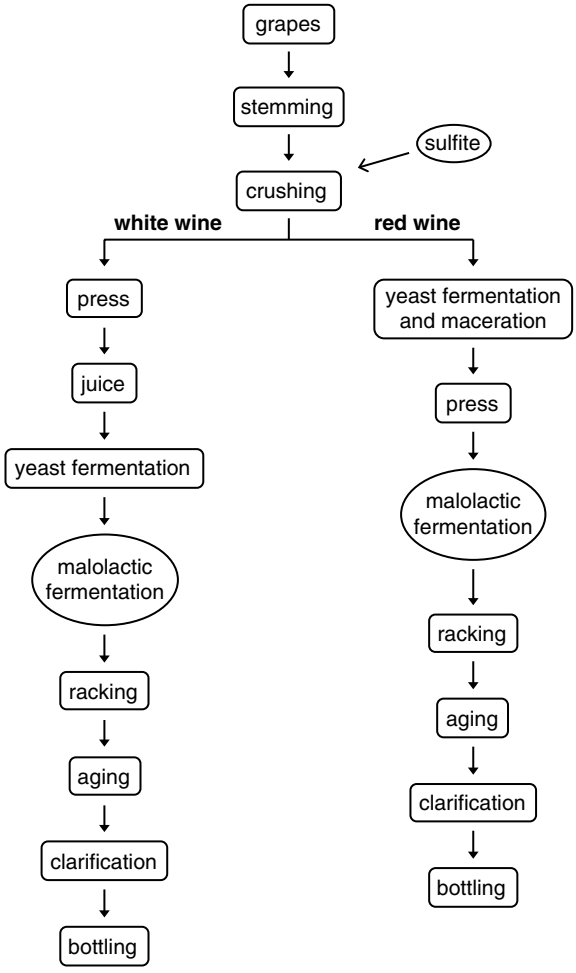


Figure 11.3 Flow chart of wine manufacture. The main difference between the manufacture of red and white wine is that the grapes for red wines are fermented in the presence of skins and seeds (maceration), whereas for white wines, these materials are removed after crushing. Although optional, sulfiting agents are added to most US wines. Whether the malolactic reaction is induced or encouraged depends on the grape composition, the style of wine, and the manufacturer's preferences.

Harvesting and preparing grapes for wine making

According to both viticulturists and enologists, the first step in wine making, harvesting the grapes, is considered to be one of the most important. Grapes must be picked at just the right level of maturity. This means that the concentrations of sugars and acids (and the sugar/acid ratio), pH, the total soluble solids, and even the phenolic constituents must be at just the right level for the particular cultivar and the type or style of wine being made. In addition, berry size and weight also influence the time at which grapes are harvested. In general, grapes should be sampled sometime before their expected harvest time and their composition assessed (at minimum, the ^{0}Brix and pH should be measured) to make sure that over-ripening does not occur.

Unfortunately, there is no exact or objective set of rules to ensure or predict the optimum time for harvesting grapes. Rather, grapes are frequently harvested based on more subjective criteria, such as color, firmness, and taste (as well as the collective wisdom and experience of the winemaker). As grapes ripen on the vine, the sugar concentration, as well as flavor and color components, increase, and acids usually decrease, so identifying the correct moment for harvesting can be a real challenge. It is possible, moreover, for grapes to over-ripen, such that the harvested grapes contain too much sugar or too little acid or be too heavily contaminated with wild yeast and molds. Once the grapes have been deemed properly mature, it is essential that they be picked and harvested quickly, since the composition can continue to change.

Even in the twenty-first century, when so much of modern agriculture has become automated and mechanized, a sizable portion of grapes for wine making is still harvested manually. Only relatively recently has mechanical harvesting begun to displace manual harvesting. In the United States, for example, the majority of grapes are now harvested by mechanical means. However, manual picking of grapes is still often done for premium quality American wines and in much of Europe. Manual harvesting is more gentle on the grapes, and bruising and breaking of the grapes is minimized. For certain wines, such as sweet wines made using noble rot-infected grapes or wines for which grape harvesting methods are regulated (e.g., French Champagne), manual harvesting is required. Anyone who has seen the odd contours and steep terrains of some of the European vineyards will also appreciate the necessity of manual grape picking. On the other hand, mechanical harvesters are faster and cheaper, and, unlike hired laborers, deployable on short notice and available around the clock.

Once the grapes are removed from the vines, they must be transported to the winery. It is important that the grapes not be bruised, crushed, or otherwise damaged either during harvesting or transport, since this encourages growth of microorganisms prior to the actual start of the fermentation. For the same reason, transportation time is also important.

Crushing and maceration

The purpose of crushing is to extract the juice from the grapes. Before the grapes are crushed, however, leaves, large stems, and stalks are removed. Some wine makers may not remove all of the stems to increase the concentration of tannins and other phenolic compounds that are present in the stems and extracted into the juice. Once the extraneous material is separated and removed, the grapes are crushed by one of several types of devices. Roller crushers consist of a pair of stainless steel cylinder-shaped rollers. Another type of crusher, called the Garolla crusher, not only performs the crushing step, but also removes stems. It consists of a

rotating shaft contained within a large horizontal stainless steel cylinder or cage. Arms on the shaft are attached to paddles or blades such that when the shaft turns, the grapes are moved and pressed against the side of the cylinder. Perforations on the walls of the cylinder allow for the juice (along with the skin, seeds, and pulpy material) to pass through into collection vats, whereas the stems gather at the end.

The crushed grapes, as noted above, contain juice, seeds, and skins. Pigments, tannins, and other phenolic compounds are located in the skins and seeds, and their extraction into the juice takes time. Endogenous pectinases and other hydrolytic enzymes within the grapes enhance extraction and must also be given time to work. This extraction step, where the crushed grape material is allowed to sit, is referred to as maceration.

Maceration conditions are not the same for all wines. For red wines, where pigment extraction is especially important, long maceration times at rather high temperatures are usually employed. In general, maceration is done at around 28°C for up to five days. The shorter the maceration times and the lower the temperature, the less phenolic and other materials will be extracted. Thus, lighter red wines, such as Beaujolais, are macerated for just a few days at no higher than 25°C. In contrast, deeper red wines, such as Bordeaux, are macerated for up to twenty-eight days at 30°C. Since fermentation begins shortly after the grapes are crushed, maceration and fermentation essentially occur at the same time. In fact, the ethanol made by fermenting yeasts enhances extraction. This situation only occurs, however, if the musts are not treated with sulfur dioxide.

Maceration at low temperatures (<15°C) ordinarily results in only moderate pigment extraction and little fermentation. However, if the must is macerated at a low temperature (between 5°C and 15°C), but for longer time, extraction of anthocyanins and aroma and flavor compounds can be enhanced. This technique, called cold maceration, simulates the natural conditions in cooler wine-producing areas.

For white wines, if a maceration step is performed, it is done at lower temperature and for much less time. Typically, only a few hours at 15°C is sufficient. For most white wines, however, the producers remove the seeds and skins immediately after crushing (i.e., sans maceration). As for red wine, the maceration conditions used for white wines influence the amount of pigments and tannins that are extracted. Wines made from Sauvignon blanc grapes where little maceration occurs typically have a low phenolic concentration, whereas Riesling and Chardonnay musts, which are often macerated in the cold, may contain higher amounts.

Sulfur dioxide treatment

This is now a convenient point at which to discuss the use of sulfur dioxide in wine making. As soon as the integrity of the grapes has been compromised by the crushing step, the sugars and other nutrients in the juice are liberated and made available for whatever microorganisms happen to be present. Ordinarily, the must is populated by epiphytic yeasts (that is, yeasts that reside on the surface of the grapes) and by adventitious yeasts that have "contaminated" the bins, crushers, presses, and other wine-making equipment. Another perhaps under-appreciated source of yeasts are those introduced via insect vectors. Thus, fruit flies, bees, and other insects are widely present in wineries may transfer yeasts from grape-to-grape in the vineyard or from grape-to-equipment in the winery. Although the surface of a single, healthy, grape may contain only about 10^2 to 10^4 yeast cells, after the grapes have been exposed to the contaminated equipment and crushed, the number of cells increases about 100-fold, to about 10^4 to 10^6 cells per ml. Whether this resident microbiota actually commences fermentation, however, depends on the intent of the winemaker.

Initiating fermentations

Two options exist for initiating the wine fermentation. First, the fermentation may proceed entirely on its own, i.e., without the addition of a yeast starter culture. Winemakers sometimes refer to these fermentations as spontaneous or indigenous. The term, "natural" is also used to describe these fermentations. None of these are really ideal – spontaneous evokes the obsolete notion of spontaneous generation, and natural implies something unnatural about adding cultures. In any case, except for temperature control, essentially no other restrictions or selective pressures are placed on the fermentation. Yeast growth, depending on the temperature, will generally occur rather quickly, with a relatively short lag phase.

The other option is to start the fermentation with a defined yeast starter culture selected by the winemaker. The latter usually requires that the indigenous or autochthonous microbiota be inactivated or controlled, such that it does not compete or possibly interfere with the added culture. This is not, however, always the case, because it is still possible to add a starter culture even in the presence of the background microbiota. The use of wine starter cultures has now become commonplace, even among many traditional wine manufacturers. Moreover, recent research on yeast ecology suggests that there may be less diversity in wines made by spontaneous fermentations than previously thought and that these natural fermentations involve relatively few strains (Box 11.5).

When starter cultures are used, the naturally occurring or so-called wild yeasts are inactivated in one of two ways. First, the must can be heat treated, usually via a high-temperature, short-time (or flash) pasteurization process. Although very effective against most organisms found in musts, even moderate heating is often detrimental to the juice and to the wine. Thus, this process is rarely used. The preferred method is to chemically pasteurize the must by adding sulfites. It should be noted that even naturally fermented wines, especially white wines, are often sulfite-treated to control undesirable organisms. The most common sulfiting agents are SO_2 gas and potassium bisulfite salts. Sulfites are cheap, effective, and multifunctional. In addition to their effectiveness against yeast and mold, these agents also inhibit growth of a wide range of bacteria, including acetic acid-producing bacteria and malolactic bacteria. Sulfites can thus be considered as serving a preservative function in wine. Importantly, they also control several deleterious chemical reactions, particularly oxidation and browning reactions.

The amount of sulfite added and when it is added varies depending on the condition of the grapes, the microbial load, must pH and acidity, and the type of wine (red or white). Usually, SO_2 or sulfite salts are added to the must just after crushing. Musts from mature grapes that often contain high levels of wild yeast require more SO_2, but in general, about 80 mg/L is sufficient. Also, the lower the pH, the less sulfite is necessary for antimicrobial activity. Due to human health concerns, however, there are also regulations that dictate how much sulfite can be present in wine. In France and most of Europe, red and white wines must contain less than 160 mg/L or 210 mg/L, respectively. In the United States, the limits are 350 mg/L for both red and white wines. Sulfite warning labels are triggered at 10 mg/L.

Yeast cultures

When pure yeast cultures are used, they are usually obtained from commercial sources. So-called house strains, although common for beer, are infrequently used for wine making, due primarily to the expertise and equipment required for strain maintenance and propagation. In contrast, commercial cultures are easy to use and require only modest technical expertise.

Box 11.5 Culture wars and microbial diversity in wine fermentations

Like most of the fermented products described in this text, wines were made for thousands of years before scientists recognized that microorganisms were responsible for the fermentation. In contrast to dairy, meat, and beer fermentations, where backslopping methods have generally been replaced by starter cultures, many wine makers continue to rely on the indigenous microflora to initiate and complete the fermentation. Even today, many of the wines produced by traditional manufacturers, especially those in Europe, are made by a "spontaneous" or natural fermentation. Thus, the source or inoculum for these fermentations are the wild autochthonous yeasts that are naturally present on the grapes, in the must, or on equipment (García-Ríos et al., 2014). Many large, modern wineries, however, have adopted yeast starter culture technology and have abandoned the traditional practice of allowing wild yeasts to initiate and perform the fermentation.

The Great Cultural Divide

Advocates of wine starter culture technology make several good arguments. First, they contend that defined yeast strains results in wine having a "pure" or cleaner flavor, as growth of wild yeasts are suppressed (Ciani and Comitini, 2015). In addition, the availability of yeast with particular physiological characteristics such as osmotolerance or the ability to grow at low temperature allows the wine maker to select yeasts particularly suited to the grape characteristics one is using or the wine type one is making (Table 11.5.1). The wine maker can also use the same yeast strains over different vintages (i.e., from one year to the next).

Importantly, the wine maker can also expect culture performance to be consistent with regard to fermentation times, flocculation properties, and flavor and end-product formation. So-called stuck fermentations are more readily avoided. In addition, nonconventional yeast strains can be used in customized cultures to provide traits and performance characteristics that suit the particular needs of the wine producer (Jolly et al., 2014; Masneuf-Pomarede et al., 2016). Finally, there may be conditions that make pure cultures almost essential, such as when the grapes or musts contain very high sugar or acid concentrations.

Vive la différence

Despite these advantages, it is undoubtedly true that naturally fermented wines have an excellent track record and many are highly regarded by wine experts. Even if one were to

Table 11.5.1 Starter cultures versus natural fermentation for wine-making.

Starter cultures	Natural
Cleaner flavor	More complex flavor
Greater consistency	Unique qualities
Faster	Slower
Low frequency of stuck fermentations	Greater frequency of stuck fermentations
Can customize strains	Cannot customize
Immune to killer yeasts	May be sensitive to killer yeast

concede that the flavor, aroma, and other organoleptic properties may be more variable in naturally fermented wines, is that necessarily such a bad thing? Certainly, one could argue that it is the variability itself that makes it possible to produce truly exceptional wines. Uniformity, created by virtue of pure cultures, goes the argument, is the problem (Mas et al., 2016). Proponents of natural wine fermentations claim that such wines are more "complex" due to the more complicated metabolic processes that occur within a complex yeast flora. In other words, it is the metabolically diverse microbiota or microbial terroir that leads to a more complex distribution of flavor components in the wine (Comitini et al., 2017).

To address this issue of metabolic diversity on a rational basis, several questions must first be raised. How many different strains are actually present during a natural wine fermentation? Are some strains more dominant than others? Are strains stable over time? What are the phenotypic properties of these strains, relevant to wine flavor generation?

Several recent studies have attempted to address these questions. In one report (Börlin et al., 2016), more than 500 yeast strains were isolated from naturally fermented musts obtained from several wineries, over several seasons, in the Sauternes appellation in southwestern France. The strains were genotyped by a technique based on microsatellite markers (repetitive DNA sequences) and their relatedness was determined by cluster analyses. The results revealed that most of the isolates were indeed wild *Saccharomyces* strains, as only 7% were related to commercial strains. There was also overlap between adjacent vineyards, indicating environmental transfer had occurred. Finally, the similarity of strains collected more than two decades earlier indicated some strains were very persistent in some of the vineyards.

Another study addressed similar questions, but in these experiments the musts were analyzed at the beginning and end of the fermentation (Demuyter et al., 2004). Thus, strain diversity could be determined at two different stages. Over a three-year period, grapes from Alsace (used to produce Gewurztraminer wines) were obtained, crushed, and fermented under three different conditions. For each year, 100 yeast isolates obtained from the beginning and end of the fermentations were identified. Grape and must handling conditions were slightly different for each of the three fermentations, such that the origins of the isolated yeasts could have been from the grapes, the press equipment, or the vat. The results for the first-year grapes showed that nearly all of the grape-associated strains, both from the beginning and end, belonged to a single group of *S. cerevisiae*. In contrast, the microbiota from grapes exposed to crushing equipment or to the vat environment was more heterogenous and contained relatively few *S. cerevisiae* isolates. Instead, these musts contained mostly *Saccharomyces bayanus* var. *uvraum.* Results for the second-year grapes showed that *S. bayanus* var. *uvraum* was the dominant organism from all three environments at all sampling times. Third-year results for grapes or grapes exposed to crushing equipment indicated that *S. cerevisiae* was dominant at the beginning and end of the fermentation, but grapes exposed to the vat environment contained *S. bayanus* var. *uvraum* as the dominant organism (as was the case for the year one results).

Collectively, this study on Alsatian wine shows that a homogeneous group of *S. cerevisiae* strains appears to dominate the grape surface, yet other non-*S. cerevisiae* yeasts still show up in the wine. The latter are associated with both the crushing equipment and the vat environment. Moreover, under the conditions established in this study, those yeasts present in the vat environment may actually be the dominant yeasts

in the wine fermentation, since they were present at the beginning and end of all three fermentations conducted over a three-year period.

Certainly, these reports also confirm previous reports that suggest wine crushing equipment and the wine environment serve as a major source of yeasts involved in the wine fermentation. Thus, it may well be that natural fermentations rely only partially on the wild yeasts that reside in the vineyard or on the surface of grapes. Rather, the major source of wild yeasts may be those that "contaminate" the wine-making equipment and that live in the confines of the winery environment.

This study points out several important features. First, it suggests that the indigenous yeasts present in wine reflect the yeast population within the vineyard, rather than the grape. Second, it would appear that only a few strains, rather than a diverse collection of strains, dominate the wines produced within a vineyard. Finally, these dominant yeasts were well-adapted to the particular local environmental conditions and shared similar physiological properties relevant to wine making. However, because the yeasts were isolated at a single point in time (at the end of the fermentation), it is possible that other strains may have been present at the beginning or at some earlier time during the fermentation.

Finally, two other interesting studies provide a reminder that it's not always about *Saccharomyces* (Brysch-Herzberg and Seidel, 2015). In a study of musts from grapes grown in Northern regions of Germany, yeasts from the genera *Hanseniaspora* and *Metschnikowia* were dominant. Indeed, *Saccharomyces* were non-detectable. These same yeasts were also found in musts from Italian grapes (Vigentini et al., 2015).

References

Börlin, M., P. Venet, O. Claisse, F. Salin, J.-L. Legras, and I. Masneuf-Pomarede. 2016. Cellar-associated *Saccharomyces cerevisiae* population structure revealed high-level diversity and perennial persistence at Sauternes wine estates. Appl. Environ. Microbiol. 82:2909–2918.

Brysch-Herzberg, M., and M. Seidel. 2015. Yeast diversity on grapes in two German wine growing regions. Int. J. Food Microbiol. 214:137–144.

Ciani, M., and F. Comitini. 2015. Yeast interactions in multi-starter wine fermentation. Curr. Opin. Food Sci. 1:1–6.

Comitini, F., A. Capece, M. Ciani, and P. Romano. 2017. New insights on the use of wine yeasts. Curr. Opin. Food Sci. 13:44–49.

Demuyter, C., M. Lollier, J.-L. Legras, and C. Le Juence. 2004. Predominance of *Saccharomyces uvarum* during spontaneous alcoholic fermentation, for three consecutive years, in an Alsatian winery. J. Appl. Microbiol. 97:1140–1148.

García-Ríos, E., A. Gutiérrez, Z. Salvadó, F.N. Arroyo-López, and J.M. Guillamon. 2014. The fitness advantage of commercial wine yeasts in relation to the nitrogen concentration, temperature, and ethanol content under microvinification conditions. Appl. Environ. Microbiol. 80:704–713.

Jolly, N.P., C. Varela, and I.S. Pretorius. 2014. Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. FEMS Yeast Res. 14:215–237.

Masneuf-Pomarede, I., M. Bely, P. Marullo, and W. Albertin. 2016. The genetics of non-conventional wine yeasts: current knowledge and future challenges. Front. Microbiol. 6:1563.

Vigentini, I., G. De Lorenzis, V. Fabrizio, F. Valdetara, M. Faccincani, C.A. Panont, C. Picozzi, S. Imazio, O. Failla, and R. Foschino. 2015. The vintage effect overcomes the terroir effect: a three year survey on the wine yeast biodiversity in Franciacorta and Oltrepo` Pavese, two northern Italian vine-growing areas. Microbiol. 161:362–373.

Mas, A., B. Padilla, B. Esteve-Zarzoso, G. Beltran, C. Reguant, and A. Bordons. 2016. Taking advantage of natural biodiversity for wine making: The WILDWINE Project. Agric. Agric. Sci. Procedia 8:4–9.

Table 11.3 Desirable properties of wine cultures.

- Able to produce high levels of ethanol
- Does not produce off-flavors
- Capable of producing unique flavors
- Ethanol tolerant
- Osmotolerant
- Ferment sugars to completeness
- Sediments or flocculates well (especially for Champagne yeasts)
- SO_2 and sulfite resistant
- Cold resistant
- Rapid fermentation rates
- Able to grow at temperatures below 10°C
- Killer toxin resistance
- Predictable and consistent and genetically stable

The main advantage of commercial cultures, however, is that they provide the wine manufacturer with important culture options. A variety of strains is available, and the choice of strain can be selected based on the needs of the customer (Table 11.3). For example, grapes with consistently high sugar concentrations may require an osmotolerant yeast culture. Yeast used to perform the secondary fermentation that occurs in Champagne manufacture must sediment well to facilitate their removal during the disgorgement step (described later in this chapter).

Yeasts are usually supplied in the form of active dry yeast, not unlike that used in the baking industry (Chapter 9). Culture suppliers ordinarily recommend inoculum levels sufficient to provide a starting cell density of about 10^6 cells per ml of must. An increase of only about two log-cycles occurs during the fermentation. The manner in which the yeast cultures are produced and preserved is important, however, because how the cells are handled during the culture production steps may influence how they grow later in the juice.

When culture companies produce yeast cultures, cells are grown under highly aerobic conditions to achieve the maximum amount of biomass. These high biomass fermentations also elicit a general stress response that enhances survival and viability during the subsequent drying steps. It appears that resistance to drying in wine yeast is promoted by synthesis of the disaccharide trehalose (similar to the cryoresistance mechanism by baker's yeast). Thus, following culture production, yeast cells are induced for aerobic metabolism and anabolic activities, whereas, when these cells are inoculated into the must, fermentative metabolism, with a minimum of biomass, is desired. Therefore, appropriate rehydration of yeasts and rapid adaptation to the new wine environment are also important.

Other pre-treatments

It is permissible to add other materials, besides sulfites, to the must to enhance extraction, modify the composition, or promote fermentation. For example, pectic enzymes can added as process aids during crushing to facilitate extraction of juice from skins and later during pressing to improve clarification. This practice is quite common for white wines manufactured in the United States. As noted above, it is not uncommon for some grapes, and juices from those grapes, to have either too low or too high of a pH. Thus, either acids, such as tartaric acid, or neutralizing salts, such as calcium carbonate, can be added to adjust must acidity. Finally, nutrients that enhance yeast growth and fermentation can be added. These yeast growth factors usually are added to white wine juices, since shorter extraction times

may result in lower nutrient concentrations. Nutrients added to juices include mainly ammonium salts and various vitamins.

Microbial ecology

In the absence of SO_2 addition, the autochthonous must microbiota is relied upon to initiate and then carry out a spontaneous fermentation. This is one of the best studied of all fermentations, and much is now known about the ecology of wine and the yeasts that participate in the wine fermentation. The yeast fermentation, however, is but one of two distinct fermentations that occur in wine making. Yeasts, of course, are indispensable, fermenting sugars to ethanol, CO_2, and small amounts of other end products. A second fermentation, called the malolactic fermentation, is carried out by specific lactic acid bacteria that are either naturally present or added for this purpose. Note that in a narrow sense, this fermentation is really a conversion of malate to lactate, but consistent with the broader definition noted earlier in this text, it is fair to consider it a fermentation. Of course, not all types or styles of wine undergo a malolactic fermentation. However, for those wines that do, it is regarded as nearly as important to wine quality as the ethanolic fermentation.

As noted above, the surface of grapes usually contain less than 10^4 yeast cells per grape (or per ml of juice). This number may increase during ripening on the vine, especially if the temperature is warm. Although ten or more yeast genera may be represented, the primary organism most frequently isolated from grape surfaces and fresh must is *Hanseniaspora uvarum* (= *Kloeckera apiculata*). In contrast, *S. cerevisiae,* the main yeast responsible for the wine fermentation, is infrequently observed initially on grapes. Rather, *S. cerevisiae* and other related strains are introduced into the must during grape handling and crushing steps directly from the equipment. The must is inoculated, in other words, by the yeasts originating from the grape surface as well as by those residing on the winery equipment. So important are the grape and environmental microbes to wine quality, that a microbial terroir, in concert with the viticultural terroir, has recently been described (Box 11.6).

Despite the large amount of available carbohydrates and other nutrients, the must is actually a selective environment. The pH is typically below that which many organisms can tolerate, and the organic acids present in the must have considerable antimicrobial activity. The high sugar concentration and high osmotic pressure can also inhibit many of the indigenous organisms. Eventually, the CO_2 formed during the early stages of fermentation makes the environment anaerobic, restricting growth of aerobic organisms. Likewise, enough ethanol is produced to provide selection against ethanol-sensitive organisms. Finally, sulfites, if added, have antimicrobial activity against a wide range of yeasts and bacteria. Thus, as the environment changes, species that are numerically dominant may be displaced by other species or strains better suited to the environment at any particular time. Microbial ecologists refer to such arrangements as successions. One reason why the outcome of a spontaneous wine fermentation is so difficult to predict is that successions rarely occur exactly the same way in different musts, since the wild microbiota is never entirely the same.

As noted above, members of the genus *Saccharomyces* represent less than 10% of the initial yeast population. Rather, the early yeast population is dominated by *H. uvarum*. This organism produces up to 6% ethanol from glucose and fructose. However, it has a low ethanol tolerance, and by the time the ethanol concentration reaches 3% to 4%, growth of *H. uvarum* is inhibited. Thus, even though *H. uvarum* is among the first to grow in the must, it is detectable only during the first several days. However, it is thought that this organism

Box 11.6 Microbial terroir

In hindsight, perhaps, it really should have been no big surprise. Microbial ecologists have long known that the milk, meat, grapes, and other raw materials used to make fermented foods and beverages were home to a large and diverse community of autochthonous (i.e., resident) microorganisms. That these organisms would contribute to the flavor, texture, and other quality attributes of the finished product would certainly be expected. Nonetheless, one of the most remarkable discoveries in understanding the microbial ecology of fermented foods, and wine in particular, was the realization that the microbiome itself is just as much a part of the terroir, as are the climate, soil, and agronomic practices. In other words, the microbes that inhabit the grapes, the press, and the barrels are associated with specific vineyards and contribute to the terroir of the wine produced at that winery. The corollary – that the use of commercial starter yeasts inoculated into grape juice reduces the microbial contribution to terroir, has also been suggested (Comitini et al., 2017).

Two papers from the lab of David Mills at the University of California, Davis illustrate the role of microbial terroir in wine-making. In the first study (Bokulich et al., 2014), more than 250 grape must samples were collected over two seasons from multiple regions in California. Both bacterial and fungal communities were sequenced. Several important findings emerged from the landmark study. First, the microbial communities that inhabit grape musts are not randomly distributed. Instead, they are shaped by region, grape variety, and climate. Thus, the Chardonnay microbiomes from Napa wineries are similar to each other, but different from those in Sonoma. The researchers also identified specific groups of organisms (including those that produce acetic or lactic acid) in the musts that could potentially portend to problems down the road.

The second study (Bokulich et al., 2016) was a longitudinal survey that involved more than 200 different wine fermentations from two California wineries. Nearly 700 samples were obtained at various steps during the fermentations. As before, both bacterial and fungal communities were sequenced. However, in this study, the chemical compositions (aka the metabolomes) of the finished wines were also determined. If these two data sets – the microbiomes and metabolomes were correlated, the implications would be very important to wine makers. The results showed that: (1) vineyard microbiomes are not unique, but rather there are microbiome patterns that are associated with particular regions; and (2) microbiomes do indeed correlate with the chemical composition of wines. However, the researchers were careful to note that correlation does not prove causation. Nonetheless, they suggested that if future studies were to show that the microbiome composition was causative for the flavor or other chemical properties of wine, then the microbiome could indeed be considered "a quantitative, definable feature of wine terroir".

References

Bokulich, N.A., J.H. Thorngate, P.M. Richardson, and D.A. Mills. 2014. Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. Proc. Natl. Acad. Sci. USA 111:E139–E148.

Bokulich, N.A., T.S. Collins, C. Masarweh, G. Allen, H. Heymann, S.E. Ebeler, and D.A. Mills. 2016. Associations among wine grape microbiome, metabolome, and fermentation behavior suggest microbial contribution to regional wine characteristics. mBio 7:1–11.
Comitini, F., A. Capece, M. Ciani, and P. Romano. 2017. New insights on the use of wine yeasts. Curr. Opin. Food Sci.13:44–49.

does produce small amounts of acids, esters, glycerol, and other potential flavor components, some of which may or may not be desirable. As the ethanol concentration increases and the Eh (or redox potential) is reduced, due to CO_2 formation, the environment begins to select for *S. cerevisiae* and other various ethanol-tolerant *Saccharomyces* spp.

Separation and pressing

After the maceration step, or in the case of most white wines, almost immediately after crushing, the juice is separated from the seeds, skins, and pulp (collectively referred to as the pomace). The juice that separates from the pomace simply by gravitational forces is called the "free run". Screens are typically used to catch any large particles. The free run juice is pumped into vats or barrels.

Since the free run juice contains less than 75% of the total juice volume and the rest is present within the pomace, the latter is usually pressed to recover the remaining juice. Several types of presses and configurations are used. Hydraulic or pneumatic wine presses squeeze the juice from the pomace. Screw- or auger-type devices force the juice against perforated cylinder walls and have the additional advantage of being continuous. The so-called first press juice can be collected and either added back to the free run juice or kept as a separate portion. The free run fraction is considered to have an appreciably higher quality and is used for premium wines. Juices containing mixtures of free run and pressed fractions are used for somewhat lower quality wines. Finally, for white wine, the juice is clarified to remove any remaining solids. Clarification is done via settling and decantation, filtration, or centrifugation.

FERMENTATION

The wine fermentation begins as soon as the grapes are crushed. However, when a starter culture is used and SO_2 is added to control the indigenous organisms, limited ethanol fermentation will occur prior to addition of the culture. In the case of white wine production, the culture is added to the must after pressing and clarification, whereas for red wine, culture addition is done prior to seed and skin removal. Thus, for red wines, fermentation occurs during maceration, just as it would for a spontaneous fermentation. The amount, concentration, and form of the culture depend on the type of wine being produced, the composition of the grapes, and other considerations specific to the wine manufacturer. Of course, the culture's main responsibility is to produce ethanol from sugars (see below), but the criteria for culture selection actually includes many other properties (Table 11.3). The culture is either prepared directly as a rehydrated dried culture or it can be propagated in an active liquid form. When added to the must, it should provide about 10^6 cells per ml.

Traditionally, fermentations were performed in open barrels or vats with a capacity of 500 L or less. Such barrels are still used today; however, enclosed stainless steel tanks are now far more common. The latter have several advantages. They are easy to clean and disinfect and often can be sterilized. Airborne microorganisms are less likely to contaminate the wine. Various control features, including temperature control and mixing and pumping activities (see below), are easily incorporated into the design, and are usually computerized. Finally, modern tanks can be quite large, with capacities of more than 250,000 L.

The temperature of incubation depends on the type of wine being produced. In general, white wines are fermented at lower temperatures than red wines. For example, many wineries control the temperature between 7°C and 20°C for white wine and between 20°C and 30°C for red wines. Some wineries prefer low incubation temperatures for all wines, because less ethanol is lost to evaporation and more of the volatile flavors are retained. In addition, low temperature incubations result in overall higher ethanol concentrations and less sugar remaining at the end of the fermentation (assuming time is not a factor). Although *S. cerevisiae* has a lower growth rate as the temperature decreases, the diversity of metabolic end products may actually increase, enhancing flavor development.

It is important to recognize that the wine fermentation is exothermic and a considerable amount of heat may be generated. Some of this heat is gradually lost or dissipated into the environment without ill effect. However, in large volume fermentations much of this heat is retained, raising the temperature of the wine. For example, if the initial temperature starts at 20°C, the temperature can increase 10°C or more. If the temperature were to rise above 30°C, the yeast may become inhibited or stop growing altogether. The wine will contain less ethanol and more residual sugar. Such fermentations are said to be "stuck". Although other factors may cause a wine fermentation to become stuck (see below), high temperature is a common reason. It is, therefore, essential that the appropriate temperature is maintained. For fermentations conducted in modern, stainless steel, jacketed vats, coolant solutions can easily be circulated externally. Alternatively, internal cooling coils can also achieve the same effect. The cooling requirement can also be met, especially for white wines, by simply locating fermentation barrels in cold rooms or cellars. However, the need for adequate cooling has led even some traditional wine manufacturers to abandon oak barrels and casks in favor of stainless steel vats.

The actual fermentation period is not long. After culture addition, the yeasts enter a short lag phase (from a few hours up to a day or two) that is then followed by a period of active log phase growth that lasts for three to five days. If the fermentation is conducted at lower temperatures (10°C to 15°C), the lag and log phases can be extended for several days. Conversely, if the yeast culture is highly active at the outset, by virtue of having been previously propagated under ideal growth conditions, the cells will almost immediately enter log phase. Although one might expect that a spontaneous fermentation would take longer, in fact, growth of the indigenous yeast begins so soon after crushing, such that the lag phase is barely noticeable.

During the log phase of growth, when an active fermentation is occurring, a layer of CO_2 forms across the surface. In red wine production, some of the pomace will float to the top and be trapped within this CO_2 layer, forming a dense blanket or cap. Since the pigments and tannins are present in this thick cap layer, a mixing step is required to return these substances back into the fermenting must. The temperature in the cap can also become elevated, supporting growth of undesirable thermophilic bacteria. Thus, mixing serves to maintain a more uniform temperature. Various techniques exist for this mixing step (called pigeage). In the "pumping over" technique, a portion of the must is periodically removed from the vat and

pumped onto the cap. Alternatively, the cap can be "punched down," either manually or via mechanical means. In modern wineries, automated pumping and punching systems are used to mix the pomace cap into the fermenting must.

The fermentation of white wine occurs after the must is pressed and clarified. After about the seventh or eighth day of fermentation, cell numbers may begin to decline, representing the end of the primary ethanolic fermentation. Some yeast strains will flocculate or clump together, causing them to settle to the bottom of the tank. This is a desirable property that enhances their removal later during the racking and clarification processes. The fermentation is considered complete when all or most of the sugars are depleted, as determined by a decrease in the Brix value. For red wines this may take as long as five to six weeks. As noted above, wines containing less than 0.5% sugar are dry, without a perception of sweetness. Of course, not all wines are intended to be dry. If sugars are present in the wine after fermentation had ended, a sweet wine results. Specialized techniques for the manufacture of sweet wines will be described later.

For red wines, much of the fermentation occurs in the presence of the pomace. When the extraction of pigments, tannins, flavor compounds, and other materials is considered sufficient, or when the desired ethanol concentration is reached, the free run juice is separated from the pomace and moved into another tank. The fermentation is then completed (if not already). In the meantime, the pomace is pressed and is either fermented separately from the free run or is mixed with the free run for the final fermentation. Since the pressed wine is rich in pigments and tannins, adding a portion back to the free run wine makes the final product richer in color and flavor.

YEAST METABOLISM

It should be evident by now that the main job of the yeasts during wine manufacture is to produce ethanol from the sugars present in the juice. However, if ethanol was the only product formed and if sugars were the only substrates metabolized by the yeast, then wine flavor and aroma would be sorely lacking. In fact, yeast growth and fermentation results in a myriad of metabolic end products that contribute, for better or worse, to the organoleptic properties of the finished wine.

The main wine yeast, *S. cerevisiae*, as well as other species found in natural fermentations, are facultative anaerobes. Thus, they have the full complement of enzymes necessary to oxidize sugars via the Krebs' or tricarboxylic acid (TCA) cycle, as well as enzymes of the Embden-Meyerhof-Parnas (EMP) glycolytic pathway. The metabolic route by which *S. cerevisiae* utilizes simple sugars depends on the environmental conditions, particularly the redox potential or Eh. Under ordinary circumstances, when *S. cerevisiae* is grown aerobically (i.e., at high Eh), metabolism is mostly by the TCA cycle. The main end product is CO_2, with a large increase in cell mass. As the Eh decreases, metabolism shifts to the glycolytic pathway, yielding equimolar concentrations of ethanol and CO_2, and a relatively modest increase in cell number. Both pathways generate ATP, but aerobic metabolism is more efficient (recall that the latter yields a net of 36 ATP molecules per glucose compared to only 2 molecules of ATP made during anaerobic glycolysis). Despite these options, the actual situation in wine, however, is different, for reasons explained below.

Regardless of the metabolic route (TCA or EMP pathways), glucose and fructose metabolism begins with transport into the cell via specific transport systems. For many years, two groups of hexose transporters (Hxt) were known to exist. For both, transport is mediated via

facilitated diffusion, driven simply by the concentration gradient. One group has low affinity (high Km) for hexose substrates, and the other group has low Km's and functions when the glucose concentration is high (i.e., at the beginning and during much of the fermentation). In the past decade, as yeast genomes were sequenced, many more putative Hxt transporters (18 as of 2017) – with varying affinities, have been identified.

Intracellular glucose and fructose feed directly into the glycolytic pathway. The accumulated glucose is phosphorylated by one of several kinases to form glucose-6-phosphate, which is then isomerized to fructose-6-phosphate. Fructose is phosphorylated to fructose-6-phosphate by a hexokinase. The fructose-6-phosphate is phosphorylated a second time to form fructose-1,6-bisphosphate (FDP), which is split by an aldolase to form triose-phosphates, and eventually pyruvate is formed. During the glycolytic pathway, a net of two molecules of ATP are synthesized per hexose by the substrate level phosphorylation reactions. Since the glyceraldehyde-3-phosphate dehydrogenase reaction generates NADH from NAD, there must be some means of replenishing the oxidized form of NAD. Otherwise, not enough NAD would be available for this reaction, and metabolism would bottleneck.

Recall that lactic acid bacteria, facing the same situation, use pyruvate itself as the electron acceptor, generating lactic acid via the enzyme lactate dehydrogenase. The route taken by *S. cerevisiae* involves two reactions. In the first, pyruvate is decarboxylated via pyruvate decarboxylase, in a reaction that requires the co-factor thiamine pyrophosphate. Carbon dioxide and acetaldehyde are formed. The acetaldehyde then serves as the electron acceptor, in a reaction catalyzed by NADH-dependent alcohol dehydrogenase, resulting in production of ethanol and NAD. No additional ATP is made by these two reactions, indicating their function is solely to replenish NAD

Factors affecting yeast metabolism

As noted above, *S. cerevisiae* has the genetic capacity to metabolize sugars via either the glycolytic or respiratory (i.e., TCA) pathways. Although oxygen availability affects expression of genes encoding enzymes of these two pathways and is an important determinant of the metabolic route, gene expression is also regulated by substrate availability. Although one might expect that in the presence of oxygen, metabolism would always be via the respiratory pathway, this is not the case. Specifically, if the glucose concentration is sufficiently high (as it is in grape must), metabolism will be fermentative. This is because transcription of catabolic genes, including genes coding for some of the TCA enzymes, is repressed by glucose, a phenomenon known as the Crabtree effect. Thus, in the wine fermentation, where sugar concentrations are high, metabolism of glucose yields mostly ethanol and CO_2, and oxidative metabolism of sugars is unlikely to occur. Only when the substrate concentration is low (less than 2 g/L), will O_2-mediated repression of glycolysis occur (the so-called Pasteur effect).

Since the wine fermentation is anaerobic, one might reasonably expect that the only end products formed from glucose and fructose would be ethanol and CO_2. If that were the case, then how could one explain the appearance of glycerol, succinic acid, acetaldehyde, acetic acid, and other products, that appear during the wine fermentation? In addition, some of the glucose carbon (albeit only about 1%) is used to form yeast biomass (i.e., cell constituents). Moreover, even after accounting for evaporative effects, the theoretical 50% yield (on a weight basis) of ethanol from glucose is never reached during the fermentation, due to byproduct formation. These byproducts can account for as much as 4% of the total products

formed. Synthesis of these byproducts occurs, in part, in response to demands on the cell to maintain Eh balance and to salvage ATP. For example, when the demand for NAD is high, a portion of the dihydroxyacetone phosphate formed from FDP via the aldolase reaction is reduced to glycerol-3-phosphate, and NAD is generated. The glycerol-3-phosphate is subsequently dephosphorylated to glycerol. The latter can be an important component of some wines, contributing sweetness and fullness.

Even under the anaerobic conditions prevailing during wine fermentation, small amounts of TCA products may still be formed to provide the cell with carbon skeletons (e.g., α-ketoglutarate) necessary for biosynthesis of amino acids. Another factor influencing sugar metabolism is SO_2, added to control wild yeasts. Sulfur dioxide can bind acetaldehyde, preventing its reduction to ethanol by alcohol dehydrogenase. As a result, NADH accumulates inside the cells and is diverted to other NAD-generating reactions, forming glycerol and other non-ethanol end products. Finally, end product formation is also influenced by the yeast strains naturally present or added to the must.

Sulfur and nitrogen metabolism

Although metabolism of carbohydrates is obviously critical to the outcome of the wine fermentation, metabolism of other must components is also important. How wine yeasts metabolize sulfur-containing compounds that are present in the must as normal grape constituents is particularly important. Most of the sulfur in grapes is in the form of elemental sulfur, sulfates, or as sulfur-containing amino acids. Since the range of sulfur-containing metabolic end products includes various sulfides, mercaptans, and other volatile compounds, sulfur metabolism can have a profound influence on wine quality. Yeasts can also produce sulfites, which, as already mentioned, have antimicrobial activity. In fact, even if sulfur dioxide or sulfite salts are not intentionally added, wine invariably contains sulfite due to its production by wine yeast.

Grapes usually contain sufficient ammonia, ammonium salts, and free amino acids to support good growth of most wine yeasts. In addition, wine yeasts can synthesize amino acids and purine and pyrimidine nucleotide from ammonia, and, therefore, have no essential requirement for amino nitrogen or pre-formed nucleotides. During the course of the fermentation, total nitrogen decreases. However, nitrogen-deficient grapes can result in an inadequate amount of nitrogen in the must, and, if not supplemented with ammonium salts, can lead to stuck and sluggish fermentations.

Stuck fermentations

Despite the apparent simplicity of the wine fermentation, as evidenced by so many successful outcomes, there are occasions when the fermentation fails. Such wines typically contain a significant amount of residual, unfermented sugar and an insufficient concentration of ethanol. The fermentation may actually still be occurring, just in a more sluggish fashion. Worse yet, the fermentation can become stuck and come to a complete standstill. Although sluggish or stuck fermentations occur infrequently, they represent a significant problem and source of economic loss for the wine producer, since wine quality will inevitably be poor. They are also especially susceptible to spoilage, since the low ethanol concentration and the availability of fermentable sugar may promote growth of undesirable bacteria and yeasts. Under more severe conditions, the wine may simply have to be discarded. Making the situation worse, the stuck vats cannot accommodate incoming grape must, presenting additional

opportunity costs to the winemaker. Therefore, despite their rare occurrence, it is important to understand the causes and to know how to prevent sluggish or stuck wine fermentations.

Among the possible causes of a stuck fermentation are those that are due to the must composition, the handling of the must and wine, or the presence of wild yeast or bacteria that inhibit desirable wine yeast. The must may contain, for example, an insufficient level of nitrogen or other nutrients necessary to support adequate yeast growth. The sugar concentration may be too high, resulting in osmotic pressures that inhibit the yeasts. Some yeasts also are inhibited by moderate ethanol concentration. As noted previously, the ethanol fermentation is exothermic, and if the temperature is not controlled or cooling is inadequate, the resulting high temperature (>30°C) may cause the fermentation to come to an abrupt halt. In contrast, too cool an incubation temperature (e.g., <10°C), as might occur during white wine production, can also result in a stuck fermentation. Most of these situations, however, are easily corrected, either by supplementing the juice with appropriate yeast nutrients, using osmotolerant or ethanol-tolerant yeast strains, or by proper temperature control.

Finally, some wild yeast strains secrete proteins called killer toxins that inhibit or kill other indigenous or starter culture yeasts. There are more than five types of killer toxins, but the most common are K1 and K2. These proteinaceous toxins first attach to cell wall receptors, then integrate and form pores within the cytoplasmic membranes of sensitive cells, thereby disrupting ion gradients and interfering with energy-transducing reactions. Producer strains resist the toxin they produce, but are sensitive to those toxins produced by other strains. Some yeast strains do not produce killer toxins, but are nonetheless resistant. Several yeast species among multiple genera include strains that are able to produce killer toxins. Importantly, killer yeasts are found throughout the wine environment, and even many of the *H. uvarum* and *S. cerevisiae* strains isolated from natural wine fermentations have the "killer" property. In fact, since yeast strains with killer toxin activity can potentially inactivate competing strains, this trait may be desirable for yeast starter cultures (Box 11.7). Certainly, starter culture strains that are immune to these toxins would not be affected by other killer strains and would not be the cause of a stuck fermentation.

Box 11.7 Killer yeasts in the winery

Wine spoilage is often mediated by growth of wild yeasts that contaminate wine while it is aging in wooden barrels. Species within the *Brettanomyces* genus (also known as *Dekkera,* the spore form) are most often involved in wine spoilage and can be very difficult to control. This is because they are naturally present not only on grapes and in musts, but also in the barrels in which the wine is aged (Smith and Divol, 2016).

Controlling spoilage organisms Growth of *Brettanomyces* may result in formation of ethylphenolic compounds, with 4-ethylphenol and 4-ethylguaiacol considered the most common (Tempere et al., 2016). These compounds are associated with the "Brett" defect in wine, which is described as "barnyard", "sweaty horse", or "wet dog" (Bisson, 2010). Needless to say, preventing these flavor and aroma defects and controlling this organism are critical in wineries. Common measures include attention to cleaning and sanitation and adequate sulfiting of the musts. It is more difficult, however, to manage *Brettanomyces* once the wine reaches the wooden barrels. This is because barrels provide good hiding places for *Brettanomyces*; they are also porous and cannot be effectively sterilized (Suárez et al., 2007).

Killer phenomenon One biological strategy to control *Brettanomyces* is based on the ability of some yeast strains to produce toxins that can kill or inhibit other yeast cells. Depending on the producer strain, these toxins vary with respect to the genetic basis and mode of action (Lei et al., 2015). In general, however, they are either encoded by double-stranded RNA or are chromosomal. Most kill target cells by creating pores in their cytoplasmic membrane, resulting in ion leakage, loss of respiratory activity, and cell death (Orentaite et al., 2016). Among the yeasts that are known to produce these so-called killer toxins are several genera associated with the wine fermentation, including *Saccharomyces*, *Kloeckera*, *Pichia*, and *Kluyveromyces*. Thus, such yeasts could be exploited as bio-control agents against *Brettanomyces* (Mehlomakulu et al., 2015).

Before describing how such an approach could work in wineries, it is important to note that the killer phenomenon cuts both ways, inhibiting spoilage as well as beneficial yeasts (Orentaite et al., 2016). That is, wild killer yeast strains are often present naturally in grape musts, thus, it is possible that they could inhibit desirable yeast strains necessary for fermentation (Maqueda et al., 2012). This could result in defective or even failed or stuck fermentations. Thus, it is essential that the killer sensitivity phenotype for wine cultures be known.

Killer strategies As noted above, strategies based on both *Saccharomyces* and non-*Saccharomyces* killer toxins have been developed to control *Brettanomyces*. In the study by Mehlomakulu et al. (2014), a collection of wine-associated yeasts were screened for killer toxin activity against *B. bruxellensis*. Two strains of *Candida pyralidae* were subsequently identified and shown to express kill toxins in wine environments. In addition, they did not inhibit *Saccharomyces cerevisiae* or malolactic bacteria used in wine fermentations. Similarly, a killer toxin (TdKT) from *Torulaspora delbrueckii* were shown to inhibit spoilage strains of *Brettanomyces* and *Pichia* in wine conditions (Villalba et al., 2016).

Another recent study assessed killer activity of *Saccharomyces* killer toxins against *Brettanomyces* as well as several other spoilage yeasts (de Ullivarri et al., 2014). As before, inhibition was observed under the low pH, high ethanol conditions that occur in wine-making. In addition, these researchers described how killer toxin delivery could be optimized by addition of nitrogen and sequential inoculation of the producer strains.

Because not all killer toxin-producing yeasts grow well in wine, and it is unlikely that killer toxin preparations could be added directly to wine, other means of applying killer toxin technology must be considered. It is conceivable, nonetheless, that the toxins could be used to treat the interior surfaces of wine barrels and thereby control the *Brettanomyces* that reside there.

References

Bisson, L. 2010. *Brettanomyces* infection in wine. In: Butzke, C.E., ed. Winemaking Problems Solved. CRC Press, Woodhead Publishing, Cambridge, pp. 190–191.

Liu, G.-L., Z. Chi, G.-Y. Wang, Z.-P. Wang, Y. Li, and Z-M. Chi. 2015. Yeast killer toxins, molecular mechanisms of their action and their applications. Crit. Rev. Biotechnol. 35:222–234.

Maqueda, M., E. Zamora, M.L. Álvarez, and M. Ramírez. 2012. Characterization, ecological distribution, and population dynamics of *Saccharomyces* sensu stricto killer yeasts in the spontaneous grape must fermentations of southwestern Spain. Appl. Environ. Microbiol. 78:735–743.

Mehlomakulu, N.N., M.E. Setati, and B. Divol. 2014. Characterization of novel killer toxins secreted by wine-related non-*Saccharomyces* yeasts and their action on *Brettanomyces* spp. Int. J. Food Microbiol. 188:83–91.

Mehlomakulu, N.N., M.E. Setati, and B. Divol. 2015. Non-*Saccharomyces* killer toxins: possible biocontrol agents against *Brettanomyces* in wine? S. Afr. J. Enol. Vitic. 36:94–104.

Orentaite, I., M.M. Poranen, H.M. Oksanen, R. Daugelavicius, and D.H. Bamford. 2016. K2 killer toxin-induced physiological changes in the yeast *Saccharomyces cerevisiae*. FEMS Yeast Res. 16.

Smith, B.D., and B. Divol, 2016. *Brettanomyces bruxellensis*, a survivalist prepared for the wine apocalypse and other beverages. Food Microbiol. 59:161–175.

Suárez, R., J.A. Suárez-Lepe, A. Morata, and F. Calderón. 2007. The production of ethylphenols in wine by yeasts of the genera *Brettanomyces* and *Dekkera*: a review. Food Chem. 102:10–21.

Tempere, S., M.H. Schaaper, E. Cuzange, R. de Lescar, G. de Revel, and G. Sicard. 2016. The olfactory masking effect of ethylphenols: characterization and elucidation of its origin. Food Qual. Prefer. 50:135–144.

de Ullivarri, M.F., L.M. Mendoza, and R.R. Raya. 2014. Killer activity of *Saccharomyces cerevisiae* strains: partial characterization and strategies to improve the biocontrol efficacy in winemaking. Antonie van Leeuwenhoek. 106:865–878.

Villalba, M.L., J.S. Sáez, S. del Monaco, C.A. Lopes, and M.P. Sangorrín. 2016. TdKT, a new killer toxin produced by *Torulaspora delbrueckii* effective against wine spoilage yeasts. Int. J. Food Microbiol. 217:94–100.

ADJUSTMENTS AND BLENDING

After the fermentation is complete, the wine will contain little or no sugar and about 12% to 14% ethanol. Still, because of differences in grape composition, microbiota, and wine manufacturing practices, variations in wine composition and sensory quality are to be expected. Therefore, adjusting the wine after fermentation (or in some cases, before) is a normal step. The pH and acidity, in particular, can vary markedly, as can the color and flavor. Therefore, some wineries adjust the acidity of wine by acidification or deacidification steps (e.g., by adding acids or neutralizing agents).

When acidity of wine or must is due to malic acid, deacidification is managed via the malolactic fermentation, which is discussed in detail later in this chapter. Adjustment to wine color and flavor can also be done, if legally permitted, by filtration and enzyme treatments, respectively. Filtration techniques, for example, are most often used to "decolorize" wine by removing undesirable pigments.

Except for very small wineries, which may have only a few vats of wine, most modern wineries have many individual vats of wine. Each one is unique, in that a particular vat may contain wine made from grapes harvested at a time or place different from the grapes in a neighboring vat. Wines within a single winery may be made from different grape varieties. Therefore, another common procedure, especially for premium wines, is to blend different wines to optimize or enhance the organoleptic properties. Blending also produces wines with consistent flavor, aroma, and color from year to year. Perhaps more so than any other winemaking step, however, blending is a particularly tricky business, and is a highly subjective process. Success relies on the imagination, creativity, and skill of the wine blending specialist.

CLARIFICATION

At the end of the fermentation, the wine contains non-soluble proteins and protein-tannin complexes, as well as living and dead microorganisms. These materials give the wine a cloudy, hazy, undesirable appearance. The clarification step is intended to remove these substances from the wine without losing desirable flavor and aroma components. It is particularly important that the cells are removed. If left in the wine, these cells can lyse, releasing enzymes that may catalyze formation of off-flavors and odors. Inducing precipitation of tartrate salts and tannin-protein complexes is also commonly done to facilitate their removal before they precipitate later during aging. It is important to recognize that, in some cases, cell lysis may be a good thing. Intracellular constituents released during cell lysis include amino acids and nucleotides, providing nutrients that are later used by bacteria in secondary fermentations or that contribute to the sensory properties.

Traditionally, wine was clarified by simply allowing the sediment, containing the yeasts and bacterial cells, as well as precipitated material, to settle naturally in barrels or vats. The wine could then be removed from the sediment (or "lees") by decantation. This process, called "racking", is usually done for the first time after three to six weeks following the end of the fermentation. Racking can be repeated several times over a period of weeks or months until the wine is nearly crystal clear. During the racking step, the wine is also aged. Racking can now be done in enclosed tanks using automated transferring systems.

Filtration is another method used to clarify wine. This can be especially effective if fining agents, such as bentonite, albumin, or gelatin, are used as filtration aids. If micro-pore filtration membranes are used, it is even possible to nearly sterilize wines. Clarification may occur after racking or after aging. This filtration process is similar to that described for beer (Chapter 10).

In contrast to removing the sediment shortly after the fermentation has ended, some wines are intentionally left in contact with the lees for an extended time before the first racking occurs. This traditional maturing practice, known as "sur lie," enhances the flavor, character, mouth feel, and complexity of the wine. It is more common for white wines than for reds.

AGING

Aging can be considered to begin just after fermentation. Thus, aging occurs when the wine is racked, as well as beyond. Aging conditions vary considerably. Some wines are aged for several years, whereas others are "aged", so to speak, for only a few weeks. Some wines are aged in expensive oak barrels, others in stainless steel, and yet others depend on bottle-aging, or a combination of all of the above. Whether a wine is aged for a long time in oak barrels or is quickly bottled and sent to market depends, in part, on marketing considerations, but also on the original composition of the grapes and how they are made into wine. Thus, long, careful aging should be reserved only for those wines that are actually intended for and improved by aging. By analogy, recall from Chapter 6 that Cheddar cheese intentionally manufactured for the process cheese market cannot be expected to develop into a flavorful, two-year Cheddar, no matter how carefully it may have been aged.

Of course, some excellent quality wines, like excellent cheeses, are meant to be consumed in a "fresh" or un-aged state, so whether or not a wine is aged does not distinguish wine quality, per se. One of the best examples of a non-aged, but well-appreciated wine is Beaujolais nouveau, a popular wine from the Burgundy area in France. Beaujolais nouveau is meant to be drunk within only a few weeks after the grapes are harvested and fermented

Table 11.4 Effects of aging on wine.

Reaction or step	Effects
Tannin precipitation	Color darkens; astringency increases initially, then decreases
Wood cooperage	Phenolic and other flavors extracted
Ester hydrolysis	Fruitiness decreases
Oxidation	Browning and flavor reactions induced
Evaporation	Concentrates non-volatile solutes; color and flavor intensifies, but aroma volatiles decrease

(released traditionally on the third Thursday of November). These fresh or "new" wines are fruity and "gulpable", and no amount of aging will lead to their improvement.

For wines that are aged, there is entire body of literature on the chemical and sensory analysis of wine. To say that the actual events that occur during aging are complicated would be quite the understatement. Hundreds of enzymatic, microbiological, and chemical reactions occur, and as many as 400 to 600 volatiles, including esters, aldehydes, higher alcohols, ketones, fatty acids, lactones, thiols, and other compounds are formed (Table 11.4). Moreover, the wine interacts with the wood and wood constituents in the barrel, oxygen in the air, and even the cork. It is important to recognize that not all of these reactions are beneficial in terms of wine quality, and some wines may actually deteriorate during aging. In fact, long aging is not good for most wines.

Ordinarily, in large wineries, fermentations occur in large tanks (exceeding 250,000 liters), and then the wine is moved into wooden barrels (about 200–220 liters) for aging. However wine can also be aged, at least initially, directly in tanks, and then later moved into oak barrels for final aging. In many European and other traditional wineries, in contrast, the entire aging period is conducted in oak barrels. The oak barrel or "cooperage" is so important to wine quality that entire industries devoted to oak tree production and cooperage construction have developed. This is because the oak barrels are not inert containers used simply to store wine, but rather they are a source of important flavor and aroma compounds. In fact, one of the major steps in barrel construction involves heating or "toasting" the barrels to promote pyrolysis. This generates a number of flavor and aroma volatiles. In the presence of wine, these compounds, along with tannins, phenolics, lignins, and lactones, are extracted from the wood and solubilized in the wine. Some of these compounds impart unique flavor notes, including vanilla and coconut. Aging wine in oak cooperage is not, however, without a downside. Oak barrels are expensive, and, even if carefully maintained, do not last forever. Loss of wine volume (and hence profit) due to evaporation can also occur. Thus, alternative materials, in particular, stainless steel, have displaced oak cooperage in many wineries. While it certainly does not contribute flavor and aroma compounds, stainless steel is less expensive, easier to clean and maintain, and can be fabricated to accommodate size and shape preferences. It is still possible for wine aged in stainless steel cooperage to obtain desirable oak-derived flavors by adding oak shavings or chips to the aging wine.

MALOLACTIC FERMENTATION

A certain amount of acidity is expected and desirable in wine. Red wines typically have a pH of 3.3 to 3.6; white wines are usually slightly more acidic. Some grapes, and the musts made from those grapes, however, may contain high levels of organic acids, such that the pH is too

low. Wines made from those grapes may suffer from excess acidity, a readily noticeable flavor defect.

Of the organic acids ordinarily present in grapes, malic acid is particularly important because of its ability to influence acidity and pH. This is because malic acid is a dicarboxylic acid, meaning it contains two carboxylic acid groups and can release or donate two protons. Thus, musts containing high concentrations (0.8% to 1.0%) of malic acid may be overly acidic and have a lower than desired pH. High malic acid concentrations are especially common in grapes grown in cooler, more northern climates. In the US, this would include several important wine regions, such as those in Oregon, Washington, northern California, and New York. Some grape cultivars ordinarily contain more malic acid than others. Although many of the vineyards in Europe are located in warmer regions and produce grapes with less malic acid (a situation that may lead to the opposite problem – too little acidity), grapes from Germany, Switzerland, and even some regions in France can still contain significantly high malic acid levels. Likewise, Australia, New Zealand, and other Southern Hemisphere wine regions also face the same challenge.

One way to reduce the malic acid levels and to "deacidify" the wine is to promote the biological decomposition of malic acid. This deacidification process occurs via the malolactic fermentation pathway that is performed by specific species and strains of lactic acid bacteria. These bacteria may be naturally present in wine and may, therefore, initiate and perform the fermentation on their own. It has now become common to add selected malolactic strains, in the form of a starter culture, directly to the must. The importance of the malolactic fermentation to wine quality is now well-appreciated. Although this fermentation is initiated in some white and rose wines (including sparkling wines), it is especially important and necessary for most red wines.

The malolactic fermentation has been the subject of extensive research. A single malolactic enzyme catalyzes the decarboxylation of malic acid directly to lactic acid (Figure 11.4). This enzyme requires NAD (and manganese), and no intermediate is formed. The net effect of the malolactic reaction is that malic acid, a dicarboxylic acid, is converted to lactic acid, a monocarboxylic acid, thereby reducing the acidity of the wine.

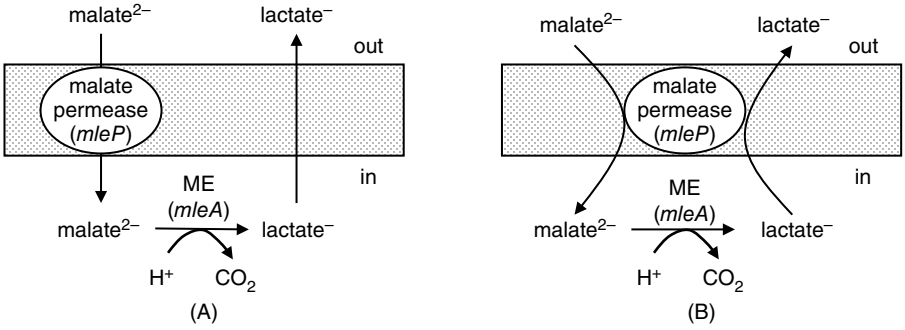


Figure 11.4 Malate transport in wine lactic acid bacteria. Transport of malate by *Oenococcus oeni* is mediated via a uniport malate permease (A). The accumulated malate is then decarboxylated directly to lactate by malolactate enzyme (ME); lactate efflux occurs via passive diffusion. The malolactic reaction also consumes a proton, such that the cytoplasm becomes more alkaline and more negatively charged. Thus, the electrochemical gradient across the membrane (the proton motive force) is increased, and ATP is conserved. In lactobacilli and lactococci, a similar process occurs (B), except that malate permease acts as an antiporter, such that lactate efflux drives malate uptake. Adapted from Konings, 2002.

In wine terms, the "harsh" flavor of malic acid flavor is converted to the "soft" flavor of lactic acid.

Several lactic acid bacteria have the enzymatic capacity to perform this fermentation. The most well-studied are species of *Oenococcus*, particularly *Oenococcus oeni* (formerly *Leuconostoc oenos*). Several species of *Lactobacillus* also have malolactic activity. Although some of these bacteria are found naturally in musts, commercial cultures are now available and are commonly used.

The malolactic fermentation has interested microbiologists, not only because of its industrial importance, but also because there seemed to be no obvious reason for bacteria to perform this conversion. In other words, of what benefit is the malolactic fermentation to the bacteria? Or more to the point, how do they gain energy from the conversion of malic acid to lactic acid? The pathway, after all, contains no substrate level phosphorylation step that would lead to ATP formation, nor is there a change in the redox potential. As it turns out, there is a means of generating ATP via the malolactic pathway, but it is indirect.

As shown in Figure 11.4, malic acid is transported into the cell via one of two ways. In *Oenococcus*, the malate permease is a uniporter, so-called because it is driven solely by the charge difference across the membrane (inside negative, outside positive). After the divalent malic acid is decarboxylated and reduced, the product, monovalent lactic acid, is effluxed. In lactococci and lactobacilli, uptake of malate is mediated by an antiporter that exchanges an incoming malic acid for an outgoing lactic acid. Importantly, no energy is spent for malate transport in either system. The exchange reaction, however, is not electroneutral. This is because one extracellular molecule of malic acid, carrying a net electric charge of –2, is exchanged for one of lactic acid that carries a net charge of only –1. This charge difference (i.e., the outside charge is now less negative) arises as a result of the intracellular decarboxylation reaction, which consumes an intracellular proton. The bottom line is that the cell is able to extrude a proton (or its equivalent) without having to spend any energy to do so. Thus, the cell conserves energy, in the form of ATP that it would ordinarily spend to pump protons from the cytoplasm to the extracellular medium. The proton gradient that forms, or the proton motive force, can then either perform other work (e.g., nutrient transport) for the cell or be used to drive ATP synthesis by the proton-translocating $F_0 F_1$ ATPase.

It is important to note that in low-acid grapes, the malolactic fermentation is undesirable, since some acidity is desired in wine. Thus, under some circumstances, the presence of naturally occurring malolactic bacteria is unwanted and the source of potential defects. In contrast, the malolactic fermentation not only is performed for deacidification, but also to promote flavor stability and balance. Moreover, malolactic bacteria often produce diacetyl from citrate, which may, at the appropriate concentration (generally between 1 mg/L and 4 mg/L), be desirable in some wines.

TYPES OF WINE

Aside from the rather broad distinction of classifying wines based on their color – red, white, or rose, there are many more descriptive means for grouping different types of wine. Thus, while the procedures used for manufacture of wine have been described in a mostly generic manner, it should be clear to the reader that there are many ways the wine maker can manipulate grape handling, fermentation, aging, and other operations to yield a wide variety of wines. It is not the intent of this section to describe styles of wine based on these treatments or even geography or cultivar (there are many excellent resources that do this).

Rather, the focus will be on the microbiological and technological principles involved in the manufacture of several well-known categories, including sweet wines, fortified wines, sparkling wines, and distilled wines.

Sweet wines

Sweet wines are simply those that contain unfermented sugar (either fructose, glucose, or sucrose). There are several ways to produce a sweet wine. The easiest (and least expensive) technique is to simply add sugar (2% to 4%) or sucrose syrup to a dry wine. This results in a wine that is definitely sweet. A similar and more common approach is to add unfermented juice, preferably from the same grapes used to make the wine. Alternatively, sweet wines can be made by stopping the fermentation before all of the glucose and fructose have been fermented. In some cases, sugar may be added to the juice prior to fermentation, such that when the fermentation is complete, residual sugar (and sweetness) remains. This is one of the more common practices in the United States; many of the sweet wines from New York State are produced this way.

Regardless of how the sugar is introduced into the wine, arresting the fermentation is the key step. Generally, this is done by rapidly cooling the wine, and then filtering out the yeast. Another method is based on encapsulating the yeasts within alginate beads, which are placed inside permeable bags. At the desired time, the bag (containing the yeast) is simply removed. Culture activity can also be stopped by adding alcohol spirits, as is the case for fortified wines (see below).

There are also several traditional approaches for making sweet wine. These are based on concentrating the sugar in the juice or grapes. The juice, for example, can be partially concentrated by heating. In contrast, the grapes can be dried via atmospheric drying, or frozen so that the ice can be removed. However, the most well-known traditional technique for concentrating sugars in grapes is to dehydrate the grapes while they are still on the vine. These so-called botrytized wines are made throughout Europe, with the Sauternes, white wines from the Loire Valley of France, being the most widely prized. There are also excellent versions made in the US and other regions.

The traditional process for making sweet botrytized wines starts in the vineyard. It is well-known to viticulturist that grapes left on the vine past their optimum harvesting will invariably be infected by fungi, including the ubiquitous fungal organism *Botrytis cinerea.* Depending on climatic conditions, there can be two outcomes to this infection. If the climate is sunny and moderately dry during the day but humid during the evening, *B. cinerea* will grow to just the right extent on the surface of the grape, resulting in what is referred to as "noble" rot (Figure 11.5). If conditions are not "just right," overgrowth of *B. cinerea* or infection by other fungi can occur, resulting in simply rotten grapes that have no value. Thus, the manufacture of these wines carries considerable risk.

Ultimately, noble rot growth of *B. cinerea* results in dehydration of the grapes and concentration of the sugars. Dehydration occurs due to secretion of pectinolytic enzymes by *B. cinerea* that degrade the pectin-containing cell walls of the grapes. Water evaporation then occurs, provided the atmosphere is sufficiently dry. The decrease in moisture is important not only because it concentrates the solids, but also because it controls growth of undesirable fungi. Eventually, the mold-covered grapes begin to take on the appearance of raisins (moldy ones at that). The moisture level of the grapes will be reduced by about 50% and, even though the mold metabolizes some of the sugars in the grapes, the sugar concentration in the juice is still increased by 20% to 40%. After the grapes are harvested and gently crushed,

Figure 11.5 Grape clusters infected with *Botrytis cinerea*, the so-called noble rot. Photo courtesy of David Mills, University of California, Davis.

portions of the free run and the pressed juice are combined. The fermentation is then generally the same as far other white wines, with a temperature range of 18°C to 20°C. At the end of the fermentation, the ethanol concentration will range from 9% to 13%, and the wine will contain as much as 10% total unfermented sugar. Because of the high sugar concentration at the outset, yeasts that can tolerate high osmotic pressure must be used.

Fortified wines

Fortified wines are those to which distilled spirits (containing as much as 95% ethanol) are added to the base wine. Thus, these wines contain higher concentrations of ethanol (more than 15%). The source of the ethanol is also important since they may contribute unique flavor compounds to the finished product. Aside from this common feature, however, a wide variety of quite different fortified wines are produced. Included are whites and reds, dry and sweet. Fortification usually occurs during or just after the fermentation. In some wines, the added ethanol may inhibit the yeast and prevent the complete fermentation of sugars, resulting in sweet dessert wines. The most well-known of the fortified wines are sherry and port.

Sherry originated in Spain around 200 years ago, and is now produced in the United States and throughout the world. One of the main features used in the traditional manufacture of Spanish sherry (but rarely outside of Spain) is a blending technique known as the solera system. According to this technique, wines of different ages are progressively and uniformly blended to achieve wines of consistent quality. Another unique feature of most types of sherry is the presence of film yeasts that grow on the surface of the wine while it is aged in special casks called "butts". The *S. cerevisiae* strains that comprise this film, called a flor, were previously thought to be the same yeasts involved in the primary alcoholic fermentation. More recently, it has been suggested that a separate group of yeasts actually comprise the flor yeast population. In either case, the secondary yeast growth

occurs under aerobic conditions, resulting in oxidation of some of the ethanol and subsequent formation of unique flavor and aroma components (e.g., esters, aldehydes, higher alcohols, and lactones).

In contrast to the traditional (and costly) solera system for manufacture of sherry, other techniques are now widely used in the United States and other countries. In the submerged flor procedure, the wine is aerated and mixed in fermentors to enhance growth of the flor-type yeasts. Baked sherries are made by heating fortified but non-aged wine to 50°C to 60°C for ten to 20 weeks. A number of flavor and aroma compounds, including acetaldehyde and furfural, are generated by baking due to caramelization and non-enzymatic browning reactions.

Port, first produced in Portugal, is another fortified wine. It is made by adding distilled wine (i.e., brandy) to the red base wine, thereby raising the ethanol concentration to nearly 20%. Since fortification occurs well before all of the sugars have been fermented, and this much ethanol inhibits yeast, this step effectively ends the fermentation. Thus, port contains as much as 10% sugars. Port and other fortified sweet wines made in this manner (e.g., Madeira and Marsala) are often referred to as dessert wines. Because fortification not only arrests the fermentation, it also reduces the time available for extraction of anthocyanin pigments and phenolic flavor compounds. Mixing pressed wine (pomace) with the free run wine, therefore, is necessary to provide adequate color and flavor in the finished wine. In the United States, heat (e.g., steam) is sometimes used to extract pigment from the grapes. Finally, as for sherry, blending and aging are also important steps for port production. Some ports are aged for as many as 50 years.

Sparkling wines

Sparkling wines are those which contain carbon dioxide, providing bubbles and effervescence. For some sparkling wines, CO_2 pressures as high as 600 kPa atmospheres can be reached (by comparison, the pressure inside a can of soda pop is less than 200 kPa). Although sparkling wines are made throughout the world, there are several manufacturing methods that are used to produce the CO_2, and these methods define, to a certain extent, the type of sparkling wine being produced. How the bubbles are formed has a major influence on the sensory properties of the wine; accordingly, the science of bubble formation is a serious matter (Box 11.8).

Clearly, the most well-known sparkling wine is Champagne, which is traditionally made, not surprisingly, via a method that originated with Champagne. For other sparkling wines, CO_2 is introduced by other methods, as described below. It is worth mentioning that Champagne, like Parmesan and Roquefort cheeses, enjoys a protected status in the European Union. Only wine produced in the Champagne region of France – about 140 km east of Paris, and made according to the centuries-old process and aged in the caves in that region can be called Champagne. All other carbonated wines, regardless of how CO_2 is introduced, must be referred to as sparkling wine. In contrast, wine makers in the United States, which are under no obligation to follow EU rules, can label any sparkling wine as a champagne. However, since the early 2000s, most have agreed to honor this labeling requirement.

The manufacture of sparkling wines starts out no differently than other wines. The main difference is that at some point carbon dioxide is introduced into the "still" (i.e., not carbonated) wine. There are several possible ways to perform this step. The first is to simply pump CO_2 directly into the wine, followed by bottling. This is not unlike soda pop production and is definitely the most economical means. At around $5 a bottle, this "champagne," bears little resemblance to the real thing.

Box 11.8 Champagne, Henry's Law, and the physics of bubbles

Evidently, it's not quite fair that microbiologists and flavor chemists should have all the fun studying Champagne and other sparkling wines. Given that it's the bubbles that distinguish these wines from others, it was inevitable that the bubble phenomenon would eventually attract the attention of physical chemists. Indeed, their formation, size, phase, and flow are all relevant to the Champagne-consumption experience. This is because bubbles or effervescence serve several important sensory functions, providing mouthfeel, enhancing aroma perception, and contributing to visual stimulation (Liger-Belair, 2014).

Bubble origins Bubbles form in the bottle from the CO_2 produced via the secondary fermentation. However, it is not quite so simple. Indeed, the CO_2 is dissolved in the wine in accordance with Henry's well-known gas law of 1803. Specifically, this law states that the concentration of a given gas (at a given temperature) that dissolves in a given type and volume of liquid is directly proportional to the partial pressure of that gas. In other words, how much CO_2 is dissolved in the wine depends on temperature and pressure.

Ordinarily, a bottle of wine Champagne contain 9 g of dissolved CO_2, with equates to an astounding volume of 5 L of gas (Perret et al., 2014). Importantly, in an unopened bottle, the wine is supersaturated with CO_2 and is indeed bubble-worthy, but does not yet exist in bubble form. This is because there is a thermodynamic energy barrier that must be overcome. Thus, it's not until the cork is removed that the dissolved CO_2 becomes organized as bubbles. This first step requires nucleation sites, small gas pockets or particles of dust or lint that entrap CO_2. When these nucleation sites attract enough CO_2 – voila, bubbles are formed. Pouring the wine from bottle to glass enhances bubble formation.

Counting bubbles This is not a trivial task, for several reasons. As noted above, bubbles form once the wine is opened and poured. However, some of the CO_2 escapes (pre-bubble) and is lost to the atmosphere. Moreover, bubble size is not constant, and changes over time and as the bubble ascend directionally. Nonetheless, a theoretical model that accounts for these factors, as well as glass shape and volume parameters, was recently proposed (Liger-Belair, 2014). Accordingly, a glass flute filled with 100 ml of Champagne, poured straight down the center, would be expected to contain about 10^6 bubbles. Disturbing that wine (i.e., by drinking), however, would reduce that number. On the other hand, pouring the wine more gently along the side of the glass would reduce CO_2 loss, retain bubbles, and tend to increase new bubble formation.

As noted above, the bubbles not only provide visual appeal and desirable mouthfeel, but upon bursting they release an aerosol jet stream of flavor and aroma compounds that touch the receptors in the nose and mouth (Ghabache et al., 2016; Liger-Belair, G. 2015). Together, this accounts for the "deliciousness" of Champagne.

References

Ghabache, E., G. Liger-Belair, A. Antkowiak, and T. Séon. 2016. Evaporation of droplets in a Champagne wine aerosol. Sci. Rep. 6:25148.

Liger-Belair, G. 2014. How many bubbles in your glass of bubbly. J. Phys. Chem. 118:3156–3163.
Liger-Belair, G. 2015. The Science of Bubbly. Scientific American. June. 24:16–21.
Perret, A., D.A. Bonhommeau, G. Liger-Belair, T. Cours, and A. Alijah. 2014. CO_2 diffusion in Champagne wines: a molecular dynamics study. J. Phys. Chem. 118:1839-1847.

All of the other processes involve a second ethanolic-CO_2 fermentation performed by yeasts. However, in contrast to the primary fermentation, which is done in ambient atmosphere (i.e., open), the second fermentation occurs in an enclosed environment such that the CO_2 is trapped and becomes supersaturated in the wine. One seemingly simple way to initiate such a secondary fermentation is to stop the primary fermentation before all of the sugar has been fermented, and then to bottle the wine and allow the remaining sugar to be fermented by the residual yeast. The difficulty with this method is that stopping and starting the fermentation is not always so easy. Still, this method has long been practiced in France, Germany, and Italy.

Rather than rely on the endogenous sugar and yeast from the primary fermentation, an alternative and more common means of initiating the second fermentation involves the direct addition of an additional source of sugar, as well as an inoculum of yeast, to the still wine. The yeast need only ferment a modest amount of substrate (usually sucrose) to generate enough CO_2 to make the wine bubbly. The secondary fermentation can be performed either in enclosed vats or directly in bottles. For either method, however, there remains the problem of the residual yeast, which, if not removed, will make the wine cloudy or turbid. Sparkling wines, especially champagnes, are prized for their absolute clarity and perfectly clear appearance. Thus, removing the yeast following the second fermentation is absolutely necessary. Three widely-used techniques have been devised to perform this step: the bulk method, the transfer method and the "Methode Champenoise" (or Champagne method).

In the bulk or Charmat method, the secondary fermentation occurs in a pressurized vat, then the wine is filtered to remove residual yeasts and filled into bottles under pressure. In the transfer process the wine is placed into thick-walled bottles, sugar and yeasts are added, and the bottle is corked. After the second fermentation in the bottle is complete, the wine is transferred into tanks, filtered to remove yeast and then re-bottled. In the Champagne method, the process is similar to the transfer system, except that no transfer occurs. Rather, the second fermentation occurs in the bottle, where the wine remains.

When the Champagne method is used, the yeasts that performed the secondary fermentation are still in the bottle. How then, if the wine does not leave the bottle, can it be clarified and made free of these residual yeasts? The answer to this question and other unique features of Champagne manufacture are described below.

Champagne

Champagne is made from Chardonnay, Pinot Noir, and Pinot Meunier grapes grown in the Champagne district. This is a northern grape-growing region and the still wines made from individual cultivars (the Pinots make red wines and the Chardonnay is used for white) are not particularly remarkable (some might call them insipid). However, when the base wines are appropriately blended (a skill first perfected by the monk Dom Perignon centuries ago), the wine assumes the best qualities of each individual cultivar. The manufacture starts, as for

other white wines, with a rather fast pressing of the grapes, such that pigment extraction is minimized. The first press juice, called the cuvée, is then inoculated with selected yeasts (these are almost always proprietary strains), and the juice is fermented at about 18°C in oak barrels (stainless steel vats are also used). Because the base wines can be rather acidic (pH as low as 3.0), a malolactic fermentation may be desirable and the appropriate cultures can be added (or growth of autochthonous malolactic strains encouraged). However, promotion of the malolactic fermentation in Champagne is not a universally accepted practice, because some bacteria-generated flavor products may affect the delicate flavor and aroma balance of the finished wine.

After fermentation to about 12% ethanol, the critical blending step is performed (using high quality wines, free of defects). Base wines from a single year may be blended to create vintage Champagne, whereas wines from different years are more often used to create non-vintage Champagne. Next, the blended wine is bottled, and the "tirage" or sugar solution (about 20 g to 25 g of sucrose per liter) and a yeast mixture are added. The yeast inoculum for the secondary fermentation usually contains strains of *S. cerevisiae* that are selected based on their ability to grow at high ethanol concentrations, tolerate low pH and low temperature, and to flocculate well. A cork closure is then inserted as a stopper. Some wineries have recently switched to simple caps. The bottles are then held on their side at about 10°C to 12°C. The bottles are also constructed differently from ordinary wine bottles (thicker, with a tapered neck), since they must be able to withstand the high CO_2 pressure.

When the secondary fermentation is complete (after about seven weeks) the wine may be allowed to age in the bottles for up to three years (or even longer). Aging or maturation occurs while the lees is still present in the wine, a critical step that distinguishes Champagne from other sparkling wines fermented by the bulk method. Next, the time to remove the yeast cells (most of which are now dead) and sediment material has arrived. The ensuing series of steps are also unique to the Champagne method.

This first step, called riddling (or remuage), involves placing bottles in special angled racks and then gradually shifting the position of the bottle, starting from near horizontal and ending up near vertical (neck down). During this daily or every-other-day jolting, turning, and twisting process, the sediment gradually slides down the bottle. After one to three months, the yeast will have collected within the neck of the bottle, specifically settling on the inside of the cork or cap. Although the riddling process is still done manually in several of Champagne houses, mechanical riddling is now common.

Once the sediment has settled on the cork, it is removed by a process called disgorgement. First, the now vertical bottles (neck down) are cooled to about 7°C, then conveyed through a freezing solution (at –20°C) such that the material in the neck, not more than about 2 cm above the cork, is quickly frozen. The bottle is inverted (neck up), and the frozen plug, containing the cork, sediment, a small amount of slushy wine, is removed. Next, about 10 to 50 ml of a sugar-wine solution (called the dosage) is then quickly added to replace the wine lost in the disgorgement process. The amount of sugar in the dosage, from less than 1.5% to more than 5%, determines whether the champagne is dry (brut), medium-dry (sec) or sweet (doux). One recent development is to force the dosage with some pressure to cause foaming. The foam occupies the head space and precludes entry of oxygen, which can otherwise be detrimental to the wine. Finally, a fresh cork is applied and fastened with a wire crown. The bottle is gently shaken to mix the wine and dosage, and the bottles are then stacked horizontally for up to three months. Champagne does not improve much beyond this aging period, and is essentially ready for consumption.

WINE SPOILAGE AND DEFECTS

Wine making is full of risks. It can reasonably be assumed that as many things that can go right during wine manufacture, just as many can go wrong. The risk is exacerbated by the considerable investment that must be made to produce the wine (hence the old joke: How does one make a million dollars in the wine business? Start with $2 million). Consider the challenge: first, grapes must be cultivated over several seasons before a reasonable crop can be harvested (a period that typically takes five years). Disease, climate, insects, and other factors can cause serious problems even before the first grape has been harvested and crushed. Once wine-making begins, growth of desirable yeast and bacteria and inhibition of others is not always easy to manage. Finally, while aging of wine may certainly result in a product that is truly spectacular, another outcome is that the wine is totally undrinkable.

Spoilage of wine, like other fermented (and even non-fermented) foods, is often due to chemical and physical activities. Oxidation reactions, whether induced by oxygen, sunlight, or metals, can be especially damaging to wine, leading not only to development of off-flavors and aromas, but also to discoloration and pigmentation defects. High temperatures are also detrimental to wine quality, catalyzing chemical and biochemical reactions that result in caramelization, browning, and oxidation reactions. Physical phenomena, such as precipitation of proteins and tannins, are responsible for cloudiness and haze formation. A particular type of haze, called casse, occurs in white wines and is caused by precipitation of metallic salts.

Despite these various chemical-physical defects, however, wine spoilage is most commonly caused by microorganisms. In fact, fungi, yeasts, and bacteria can all be responsible for spoilage at some point during the wine manufacturing process.

Spoilage by molds

Fungal growth and spoilage rarely occurs during the wine fermentation, since most fungi are aerobic and sensitive to ethanol. Rather, mold is most important before and after the wine is made. Fungal growth on grapes is one of the most serious problems encountered in grape viticulture, causing considerable loss of crop. If not controlled, rots, mildews, and other fungal diseases can destroy an entire vineyard. As noted earlier for sweet botryized wines, a fine line separates spoiled, rotten grapes from desirable, noble rot growth of *B. cinerea.* Some fungi, such as *Penicillium*, *Aspergillus*, *Mucor*, and *Rhizopus*, can grow on freshly harvested grapes during transport to the winery. Pesticides, sulfiting agents, and other antimycotics can be applied to help control this problem, but care must be exercised to minimize their impact on the wine and during fermentation.

Post-fermentation problems with mold are usually due to contaminated cork, leading to a defect known as cork taint. According to many manufacturers and retailers, this is one of the most serious defects in bottled wine and one which has attracted wide attention among cork producers, wineries, and consumers (Box 11.9). The defect is now thought to occur as a result of growth of various fungi (including *Penicillium, Aspergillus,* and *Trichoderma*). Visible mold growth is rarely evident (those corks would not be used), so cork taints occur earlier in the cork making process, when exposure to fungi is common. These fungi then produce musty- or mushroom-smelling compounds that diffuse into the wine after the corks are inserted into the bottles. Wine corks can now be treated to remove the offending taint. However, there has been a strong trend toward the use of plastic and other alternative wine enclosures, the use of which may significantly reduce the incidence of cork-related defects.

Box 11.9 Corks, composites, caps, and other closures used for wine

The manufacture of high quality wine begins in the vineyard, with the care of the vines and grapes and their harvesting at just the right time. How the grapes are subsequently crushed and pressed also have major impacts. Of course, fermentation and aging profoundly influence wine quality. Collectively, it would be a considerable understatement to say that by the time the wine is bottled, a lot of time and hard work has occurred.

Cork taint consequences Imagine performing all these steps with meticulous attention, only to discover later upon removal of the cork, that the wine has developed an objectionable musty, moldy, damp cardboard-like aroma that renders it undrinkable. As unfortunate as this scenario might be, it is by no means rare. In fact, cork taint, the most likely cause of this particular defect, is considered to be one the most common causes of wine spoilage, affecting millions of bottles. Although the incidence of cork-taint has reportedly decreased in the past decade, it remains a major concern among wine-manufacturers. Indeed, according to a 2015 Wine Spectator magazine survey, more than 2% of wines from the US (California, Oregon, and Washington), Australia, and New Zealand were cork-tainted.

How often consumers actually return or even reject cork-tainted wine is not known, but it seems likely they will remember their experience with that particular label or vineyard when they purchase their next bottle of wine. Not surprisingly, given the frequency with which this problem occurs, cork taint has become one of the most important (and most controversial) issues facing the wine industry. Annual economic losses of more than $10 billion have been estimated (Corsi et al., 2016). Therefore, understanding what causes cork taint and developing strategies to prevent it are the focus of considerable academic and industry-sponsored research.

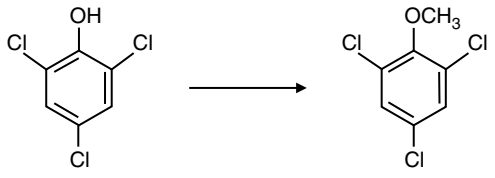
Cork Technology and Microbiology Corks have been used as closures for wine bottles at least since the 1600s. Not only are cork stoppers effective at preventing leaking, they also permit a very small amount of gas exchange that is believed to promote flavor and aroma development in aged wine. Cork is obtained from the bark of the *Quercus suber* oak tree, which grows in regions around the western Mediterranean Sea. Portugal is the main producer of cork (and has the biggest stake in the cork taint problem).

The manufacture of cork follows a traditional process that starts with its harvest (Pereira, 2007). The first, or virgin bark, stripped from the tree when it is about 20 to 30 years old, is not used; neither is the second growth obtained after another seven to ten years. Bark is not cork-worthy for another nine years, when it is called reproduction bark (subsequent strippings can occur every nine years). The harvested cork slabs are then boiled, cut into strips, and the cylindric cork units are punched out and washed in water. Next, the corks can be bleached using hydrogen peroxide, rather than hypochlorite solutions, as had been previously done (Pereira, 2007). The latter compounds could inactivate microorganisms, but also caused more problems than they prevented (see below). The corks are then dried, sorted, and packaged. The name of the winery or vintage can also be etched onto the corks.

Among the microorganisms associated with cork, fungi, and *Penicillium* and *Chrysonilia* species, in particular, appear to be the most common (Pereira et al., 2006). Other fungi include *Trichoderma*, *Aspergillus*, *Mucor*, and *Monillia*. Yeast may also be present. The boiling and bleaching steps effectively reduce the microbial load. However, fungi growth is possible prior to these steps or after, if re-contamination occurs. In fact, mold growth on cork has long been thought to be necessary for cork maturation and is, therefore, encouraged as part of traditional cork processing (Pereira et al, 2007). Still, fungi have long been suspected of being involved in wine taint formation, although direct evidence establishing this link has only recently emerged (Simpson and Sefton, 2007).

Cork taint chemistry The actual chemical agent responsible for cork taint has been identified as 2,4,6-trichloroanisole or TCA (Figure 11.9.1; Peña-Neira et al., 2000). Not only does TCA have a musty, disagreeable odor, but its threshold for detection is extremely low. Sensitive tasters can detect TCA at levels as low as a few parts per trillion, although for most individuals, higher concentrations must be present before TCA is noticed (Cravero et al., 2015, Tempere et al., 2016). Although other chloroanisoles, as well as other phenol- and pyrazine-containing compounds, may also contribute to cork taint (Cravero et al., 2015; Peña-Neira et al., 2000; Simpson et al., 2004), TCA is invariably present in tainted wine (Silva et al., 2011).

Biochemical evidence suggests that TCA is synthesized from chlorophenol precursors by a methylation reaction (Alvarez-Rodriguez et al., 2002). Chlorophenols are highly toxic to fungi, and this methylation reaction has been thought to be involved in detoxification (TCA, although odorous, is non-toxic). It has been reported that cork-associated fungi can perform this reaction and produce TCA in cork, provided 2,4,6-trichlorophenol (TCP) is present as a substrate (Alvarez-Rodriguez et al., 2002). In this particular study, a strain of *Trichoderma longibrachiatum* produced the highest level of TCA (nearly 400 ng/g of cork), and several other fungi produced more than 100 ng/g of TCA. It is important to emphasize that TCA synthesis required TCP as a substrate and that no TCA was formed in these experiments when other chlorinated phenols were present. Thus, although fungi are considered to be responsible for TCA and cork taint formation, the reaction depends on the presence of TCP. The latter were once used as agricultural herbicides and fungicides and were likely applied in cork-producing areas. It has been suggested that chlorine treatment of harvested cork may further increase the trichlorophenol content, and subsequently, the TCA level.



2,4,6-trichlorophenol (TCP) 2,4,6-trichloroanisole (TCA)

Figure 11.9.1 Conversion of 2,4,6-trichlorophenol (TCP) to 2,4,6-trichloroanisole (TCA).

Despite these recent findings, there is still considerable debate regarding the true incidence of cork-taint in wine, as well as the source of the tainted aroma. Cork producers and some cork researchers have argued that cork taint occurs far less often than the reported frequencies (Pereira, 2007). They also claim that TCA can arise from other sources, such as barrels, pallets and cellar environments. In addition, other non-cork taints continue to be referred to as cork taint. Poor storage and handling of corks can indeed result in other off-flavors; however, these defects are controllable. When correctly processed, cork stoppers, their proponents contend, are still the best way to seal bottles and preserve the quality of wine (Pereira et al., 2007).

Remedies and alternatives Regardless of the true frequency of cork taint, whether 1% or 10% or somewhere in between, this can hardly be considered an acceptable defect rate. No industry could long tolerate a situation where even one unit out of 100 fails to meet quality standards. The wine industry is no different and that is why non-cork alternatives have become so popular in the past twenty years. Efforts by the cork industry to improve cork and prevent cork taint are also under way. For example, supercritical CO_2 treatment and other physical methods can reportedly extract or remove TCA from cork to undetectable levels (by sensory analysis). Importantly, analytical methods have also been developed to screen corks and cork materials for TCA so that tainted corks are not used. Indeed, certification, screening, and other testing programs now make it possible for cork manufacturers and wine makers to have adopted a zero-tolerance policy.

Despite the reduced incidence of cork-tainted wine, a wide variety of cork alternatives have been developed and are widely used. These include cork agglomerates and hybrids, plastic enclosures, and metal screw caps (boxed and bag-in-the-box packages might also qualify, but will not be further discussed). Agglomerates consists of cork granules (also called "dust") stuck together with glue and extruded into cork shapes. They have long been used by sparkling wine manufacturers and have many of the desirable features of cork, but there are reports they may also contain TCA (presumably from cork particles contaminated with TCA). However, agglomerates are now available that are reportedly TCA-free. Hybrid corks are similar to the agglomerates, but contain plastic particles mixed with natural, ground cork. Plastic or synthetic corks are comprised of ethylene vinyl acetate. They have resilience similar to cork, but contain no cork material (and no TCA). Detractors have suggested that plastic corks impart plastic flavors to the wine (i.e., plastic taint).

Metal screw caps for wine were introduced in the 1990s. They are similar to those used for carbonated beverages. Screw caps are easy to open and re-close, and are TCA-free. They have all of the attributes of an ideal enclosure with no real technical downside. Wines from screw-capped bottles generally score well in taste tests (although rubber-like flavors, from the enclosure seal, have been reported to migrate into the wine). For sure, screw cap wines are not defect-free, and according to one recent study, are just as likely as cork-enclosed bottles to have aroma or other chemical defects (Eisermann-Ctercteko, 2013).

Ultimately, the type of enclosure used by a particular winery depends only in part on the performance characteristics of the particular material. For example, although

the stopper is intended to keep oxygen out of the wine, a small amount of oxygen transfer might be a good thing (Silva et al., 2011). Nonetheless, perhaps the more important determining factor is the attitude or perception consumers have toward specific types of wine enclosures. Surveys indicate that consumers prefer cork – they expect to hear a "pop" when the bottle is opened, and that tradition is a key feature of the wine-drinking experience. Many wine drinkers may not even recognize off-flavors in wines, including cork and other taints. Moreover, plastic corks, and especially screw cap enclosures, are often associated with economy wines, regardless of what is actually in the bottle (Marin and Durham, 2007).

Despite these attitudes, non-cork enclosures continue to gain popularity, and now are used for as much as 50% or more of the wine produced in some countries. For example, Australia and New Zealand have championed the use of screw cap enclosures. Many California wineries use cork only for their premium wines, and synthetic composite or screw caps for their other wines. Some experts have predicted that the trend away from cork will continue.

References

Alvarez-Rodriguez, M.L., L. Lopez-Ocana, J.M. Lopez-Coronado, E. Rodriguez, M.J. Martinez, G. Larriba, and J.-J. R. Coque. 2002. Cork taints of wines: role of the filamentous fungi isolated from cork in the formation of 2,4,6-trichloroanisole by O methylation of 2,4,6-trichlorophenol. Appl. Environ. Microbiol. 68:5860–5869.

Corsi, A.J., F.C.R. Hernandez, G.C. y Cruz, and J.A. Neal. 2016. The effectiveness of electron beam irradiation to reduce or eliminate mould in cork stoppers. Int. J. Food Sci. Technol. 51:389–395.

Cravero, M.C., F. Bonello, M.D.C. Pazo Alvarez, C. Tsolakis, and D. Borsa. 2015. The sensory evaluation of 2, 4, 6-trichloroanisole in wines. J. Inst. Brew. 121:411–417.

Eisermann-Ctercteko, A. 2013. Let's not be screwed! Screwcap damage levels greater than cork taint: Implications for producers, the retail sector and consumers. Wine & Viticulture Journal. 28:38, 40, 42–44.

Marin, A.B., and C.A. Durham. 2007. Effects of wine bottle closure type on consumer purchase intent and price expectation. Am. J. Enol. Viticult. 58:192–201.

Peña-Neira, A., B.F. de Simon, M.C. García-Vallejo, T. Hernández, E. Cadahía, and J.S. Suarez. 2000. Presence of cork-taint responsible compounds in wines and their cork stoppers. Eur. Food Res. Technol. 211:257–262.

Pereira, C.S., J.J.F. Marques, and M.V.S. Romão. 2000. Cork taint in wine: scientific knowledge and public perception - a critical review. Crit. Rev. Microbiol. 26:147–162.

Pereira, C.S., G.A.M. Soares, A.C. Oliveira, M.E. Rosa, H. Pereira, N. Moreno, M.V.S. Romão. 2006. Int. Biodeterior. Biodegradation 57:244–250.

Pereira, H. 2007. Cork: Biology, Production and Uses. Elsevier Science, Amsterdam.

Silva, M.A., M. Julien, M. Jourdes, and P.-L. Teissedre. 2011. Impact of closures on wine post-bottling development: a review. Eur. Food Res. Technol. 233:905–914.

Simpson, R.F., D.L. Capone, and M.A. Sefton. 2004. Isolation and identification of 2-methoxy-3,5-dimethylpyrazine, a potent musty compound from wine corks. J. Agric. Food Chem. 52:5425–5430.

Simpson, R.F., and M.A. Sefton. 2007. Origin and fate of 2,4,6-trichloroanisole in cork bark and wine corks. Aust. J. Grape Wine Res 13:106–116.

Tempere, S., M-L. Hamtat, G. Revel, and G. Sicard. 2016. Comparison of the ability of wine experts and novices to identify odorant signals: a new insight in wine expertise. Aust. J. Grape Wine Res. 22:190–196.

Spoilage by yeasts

Wild yeasts represent a major cause of wine defects and spoilage. Moreover, since yeasts are an expected part of the natural flora of grapes and must, their growth before, during, and after the wine fermentation is often difficult to control. For example, *K. apiculata*, one of the yeasts involved in the early stages of a natural fermentation, can produce high enough levels of various esters, mainly ethyl acetate and methylbutyl acetate. These products impart an ester taint, which has a vinegar-like aroma. Once vigorous growth of *S. cerevisiae* begins, other yeasts are generally unable to compete and grow. However, if *S. cerevisiae* does not become well-established (i.e., during spontaneous fermentations), other yeasts can grow and produce undesirable end-products, included acetic and succinic acids.

Growth of yeasts during aging of wine, either in barrels or bottles, is a particularly serious spoilage problem. The main culprits are species of *Brettanomyces/Dekkera,* and *Brettanomyces bruxellensis* in particular. Note that *Brettanomyces/Dekkera* exists in one of two forms, where *Brettanomyces* is the asexual, non-sporulating form and *Dekkera* is the sexual, sporulating form of the same yeast. These yeasts are common contaminants of wineries and the oak barrels used for aging. Growth of these organisms may lead to volatile phenol-containing compounds that give the wine a disagreeable "mousy" aftertaste. Importantly, mousy taints may also be caused by bacteria, including heterofermentative lactic acid bacteria.

Brettanomyces may produce a variety of other taints, descriptively described as "barnyard", "horse sweat", "band-aid", and "wet dog". Indeed, these are probably the more common and troublesome taints produced by these yeasts. Common chemical markers or signature molecules for "Brett" spoilage are 4-ethyl phenol and 4-ethyl guaiacol. Their presence in suspect wines can be routinely monitored. There are also various film yeasts, including species of *Pichia* (= *Hansenula*) *and Candida*, and that grow on the surface of wine during barrel aging, much like the flor yeast whose growth in sherry making is intentional and desirable. Under ordinary circumstances, however, unintentional growth of film yeast can lead to oxidation of ethanol and formation of acids, esters, acetaldehyde, and other undesirable end products. Ultimately, most of the spoilage yeast that cause problems in barrels can be controlled or managed by SO_2 addition, maintenance of proper anaerobic conditions (topping off of barrels), barrel management, and especially good sanitation practices.

Spoilage by bacteria

As troubling as are yeast and molds, an arguably more common and perhaps more serious form of microbial spoilage is caused by bacteria. Two distinct groups are of importance: the acetic acid bacteria and the lactic acid bacteria, both of which contain species able to tolerate the low pH, high ethanol conditions found in wine. These bacteria are responsible for acidic and other end products that seriously affect wine quality. They gain access to wine via transfer from grapes, air, and equipment, but fruit flies and other insects are now regarded as a major reservoir of these bacteria.

The acetic acid bacteria that are most important in wine spoilage belong to one of three genera: *Acetobacter, Gluconoacetobacter*, and *Gluconobacter*. The main species involved in wine spoilage are *Acetobacter aceti*, *Acetobacter pasteurianus*, and *Gluconobacter oxydans.* They are Gram-negative, catalase-positive rods capable of oxidizing alcohols to acids. These bacteria also are considered as obligate aerobes; however, it now appears that limited growth

and metabolism can occur even under the mostly anaerobic conditions that prevail during wine making.

Although acetic acid bacteria are generally found at relatively low levels in vineyards and in must (<100 cells per g), moldy or bruised grapes can contain appreciably higher levels. Insect vectors also contribute acetic acid bacteria. If the ethanol fermentation occurs soon after harvesting and crushing, then growth of these organisms, especially *G. oxydans,* is inhibited and numbers may actually decline. When the fermentation is complete and the wine is drawn off and subsequently transferred and racked, aeration inevitably occurs, activating growth of these bacteria.

Aging in oak barrels may also promote growth of acetic acid bacteria, in part because barrels may be contaminated with these bacteria, but also because oxygen diffusion into small oak barrels can be significant. Under these conditions, acetic acid bacteria can oxidize ethanol in the wine, producing enough acetic acid (>0.7 g/L) to give the wine a pronounced vinegar flavor and aroma. Although lesser amounts can sometimes be tolerated, other end products resulting from growth of acetic acid bacteria, including ethyl acetate, acetaldehyde, and dihydroxyacetone, may also contribute to spoilage defects. Low sulfur dioxide levels will further enhance growth of acetic acid bacteria.

The other group of bacteria associated with microbial spoilage of wine is the lactic acid bacteria. This cluster of Gram-positive, facultative rods and cocci consists of 12 genera, however, only four, *Lactobacillus*, *Oenococcus*, *Leuconostoc,* and *Pediococcus*, are usually involved in wine spoilage. Due to their saccharolytic metabolism, it is not surprising that lactic acid bacteria are found on intact grapes, albeit at low populations (10^2/g to 10^3/g) that eventually increase 10- to 100-fold during harvesting and crushing. Although most lactic bacteria do not grow during the ethanolic fermentation (the exception being the malolactic bacteria), some strains are tolerant of high ethanol concentrations and are able to grow later during post-fermentation steps. High temperature and pH and low SO_2 concentrations favor growth of lactic acid bacteria. Growth of these bacteria in wine can result in several spoilage conditions, including acidification and deacidification, as well as production of various metabolites that cause off-flavors, aromas, and other defects.

Lactic acid bacteria have either a homofermentative or heterofermentative metabolism, or in some strains, the metabolic capacity for both. When homofermentative lactic acid bacteria, such as *Pediococcus* and *Lactobacillus plantarum*, grow in wine and ferment glucose, they produce lactic acid. This acid causes the wine to become excessively sour. In contrast, metabolism of pentose sugars, present in the grapes as well as extracted from oak barrels, occurs via the pentose phosphate pathway, yielding acetic acid, ethanol, and CO_2. These same end-products are also produced by several heterofermentative lactic acid bacteria found in wine, including *Leuconostoc mesenteroides*, *Oenococcus oenus*, and *Lactobacillus brevis*. Although these bacteria produce considerably less acetic acid than *Acetobacter* or *Gluconobacter*, enough may be produced to impart a detectable vinegar-like flavor to the wine. There are also legal limits in the US, Europe, and Australia for volatile acidity.

Several lactic acid bacteria have the capacity to convert malic acid to lactic acid via the malolactic pathway. In musts containing high malic acid concentrations and having low pH, the malolactic bacteria perform a desirable, even essential function, by deacidification of the wine. However, if the must acidity is already low and the pH high, the malolactic fermentation may result in an increase in wine pH such that too little acidity remains. The wine also will be more susceptible to spoilage since a major barrier to microbial growth, low pH, is diminished.

Spoilage by lactic acid bacteria can also be caused by other metabolic end products. Metabolism of fructose by heterofermentative *L. brevis*, for example, results in formation of mannitol and generates acetic acid as a side reaction. In a fermentation analogous to the malolactic pathway, *L. brevis* can metabolize tartaric acid, causing an increase in wine pH and formation of volatile acids. Mousy taints, similar to those produced by the spoilage yeast *Brettanomyces*, can also be produced by *Lactobacillus* sp., *Leuconostoc* sp., and other heterofermentative lactic acid bacteria. A flowery off-odor referred to as geranium taint also may be produced by lactobacilli.

Glycerol oxidation by *Leuconostoc mesenteroides*, *Lactobacillus brevis,* and other lactic acid bacteria generates acrolein that reacts with tannins and anthocyanin phenolics to form bitter compounds. This defect, referred to as amertume, is more common in red wines, due to the higher tannin and phenolic levels in red wines. Its microbiological cause was first noted by Pasteur.

Many lactic acid bacteria, including strains of *Pediococcus* and *Lactobacillus,* produce diacetyl, which imparts a buttery aroma in wine that, at high concentrations (generally above 5 mg/L), is considered undesirable. Finally, the formation of glucose-containing polysaccharides, such as dextrins and glucans, can give an oily, viscous and objectionable mouth feel. Ropiness usually occurs only in sweet wines and is caused by *Pediococcus, Oenococcus,* and *Leuconostoc* spp.

Finally, spoilage of another sort is also caused by biogenic amine-producing lactic acid bacteria. These biogenic amines are associated more with a form of food poisoning, rather than spoilage per se, but are mentioned here because their presence in wine is often correlated with other spoilage products. In recent years, the presence of these bioactive nitrogen-containing compounds in wine has attracted considerable concern. Regulatory agencies are even considering imposing limits for these compounds in wine and other foods.

Biogenic amines are formed when various amino acids, including histidine, tyrosine, and ornithine are decarboxylated by specific decarboxylase enzymes produced by wine bacteria. In addition to carbon dioxide, histamine, tyramine, and putrescine, respectively, are formed. Depending on the dose and individual sensitivity, these biogenic amines can cause headaches, nausea, and allergic-type reactions. These biogenic amines also form in other fermented foods, including cheese, sausage, and beer. Another compound, ethyl carbamate, can be formed via a chemical reaction between urea and ethanol. Ethyl carbamate is suspected of being a carcinogen**,** and its presence in wine is increased by heating steps, such as pasteurization, and by high urea concentrations.

BIBLIOGRAPHY

Bisson, L.F. 1999. Stuck and sluggish fermentations. Am. J. Enol. Vitic. 50:107–119.

Briggs, D.E., C.A. Boulton, P.A. Brookes, and R. Stevens. 2004. Brewing: Science and Practice. Woodhead, Cambridge.

Betteridge, A., P. Grbin, and V. Jiranek. 2015. Improving *Oenococcus oeni* to overcome challenges of wine malolactic fermentation. Trends Biotechnol. 33:547–553.

Fleet, G.H. 2008. Wine yeasts for the future. FEMS Yeast Res. 8:979–995.

Fugelsang, K.C. and C.G. Edwards. 2006. Wine microbiology: practical applications and procedures. Springer Science, New York.

González-Barreiro, C., R. Rial-Otero, B. Cancho-Grande, and J. Simal-Gándara. 2013. Wine aroma compounds in grapes: A critical review. Crit. Rev. Food Sci. Nutr. 55: 202–218.

Konings, W.N. 2002. The cell membrane and the struggle for life of lactic acid bacteria. Antonie van Leeuwenhoek 82:3–27.

Malfeito-Ferreira, M. 2011. Yeasts and wine off-flavours: a technological perspective. Ann. Microbiol. 61:95–102.

Mendoza, S.N., P.M. Cañón, A. Contreras, M. Ribbeck, and E. Agosin. 2017. Genome-scale reconstruction of the metabolic network in *Oenococcus oeni* to assess wine malolactic fermentation. Front. Microbiol. 8.

Parish M., and G. Fleet. 2013. Wine. In: Doyle, M. and Buchanan R., eds. Food Microbiology. ASM Press, Washington, DC, pp. 915–947.

Pérez-Serradilla, J.A. and M.D. Luque de Castro. 2008. Role of lees in wine production: A review. Food Chem. 111:447–456.

Ribéreau-Gayon, P., D. Dubourdieu, B. Donèche, and A. Lonvaud, eds. Handbook of enology, the microbiology of wine and vinifications. Vol. 1. John Wiley, Oxford, 2006.

Robinson, A.L., P.K. Boss, P.S. Solomon, R.D. Trengove, H. Heymann, and S.E. Ebeler. 2014. Origins of grape and wine aroma. Part 1. Chemical components and viticultural impacts. Am. J. Enol. Viticult. 65:1–24.

Steensels, J., L. Daenen, P. Malcorps, G. Derdelinckx, H. Verachtert, and K.J. Verstrepen. 2015. *Brettanomyces* yeasts – From spoilage organisms to valuable contributors to industrial fermentations. Int. J. Food Microbiol. 206:24–38.

Styger, G., B. Prior, and F.F. Bauer. 2011. Wine flavor and aroma. J. Ind. Microbiol. Biotechnol. 38:1145–1159.

Waterhouse, A.L., G.L. Sacks, and D.W. Jeffery. 2016. Understanding Wine Chemistry, 1st edn. Wiley, Oxford.

12 Vinegar

Vinegar, son of wine.

Hebrew proverb

HISTORY

As noted throughout this text, it is not possible to know precisely when human beings first began to produce and consume fermented foods. Nonetheless, the origin for some products can reasonably be estimated. Vinegar, for example, was likely discovered shortly after (like about a week) the advent of the first successful wine fermentation. As excited as that first enologist must have been to have successfully turned grape juice into wine, one can imagine the disappointment that must have followed when the wine subsequently turned into a sour, unpalatable liquid, seemingly devoid of any redeeming virtues. Even though that sour wine, *vin aigre* in French, obviously couldn't be consumed with the same enthusiasm as the wine, it was not without value. Indeed, vinegar has a long history of use, and is now one of the most widely used ingredients in the food industry. According to the 2017 Ullmann's Food and Feed, annual world-wide production of vinegar is over 4 billion L, with Europe, the US, and Japan accounting for about 60%.

The documented history of vinegar consumption dates back several thousand years. Although other fermented foods, such as wine, beer, and cheese, evolved, in part, because of their enhanced preservation status, vinegar was likely used for its ability to preserve other non-fermented, perishable foods, such as meats and vegetables. Thus, vinegar can be considered as the first biologically-produced preservative. However, in addition to its use as a so-called pickling agent, it was also consumed directly as a flavoring agent, and, in a diluted form, as a beverage.

Vinegar consumption was first noted in the Bible ("come here and partake of the bread, and dip your morsel in the vinegar"), and, according to the New Testament, was given to Jesus during the crucifixion. Diluted vinegar was a popular beverage throughout the Greek

Microbiology and Technology of Fermented Foods, Second Edition. Robert W. Hutkins.

and Roman eras, where it gained favor as a therapeutic beverage. The production of vinegar was not confined to Europe – it was also widely produced and consumed throughout Asia. Although the substrates were different, the processes were remarkably similar to those that evolved in Europe. Finally, in addition to its use as a food or food ingredient, vinegar has also long been used as a topical disinfectant, as a cleaning agent, and as an industrial chemical due to its strong demineralization properties.

In the food industry, vinegar is used mainly as an acidulant, a flavoring agent, or a preservative, but it also has many other food processing applications. It is found in hundreds of different processed foods, including salad dressings, mayonnaise, mustard and ketchup, bread and bakery products, pickled foods, canned foods, and marinades and sauces. It is used in almost every culture and is part of nearly every cuisine. Although many of the vinegars produced around the world are made from ordinary substrates and often have rather nondescript sensory properties, others are produced from premium wines, carefully aged, and prized (and priced) based on their unique organoleptic attributes.

Returning to our early wine maker, there is a biological reason, of course, to account for the unfortunate fate of that ancient wine. Wine, we now know, can turn into vinegar due to the growth of naturally-occurring bacteria that oxidize the ethanol in the wine to form acetic acid. In fact, any ethanol-containing material can serve as a substrate for the vinegar fermentation, and, as will be discussed below, there are numerous types of vinegars that are produced from a variety of ethanolic substrates. While wine producers take special measures to prevent contamination by acetic acid-producing bacteria and to avoid the oxygen supply that is necessary for the ethanol-to-acetic-acid conversion, manufacturers of vinegar do just the opposite. In other words, the vinegar fermentation is conducted in the presence of suitable acetic acid-producing bacteria and under conditions that favor growth and oxidative metabolism.

DEFINITIONS

There is no standard of identity for vinegar in the United States. However, there are so-called "guidelines" that define the starting material, the finished specifications, and the labeling declaration. Moreover, as will be described in more detail later, vinegar must be made from one of various types of ethanol-containing solutions. The most common starting materials, whose identity must be indicated on the label, are grape and rice wine, fermented grain or malt mashes, and fermented apple cider. Distilled ethanol is also permitted as a substrate for vinegar manufacture. Importantly, according to the US Code of Federal Regulations, vinegar must result from the "acetous fermentation" of ethanol. In other words, acetic acid made via chemical synthesis cannot be labeled as vinegar. Vinegar must contain at least 4% acetic acid or at least 40 grains (where one grain = 0.1% acid). Usually, the ethanol concentration is less than 0.5%, and the pH is between 2.0 and 3.5. In countries where identity standards do exist, they generally are consistent with the US definition. However, regional standards may also be required.

As noted above, the raw material determines the name of the vinegar (e.g., red wine vinegar, apple cider vinegar, malt vinegar). However, the starting material also has a profound influence on the flavor and overall quality attributes of the vinegar. Although the predominant flavor of all vinegars is due to acetic acid, other flavors, specific to the ethanol source or the means of its manufacture, may also be present. In addition, some vinegars may also contain herbs and spices (e.g., tarragon, mustard), added before or after the fermentation.

VINEGAR MANUFACTURING PRINCIPLES

The manufacture of vinegar consists of two distinct processes. The first step is an ethanolic fermentation performed mostly by yeasts. In the second step, an acetic acid fermentation is carried out by acetic acid bacteria. Whereas the ethanol fermentation is anaerobic, the latter is conducted under highly aerobic conditions. In fact, many of the technological advances in the vinegar industry have focused on ways to introduce more air or oxygen into the fermentation system.

Microorganisms

Aside from those bacteria that produce small amounts of acetic acid as an overflow or side reaction from sugar metabolism (e.g., lactic acid bacteria), there are several genera of bacteria that produce acetic acid as the primary metabolic end product. It is important, however, to distinguish between those anaerobic bacteria and archaea that produce acetic acid from metabolism of one-carbon precursors and the aerobic bacteria that produce acetic acid via oxidation of ethanol. The former bacteria are referred to as acetogens and include species of *Clostridium*, *Eubacterium*, *Acetobacterium*, *Peptostreptococcus*, and other Gram-positive anaerobic rods and cocci. Acetogens rely on the acetogenesis or Wood–Ljungdahl pathway, in which energy is primarily derived from other sources, and CO_2 serves as the electron acceptor. The latter becomes reduced to acetic acid under strict anaerobic conditions.

In contrast, acetic acid bacteria transfer electrons from ethanol to the electron transport chain, with oxygen serving as the terminal electron acceptor. Thus, although the same product, acetic acid, is formed, two totally different biochemical processes and pathways are used. The acetic acid bacteria have been represented primarily by four genera: *Acetobacter, Gluconobacter*, *Gluconoacetobacter*, and *Acidomonas.* In just the past decade, however, many new genera (the total is 17, as of 2017) have been described (Table 12.1), but only a few are relevant for commercial vinegar production. In particular, several vinegar-producing strains have been reclassified as members of the new genus, *Komagataeibacter.* All of these genera are members of the sub-phylum alpha-*Proteobacteria* and the family *Acetobacteraceae.* The phylogeny of these and related bacteria is illustrated in Figure 12.1.

Table 12.1 Acetic acid bacteria: genera and common species[1].

Acetobacter	*Gluconobacter oxydans*
Acetobacter pasteurianus	*Asaia bororensis*
Acetobacter aceti	*Acidomonas methanolica*
Acetobacter peroxydans	*Neokomagataea thailandica*
Acetobacter orleanensis	*Neoasaia chiangmaiensis*
Acetobacter cerevisiae	*Kozakia baliensis*
Acetobacter orientalis	*Saccharibacter floricola*
Gluconoacetobacter	*Swaminathania salitolerans*
Gluconoacetobacter xylinus	*Nguyenibacter vanlangensis*
Gluconoacetobacter liquefaciens	*Endobacter medicaginis*
Komagataeibacter	*Granulibacter bethesdensis*
Komagataeibacter europaeus	*Swingsia samuiensis*
Komagataeibacter hansenii	*Ameyamaea chiangmaiensis*
Komagataeibacter xylinus	*Tanticharoenia sakaeratensis*

[1] Adapted from Yamada, 2016 and Cleenwerchk and de Vos, 2008.

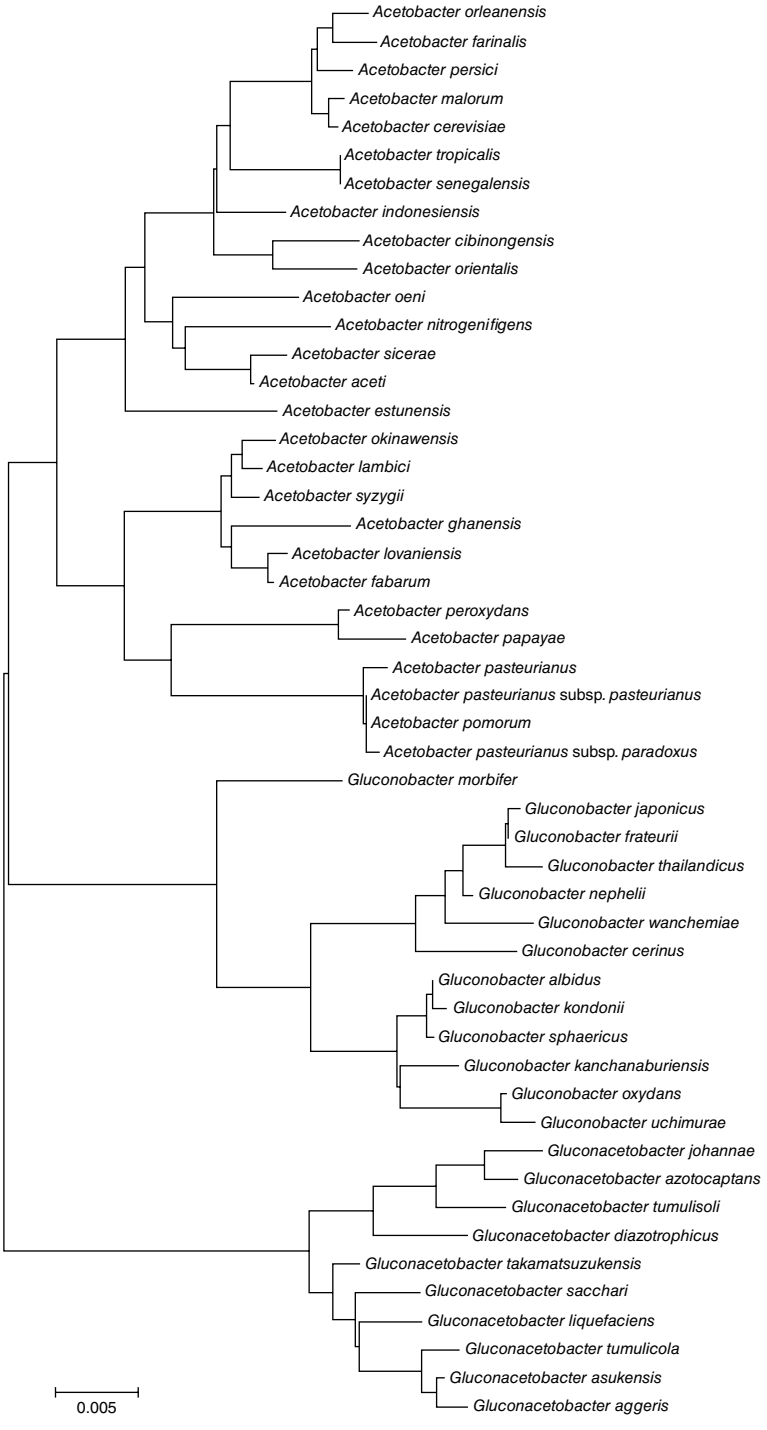


Figure 12.1 Phylogeny of acetic acid bacteria, based on 16S rRNA sequences.

General properties

Acetic acid bacteria are Gram-negative, obligate aerobes, motile or non-motile, and have an ellipsoidal to rod-like shape that appear singly, in pairs or in chains. They have a G+C base composition of 53% to 66%. Acetic acid bacteria are widely distributed in plant materials rich in sugars, such as fruits and nectars. They are also common inhabitants of alcohol-containing solutions, including wine, beer, hard cider, and other ethanolic beverages. Not surprisingly, acetic acid bacteria often share habits with ethanol-producing yeasts. Some acetic acid bacteria, notably *Acetobacter,* are more efficient at oxidizing ethanol, rather than glucose, to acetic acid. In contrast, *Gluconobacter* oxidizes and grows especially well on glucose compared to ethanol.

Cultures

Although cultures for craft or home-made vinegar are available, these are essentially wild cultures produced on a very small scale. Commercial, purified starter cultures for industrial vinegar manufacture are not ordinarily produced. In part, this is because traditional processes for introducing cultures work and are easy (see below). In addition, vinegar is generally an inexpensive product, and the economics of vinegar production do not warrant the cost of a culture.

Instead of commercial cultures, therefore, vinegar fermentations are conducted via natural fermentation or backslopping. This accounts for the large number of strains isolated from different production facilities. Most of the species used industrially for vinegar manufacture belong to one of three genera, *Acetobacter, Gluconobacter*, and *Gluconoacetobacter* (see below). Although *Acetobacter aceti* has long been considered to be the primary organism involved in the vinegar fermentation, many other species have been identified in vinegar and in production facilities, including *Acetobacter pasteurianus*, *Gluconacetobacter europaeus* (formerly *Acetobacter europaeus*), *Gluconacetobacter xylinus* (formerly *Acetobacter xylinus*), and *Gluconobacter oxydans*.

In general, specific bacterial strains are associated with particular substrates, processes, and geographic locations. For example, *A. pasteurianus* was identified as the main species involved in production of rice wine vinegars produced in Japan, whereas in Germany, *A. europaeus* is considered to be the most common organism used for submerged-type fermentation processes (see below). It seems clear, therefore, that no single species can be considered to be solely responsible for the vinegar fermentation.

In general, the criteria used to distinguish between species have been based on those biochemical and physiological properties most relevant to the acetic acid fermentation (Table 12.2). Although all species of *Acetobacter, Gluconacetobacter,* and *Gluconobacter* produce acetic acid from ethanol, they differ, metabolically, in several respects. First, whereas metabolism of ethanol by *Gluconobacter* stops at acetic acid, *Acetobacter* can, under certain conditions, completely oxidize ethanol to CO_2 (discussed below). In contrast, oxidative metabolism of glucose and other sugars is much greater in *Gluconobacter* compared to *Acetobacter,* which produces little acid from sugars. Finally, these organisms can be distinguished on the basis of the molecular architecture involved in the acetic acid fermentation. Although both rely on quinone-containing co-factor systems as electron acceptors during the ethanol oxidation reactions, *Gluconobacter* possesses a G_{10} type quinone system and *Acetobacter* uses a Q_9 system.

Table 12.2 Physiological properties and characteristics of acetic acid bacteria.

Property	*Acetobacter*	*Gluconobacter*	*Gluconoacetobacter*
Temperature optimum (C)	25–30	25 – 30	25–30
pH optimum	5.4–6.3	5.5 – 6.0	5.4–6.3
Acetic acid oxidation	+	–	+/–
Lactic acid oxidation	+	–	+/–
Acid from glucose	+	+	+
Ubiquinone type	Q-9	Q-10	Q-10

Adapted from Yamada et al., 2000 and Holt et al., 1994.

METABOLISM AND FERMENTATION

The acetic acid pathway used by *Acetobacter, Gluconobacter* and other acetic acid bacteria is an example of what biochemists refer to as an incomplete oxidation. In most oxidative pathways (e.g., the Krebs or citric acid cycle), organic substrates are ordinarily oxidized all the way to CO_2 and H_2O. However, in the vinegar fermentation, acetic acid bacteria usually oxidize the substrate, ethanol, only to acetic acid. There are, as described below, exceptions that exist where complete oxidation to CO_2 can occur.

The actual acetic acid pathway consists of just two main steps (Figure 12.2). First, ethanol is oxidized to acetaldehyde. Then, the acetaldehyde is oxidized to acetic acid. An intermediate step, in which acetaldehyde hydrate is formed, may also occur. Oxygen is required as the terminal electron acceptor. It should also be noted that the conversion of ethanol to acetic acid occurs on an equimolar basis, such that after the reactions are complete, the final concentration of acetic acid will be equal to that of the ethanol in the starting material (assuming negligible loss from evaporation). Only a minor amount of the carbon from ethanol is used for biomass or converted to other products.

The biochemical and physiological processes involved in the acetic acid fermentation are quite unlike those described for the lactic and ethanolic fermentations. The latter are anaerobic fermentations, whereas the acetic acid fermentation is performed by obligate aerobes. The acetic acid pathway yields energy, but not by substrate level phosphorylation. Instead, the oxidation reactions are coupled to the respiratory chain that generates ATP via electron transport and oxidative phosphorylation reactions. Finally, the acetic acid fermentation is unique in that both the substrate (ethanol) and product (acetic acid) are toxic. This no doubt contributes to the relative lack of competitors during what is essentially an open fermentation. In fact, acetic acid bacteria are rather remarkable for their ability to tolerate low pH and high ethanol and acetic acid concentrations (Box 12.1). In large part, this is because metabolism occurs not in the cytoplasm, but rather within the periplasmic space and cytoplasmic membrane.

The two main enzymes involved in the acetic acid fermentation are alcohol dehydrogenase (AlDH) and acetaldehyde dehydrogenase (AcDH. These enzymes are the primary dehydrogenases responsible for nearly all of the detectable oxidation activity. They are located within the cytoplasmic membrane and face into the periplasm (Figure 12.2). This means that (i) the ethanol and acetaldehyde oxidation reactions occur in the periplasm; and (ii) ethanol can be metabolized without having to be accumulated within the cytoplasm.

The importance of these enzymes warrants additional discussion. Both AlDH and AcDH also contain pyrroloquinoline quinone (PQQ) as a prosthetic group. The latter serves as the

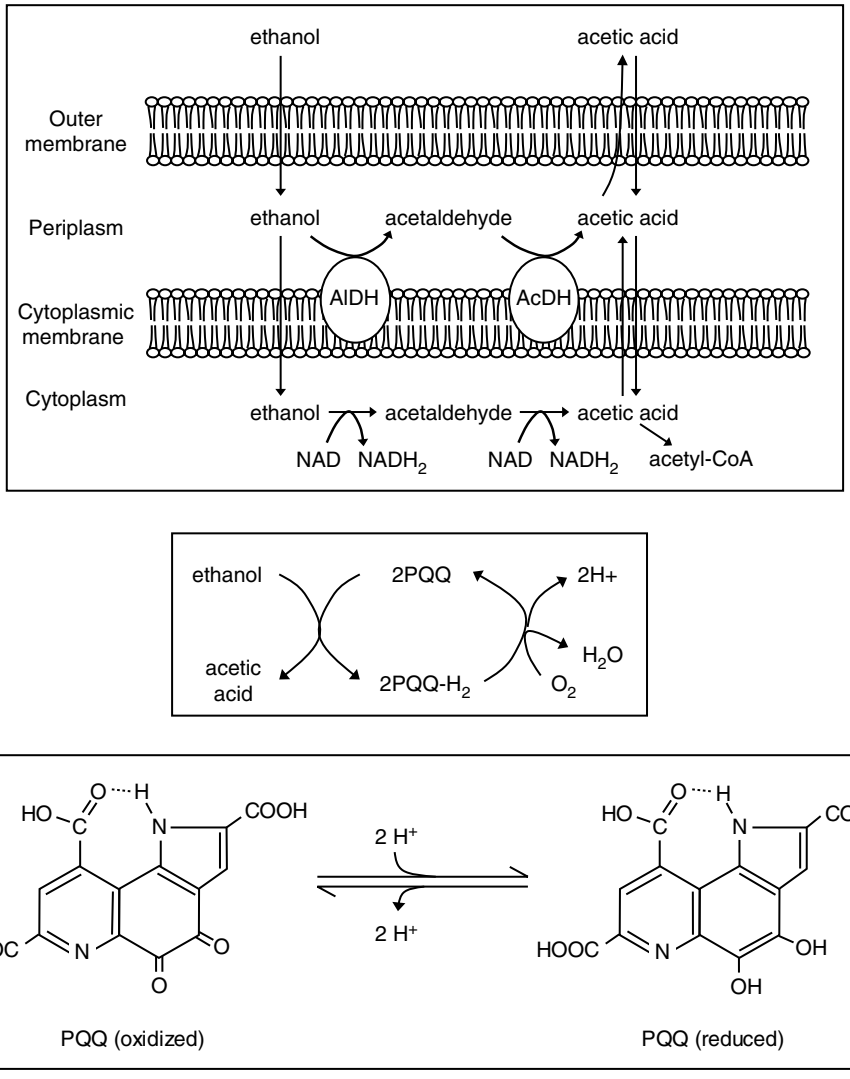


Figure 12.2 Membrane biology, oxidation of ethanol, and acetate assimilation by acetic acid bacteria. The oxidation reactions occur in the periplasm (upper panel) and are catalyzed by the PQQ-dependent enzymes, alcohol dehydrogenase (AlDH) and acetaldehyde dehydrogenase (AcDH). These reactions also occur in the cytoplasm by NAD-dependent dehydrogenases, but at very low rates. Acetic acid assimilation to acetyl Co-A and subsequent oxidation to CO_2 via the citric acid cycle, occurs only when ethanol is absent. The oxidation reactions are accompanied by transfer of electrons from PQQ to cytochromes of the respiratory chain (middle panel), leading to formation of a proton motive force and synthesis of ATP via oxidative phosphorylation. Oxygen serves as the terminal electron acceptor. The structure of oxidized and reduced forms of the pyrroloquinoline quinone (PQQ) co-factor are shown in the lower panel. Adapted from Saichana et al., 2015.

primary electron acceptor during the oxidation reactions and is responsible for the transfer of electrons to the cytochromes of the respiratory chain. Protein analyses of purified PQQ-dependent AlDH and AcDH from several *Acetobacter* and *Gluconobacter* species have shown that these enzymes consist either of two or three subunits. These subunits contain

Box 12.1 Acetic acid tolerance in acetic acid bacteria

As noted throughout this text, food fermentation organisms produce a wide range of metabolic end-products, including alcohols, acids, and peroxides, that are inhibitory to other organisms. These compounds are presumably produced, in part, for ecological reasons, namely to provide the producer with a competitive advantage in that particular environment. However, the producer organisms must also be able to tolerate these same inhibitory products, at least more so than their competitors. In the case of acetic acid bacteria, tolerance to both ethanol and acetic acid is a necessary feature of their physiology. Accordingly, acid tolerance is also reflected in their genomes, which are replete with genes encoding for acetic acid tolerance (Wang et al., 2015).

During the vinegar fermentation, acetic acid producing cells typically encounter ethanol at concentrations above 12% (about 2 M). As ethanol is oxidized to acetic acid, vigorous growth of these organisms is maintained even when the concentration of acetic acid reaches more than 10%. Some strains of *Acetobacter*, *Gluconacetobacter*, and *Komagataeibacter* can produce (and tolerate) as much as 20% acetic acid or 3.3 M (Trček et al., 2015). To provide some perspective, most *Escherichia coli* strains are inhibited by less than 1% acetic acid (Lasko et al., 2000). Thus, the means by which acetic acid bacteria deal with acetic acid stress is not only of fundamental interest, but it also has practical implications. Strains that are more acid tolerant, for example, would be expected to be more productive during commercial vinegar fermentations.

To fully appreciate the physiological challenge faced by acetic acid bacteria, consider the actual problem that exists when they grow during the acetic acid fermentation. In a 1 M (6%) acetic acid solution at a pH of 4.5, most of the acetic acid will be in the undissociated or acid form. Since the cell membrane is permeable to the acid form, cells at that pH could accumulate, in theory, more than 0.6 M acetic acid within the cytoplasm (via simple diffusion). In the absence of an efflux system, accumulation of the acid will continue until the inside and outside concentrations are equal. Because the physiological pH within the cytoplasm is higher than the external pH (at least for most bacteria), the accumulated acid will immediately dissociate to form the anion and a free proton (in accordance to the Henderson–Hasselbalch equation). This will further shift the $[acid_{in}]/[acid_{out}]$ ratio such that even more acid will be accumulated.

At some point the accumulated acetic acid will eventually cause the intracellular pH to decrease until a critical inhibitory threshold is reached (Trček et al., 2015). If the cells relied on proton efflux to maintain pH homeostasis, then substantial amounts of ATP (or its equivalent) would be required to drive the proton pumping apparatus. This would lead to a major drain on the energy resources available to the cell.

Despite these apparent hurdles, researchers have identified several mechanisms that enable *Acetobacter* and other acetic acid bacteria to tolerate high acetic-acid and low-pH environments (Figure 12.1.1). In general, there are five strategies bacteria have at their disposal (Nakano and Ebisuya, 2016; Wang et al., 2015). These mechanisms involve both physiological and genetic responses and are summarized below.

1. **Keep out.** In other words, cells can reduce acetic acid diffusion into the cytoplasm by building less permeable membranes. As noted above, at low pH, undissociated acetic acid diffuses across the cytoplasmic membrane. This diffusion depends in part on the composition of this barrier. In particular, the presence of

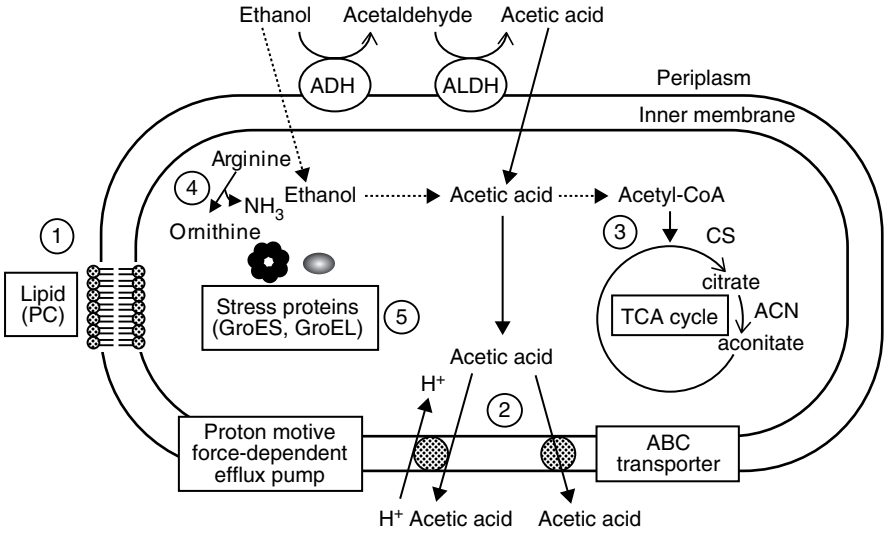


Figure 12.1.1 Molecular strategies that contribute to acetic acid tolerance in *Acetobacter* and *Gluconacetobacter*. Shown are: (1) keep out; (2) kick out; (3) metabolize; (4) neutralize; (5) protect and repair. Modified from Nakano and Fukaya, 2008, with permission.

phosphatidylglycerol and phosphatidylcholine in the membrane have a major influence on the diffusive properties (Trček et al., 2015). The latter is formed from phosphatidylethanolamine, via a methylase encoded by the *pmt* gene. These phospholipids, along with cis-vaccenic acid, an unsaturated fatty acid, and various sphingolipids alter cell membrane such that diffusion of acetic acid is reduced (Trček et al., 2007; Barja et al., 2016).

2. **Kick out.** Intracellular acetic acid can also be pumped out directly, provided sufficient energy sources are available. In *Acetobacter aceti*, at least two acid efflux system are known (Nakano and Ebisuya, 2016). One acetic acid efflux system is energized by a proton motive force that is itself dependent on respiration activity (Matsushita et al., 2005). A second ABC-type system has also been described that relies on ATP hydrolysis (Nakano et al., 2006).
3. **Metabolize.** Pathways exist for assimilating acetic acid or metabolizing it to CO_2 and other non-inhibitory products. In particular, the *aar* (*a*cetic *a*cid *r*esistance) operon consists of three genes, *aarA*, *aarB*, and *aarC* that encode for proteins with homology to enzymes involved in the citric acid pathway (Mullins et al., 2008; Mullins and Kappock, 2013). Specifically, *aarA* encodes for the citrate-forming enzyme citrate synthase, and *aarC* encodes for coenzyme A transferase, an enzyme also involved in acetate assimilation. This pathway would be one way to not only de-toxify the acid, but also enable the cell to use acetate as an energy source.
4. **Neutralize.** Another way cells can compensate for the potential acidification of the cytoplasm by acetic acid is to raise the cytoplasmic pH. This can be done by consumption of protons that cause acidification or production of alkaline molecules (Wang et al., 2015). One such system (proposed based on the presence of genes

encoding this putative pathway) is the ornithine decarboxylase-antiporter pathway in *Komagataeibacter europaeus.* This pathway consumes intracellular protons and effluxes ammonia.

5. **Protect and repair.** Proteomic analyses have shown that *Komagataeibacter* and *Acetobacter* express several proteins that protect the cells against various stresses (Andrés-Barrao et al., 2012; Andrés-Barrao et al., 2016; Zhang et al., 2015). In particular, the GroES and GroEL proteins (encoded by *groESL*) are part of the chaperonin family of stress-induced proteins that protect cells by stabilizing proteins and preventing their mis-folding. In *A. aceti*, *groESL* is induced by acetic acid (as well as heat and ethanol). Furthermore, *groESL* over-expressing strains are even more resistant to these stresses, suggesting that these gene products contribute to overall acetic acid resistance. Other systems also exist that are intended to serve as repair systems, i.e., to fix damaged DNA, such as the recently described UvrA,-encoded nucleotide excision repair system in *Acetobacter pasteurianus* (Zheng et al., 2015).

References

Andrés-Barrao, C, M.M. Saad, M.-L. Chappuis, M. Boffa, X. Perret, R. Ortega Pérez, and F. Barja. 2012. Proteome analysis of *Acetobacter pasteurianus* during acetic acid fermentation. J. Proteomics 75:1701–1717.

Andrés-Barrao, C., M.M. Saad, E.C. Ferrete, D. Bravo, M.-L. Chappuis, R. Ortega Pérez, P. Junier, X. Perret, and F. Barja. 2016. Metaproteomics and ultrastructure characterization of *Komagataeibacter* spp. involved in high-acid spirit vinegar production. Food Microbiol. 55:112–122.

Barja, F., C. Andrés-Barrao, R. Ortega Pérez, E.M. Cabello, and M.-L. Chappuis. 2016. Physiology of *Komagataeibacter* spp. during acetic acid fermentation. In: K. Matsushita, H. Toyama, N. Tonouchi, and A. Okamoto-Kainuma, eds. Acetic Acid Bacteria. Springer, Japan, pp. 201–221.

Lasko, D.R., N. Zamboni, and U. Sauer. 2000. Bacterial response to acetate challenge: a comparison of tolerance among species. Appl. Microbiol. Biotechnol. 54:243–247.

Matsushita, K., T. Inoue, O. Adachi, and H. Toyama. 2005. *Acetobacter aceti* possesses a proton motive force-dependent efflux system for acetic acid. J. Bacteriol. 187:4346–4352.

Mullins, E.A., J.A. Francois, and T.J. Kappock. 2008. A specialized citric acid cycle requiring succinyl-coenzyme A (CoA): acetate CoA-transferase (AarC) confers acetic acid resistance on the acidophile *Acetobacter aceti*. J. Bacteriol. 190:4933–4940.

Mullins, E.A. and T.J. Kappock. 2013. Functional analysis of the acetic acid resistance (aar) gene cluster in *Acetobacter aceti* strain 1023. Acetic Acid Bacteria 2(s1):e3.

Nakano, S., M. Fukaya, and S. Horinouchi. 2006. Putative ABC transporter responsible for acetic acid resistance in *Acetobacter aceti*. Appl. Environ. Microbiol. 72:497–505.

Nakano, S. and M. Fukaya. 2008. Analysis of proteins responsive to acetic acid in *Acetobacter*: molecular mechanisms conferring acetic acid resistance in acetic acid bacteria. Int. J. Food Microbiol. 125:54–59.

Nakano, S. and H. Ebisuya. 2016. Physiology of *Acetobacter* and *Komagataeibacter* spp.: acetic acid resistance mechanisms in acetic acid fermentation. In: K. Matsushita, H. Toyama, N. Tonouchi, A., and Okamoto-Kainuma, eds. Acetic Acid Bacteria. Springer, Japan, pp. 223–234.

Trček, J., K. Jernejc, and K. Matsushita. 2007. The highly tolerant acetic acid bacterium *Gluconacetobacter europaeus* adapts to the presence of acetic acid by changes in lipid composition, morphological properties and PQQ-dependent ADH expression. Extremophiles. 11:627–635.

Trček, J., N.P. Mira, and L.R. Jarboe. 2015. Adaptation and tolerance of bacteria against acetic acid. Appl. Microbiol. Biotechnol. 99:6215–6229.

Wang, B., Y. Shao, T. Chen, W. Chen, and F. Chen. 2015. Global insights into acetic acid resistance mechanisms and genetic stability of *Acetobacter pasteurianus* strains by comparative genomics. Sci. Rep. 5.
Zhang, Z., H. Ma, Y. Yang, L. Dai, and K. Chen. 2015. Protein profile of *Acetobacter pasteurianus* HSZ3-21. Curr. Microbiol. 70:724–729.
Zheng, Y., X. Chen, J. Wang, H. Yin, L. Wang, and M. Wang. 2015. Expression of gene *uvrA* from *Acetobacter pasteurianus* and its tolerance to acetic acid in *Escherichia coli*. In: T.-C. Zhang and M. Nakajima, eds. Advances in Applied Biotechnology, Springer-Verlag, Berlin Heidelberg.

heme-binding domains that mediate intramolecular transport of electrons, in addition to the PQQ prosthetic groups.

Acetic acid bacteria also have NAD- (or NADP)-dependent dehydrogenases present within the cytoplasm. However, the specific activities of these enzymes are up to 300 times lower than the membrane-bound, PQQ-dependent dehydrogenases. In addition, the pH optima of the latter enzymes in *Acetobacter* is between 4.0 and 5.0, which is much closer to the normal physiological pH than the NAD-dependent dehydrogenase enzymes, whose pH optima is above 7.0. Although the function of these enzymes has not yet been established, it has been suggested that they are involved in acetaldehyde and acetate assimilation.

It should be noted that alcohols and aldehydes other than ethanol and acetaldehyde can serve as substrates for both of these dehydrogenases. Thus, primary alcohols such as propanol, and secondary alcohols and polyols, such as isopropanol and glycerol, all of which can be present in mashes used for vinegar production, can be oxidized and converted into acids, ketones, and other organic end products. Many of these compounds make important contributions to the aroma and flavor characteristics of vinegar.

Finally, as noted earlier, the acetic acid pathway is usually considered an incomplete oxidation. This is because the substrate, ethanol, is only partially oxidized and the acetic acid that is formed is not oxidized further. However, while this is true for some acetic acid bacteria, such as *Gluconobacter* (a member of the so-called suboxydans group), most species of *Acetobacter* can oxidize acetic acid, provided conditions are suitable. The latter include bacteria of the oxydans group, represented by the common vinegar-producing species *A. aceti* and *A. pasteurianus*. The absence of ethanol in the medium is the main condition necessary for acetic acid oxidation. Ethanol apparently represses synthesis of citric acid cycle enzymes. When ethanol is absent, those enzymes are induced and complete oxidation of acetic acid to CO_2 and H_2O can occur. Of course, in actual vinegar production, this so-called over-oxidation of acetic acid is undesirable, because it causes the literal disappearance of the end product to the atmosphere. Thus, this is likely one reason why vinegar fermentations have historically been conducted in a semi-continuous mode, in which a minimum amount of ethanol is always present.

CULTURE GENETICS

Interest in the genetics of acetic acid bacteria has been motivated by several issues, including sensitivity to acid and ethanol, production instability, over-oxidation, and flavor formation. Nonetheless, the genomes of several acetic acid bacteria, including *A. aceti*, *A. pasteurianus* and other industrial species, have only recently been sequenced. As expected, these genomes

contain genes encoding for the relevant physiological traits associated with ethanol oxidation and growth at low pH. They are also rich in transposable elements, and some strains contain hyper-mutable regions. Industrial strains of *Acetobacter* often contain plasmids. Collectively, these properties may account for the observed strain instability and changes in phenotypic properties.

VINEGAR TECHNOLOGY AND PRODUCTION

The first step in the manufacture of vinegar is the production of an ethanolic substrate, whether from wine, cider, malt or rice mashes, or other materials. However, regardless of the source, the ethanol substrate is usually prepared with the eventual end product (i.e., vinegar) in mind. Thus, the starting materials do not necessarily have to be of the very highest quality. However, this does not mean that poor quality materials should be used, since many of the flavor and aroma properties of the finished vinegar are derived from the starting material. Of course, if distilled ethanol is used as the substrate, then the resulting vinegar will lack most of these flavor and aroma characteristics.

The second step of vinegar production, conversion of the ethanol substrate to acetic acid, can be performed by one of several methods, all of which rely on oxidative fermentation of ethanol by acetic acid bacteria. These processes are generally referred to as the open (or slow) method, the trickling generator process, and the submerged fermentation process. The latter two are more widely used, especially for larger vinegar manufacturers, since they can operate in a continuous mode and can reduce the fermentation time from several weeks to a matter of days. However, even the traditional open methods can be performed in a semi-continuous fashion and are still used in many parts of the world. Moreover, the open vat process is the method of choice for the premium types of vinegar, including various wine vinegars, such as balsamic. Although the latter has become very popular as a "specialty" vinegar, only the authentic versions are made via long barrel aging (Box 12.2).

Box 12.2 The appeal of specialty vinegars (or, "You paid how much for that vinegar?")

By far, most of the vinegar produced in the United States is used by the food industry in the manufacture of salad dressings, pickles, catsup, mustard, and a variety of other processed foods. In general, the vinegar used for these products should be inexpensive and plain-tasting. Even the more flavorful wine, malt, and cider vinegars available at the local grocery store and used as table vinegar carry rather modest retail prices.

Some gourmet food sections, however, may offer specialty vinegar products that are as expensive and exceptional as very fine wines. Among the most well-known of the specialty vinegars are the sherry and balsamic vinegars. They are made, respectively, in the Jerez region of Spain and the Modena region of Italy. Although sherry- and balsamic-like versions are now made in the United States and elsewhere, these are protected names in Europe, where their production and authenticity are regulated by the European Union (Giudici et al., 2009).

Authentic balsamic vinegar is striking in several respects. It has a very dark brown appearance, a strong complex aroma, and a sour, but slightly sweet flavor. Importantly,

the fermentation of balsamic vinegar is quite different than traditional vinegars. The starting material is musts obtained from a variety of grapes, mainly Trebbiano, but also Lambrusco and Sauvignon. The musts are concentrated two-fold by raising the temperature to near boiling, followed by simmering until the mixture is syrup-like. This step-wise process can take as long as three days and results in a sweet solution with a sugar concentration of 20% to 24%. Next, the material is inoculated with a "mother culture" (from a previous batch) and transferred into premium quality wooden barrels (examples include oak, chestnut, mulberry, and cherry wood). These wild cultures contain various osmophilic yeasts, including strains of *Saccharomyces* and *Zygosaccharomyces*, and the acetic acid bacterium *Gluconobacter*. An alcoholic fermentation and the acetic acid fermentation essentially occur at very near the same time, since *Gluconobacter* can oxidize sugars directly to acetic acid.

Another unique feature of the balsamic fermentation process concerns the manner in which the vinegar is aged. Rather than aging the product in bulk in a single barrel, balsamic vinegar is aged in a series of steps in which the product is transferred, via decantation, from barrel to barrel. Each barrel is usually constructed from different woods, which contributes to the flavor and aroma of the finished product. During each transfer step, about half of the volume from the preceding barrel is moved to the next barrel.

The barrels (usually in sets of five or seven) are held in attics that are exposed both to very warm and very cool temperatures, depending on the season. Warm temperatures stimulate fermentation, whereas cool temperatures promote sediment formation (which improves clarity). The rich and aromatic flavors associated with balsamic vinegar are due, in part, to the grape constituents and the volatile and non-volatile components extracted from the wood, but also to the acids, esters and other metabolic end products produced by the microbiota. To achieve Denominazione di Origine Controllate (DOC) status and Protected Designation of Origin (PDO) certification, the vinegar must be aged for at least 12 years, but some balsamic vinegars are aged for 25 years or even longer. Less than 12,000 liters of authentic balsamic vinegar (Aceto Balsamico Tradizionale) are released for sale per year. Thus, it is not surprising that retail prices for 90 ml bottles of 25-year-old PDO balsamic are about $150. While more moderately priced versions exist (Aceto Balsamico di Modena), they usually are diluted with wine vinegars or aged less.

Authentic sherry vinegars (vinagre de Jerez) are produced via the "solera" process in a manner similar to that used for Sherry wines (Tesfaye et al., 2009). Specifically, a portion (less than one-third of the total volume) of the vinegar is transferred from cask (or "butt") to cask. Each successive portion is more aged than the preceding material, such that the final product has a uniform and consistent quality. Aging typically occurs for a minimum of six months, to as long as two years or more (to earn "Reserva" status). There is now a PDO for ten-year sherry vinegar, called "Gran Reserva", and many are aged for even longer.

Flavors for the sherry vinegars, as for balsamic vinegar, are derived from fermentation, as well as the wood used during aging (Callejón et al., 2008). The latter contribute important volatile compounds associated with aged sherry vinegar. Indeed, there is an extensive chemical diversity in these vinegars that account for the flavor and aroma (Durán-Guerrero et al., 2015). Not surprisingly, given their economic value,

there are adulterated sherry and balsamic products in the marketplace. The same sophisticated chemical and spectroscopic methods used to characterize these vinegars can also be used to authenticate their PDO status (Chinnici et al., 2016; Graziosi et al., 2017; Tesfaye et al., 2009).

Finally, it should be noted that distinctive specialty wine vinegars are not just produced in Modena, Jerez, or Orleans, but also in the United States. A growing market for varietal vinegars, made from single variety wines, has emerged, especially in the wine-producing regions in California. These vinegars are fermented in barrels; some producers have adopted traditional Orleans-style fermentors and long aging periods.

References

Callejón, R.M., M.L. Morales, A.C. Silva Ferreira, and A.M. Troncoso. 2008. Defining the typical aroma of Sherry vinegar: sensory and chemical approach. J. Agric. Food Chem. 56:8086–8095.

Chinnici, F., E. Durán-Guerrero, and C. Riponi. 2016. Discrimination of some European vinegars with protected denomination of origin as a function of their amino acid and biogenic amine content. J. Sci. Food Agric. 96:3762–3771.

Durán-Guerrero, E., F. Chinnici, N. Natali, and C. Riponi. 2015. Evaluation of volatile aldehydes as discriminating parameters in quality vinegars with protected European geographical indication. J. Sci. Food Agric. 95:2395–2403.

Giudici, P., M. Gullo, and L. Solieri. 2009. Traditional Balsamic vinegar. In: Solieri, L. and P. Giudici, eds. Vinegars of the World. Springer, New York, pp. 157–179.

Graziosi, R., D. Bertelli, L. Marchetti, G. Papotti, M.C. Rossi, and M. Plessi. 2017. A novel 2D-NMR approach for the classification of balsamic vinegars of Modena. J. Agric. Food Chem. 65:5421–5426.

Tesfaye, M., M.L. Morales, M.C. García-Parilla, and A.M. Troncoso. 2009. Jerez vinegar. In: Solieri, L. and P. Giudici, eds. Vinegars of the World. Springer, New York, pp. 179–195.

Open vat process

The open vat process relies on surface growth of acetic acid bacteria in vats, barrels, jars, or trays. This was undoubtedly the first method used for vinegar production, since it can be easily performed and involves naturally-occurring or wild cultures. Left alone and exposed to the air, wine or other ethanol-containing materials will likely turn into vinegar. Although many variations exist, barrel-type fermentations are typical of the processes that evolved in Europe and that are still used today. The most well-known example is the Orleans process, which takes its name from the French city where this particular method was originally developed. Similar processes also evolved independently in the Far East, but the latter were based on rice wine and other types of alcohol-containing substrates, rather than on grape wine or cider. The principle of the process is simple and straightforward. The ethanolic substrate is placed in a suitable vessel, and the fermentation is initiated either by acetic acid bacteria that naturally contaminate the vessel or by a portion of vinegar from a recent batch (i.e., a form of backslopping). The inoculated material is exposed to the atmosphere, but is otherwise left undisturbed. Hence, this process is sometimes referred to as the "let alone" method.

In the open vat process, acetic acid bacteria grow only at the surface. Growth is accompanied by production of a polysaccharide-containing film or pellicle that forms at the liquid-air interface (Box 12.3). Although most strains of *Acetobacter* are capable of pellicle formation, this property can occasionally be lost, especially if cells are grown under

Box 12.3 Vinegar bacteria and synthesis of cellulose pellicles

For vinegars made by barrel, Orleans, and other "stand alone" methods, the acetic acid bacteria grow at the liquid–air interface. At that interface, a surface film forms that is essential for successful fermentation. If disturbed, the bacteria must re-establish the film before fermentation can proceed.

The formation of this film, and in particular its function and structure, has long been a rather curious phenomenon. Pasteur first observed the presence of a "gelatinous and slippery" material as a vinegar by-product (Iguchi et al., 2000). The chemical structure was identified in 1886 as cellulose, a polysaccharide produced by plants, but not usually by bacteria (Brown, 1886a, 1886b). Indeed, several vinegar bacteria are now known to produce a very pure form of cellulose as part of the surface pellicle. Included are *Acetobacter, Gluconacetobacter*, and the recently named genus, *Komagataeibacter*. The latter is also found in kombucha, the fermented tea product that has recently become very popular (see Box 12.4).

More recently, the pathways and genes for cellulose synthesis by vinegar bacteria have been established (Römling and Galperin, 2015). Ultimately, however, the question that is perhaps most intriguing is why these bacteria produce this pellicle or film in the first place. Several possibilities have been proposed (Iguchi et al., 2000). First, the film provides a positional advantage for aerobic organisms. Second, by growing just under the film they are protected against ultraviolet. Similarly, the film offers protection against predators and large molecular weight antimicrobial agents. Of course, for manufacturers of traditional Orleans and other barrel-type products that depend on film-forming bacteria, the cellulose pellicle is the *raison d'être*.

References

Brown, A.J. 1886a. The chemical action of pure cultivations of *Bacterium aceti*. J. Chem. Soc. 49:172–187.

Brown, A.J. 1886b. On an acetic ferment which forms cellulose. J. Chem. Soc. 49:432–439.

Iguchi, M., S. Yamanaka, and A. Budhiono. 2000. Bacterial cellulose – a masterpiece of nature's arts. J. Materials Sci. 35:261–270.

Römling, U., and M. Y. Galperin. 2015. Bacterial cellulose biosynthesis: diversity of operons, subunits, products, and functions. Trends Microbiol. 23:545–557.

non-static conditions. This is important during open vat processes, because an intact film is essential to the success of the fermentation. Any disturbance or disruption of the film may delay the fermentation, since the pellicle must re-form to continue the fermentation. Thus, open vat processes may take several weeks before the fermentation is established, and several months before the fermentation is complete.

Traditional open vat or surface film fermentations ordinarily operate in batch mode. After the fermentation is complete, the vessel is emptied, and is then refilled with substrate (i.e., fresh wine). The acetic acid bacteria, left over or added, must re-establish surface growth. It is possible, nonetheless, to perform this fermentation in a semi-continuous mode, provided steps are taken to maintain the surface film during substrate addition and product removal steps.

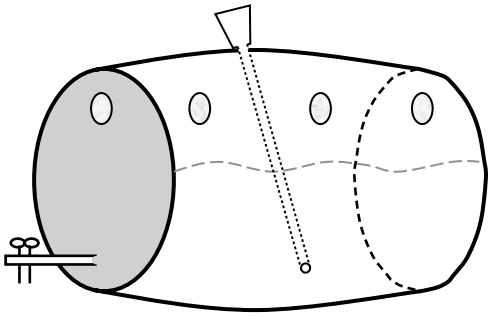


Figure 12.3 Schematic illustration of vinegar barrel used for the Orleans process. Adapted from Adams and Moss, 1995.

In the Orleans process, for example, the barrels are constructed such that a filling device (i.e., a pipe) extends from just outside the top of the barrel all the way near to the bottom (Figure 12.3). Aeration is provided by holes (covered with cheesecloth) drilled into the sides of the vessel. A tap is positioned at the bottom end that is used to withdraw the product. The barrel is filled with wine to about 60% to 70% capacity and inoculated with a fresh vinegar culture, commonly called "mother of vinegar". The culture forms a pellicle as described above and begins to oxidize the ethanol. When the acetic acid fermentation is completed, it is then possible to remove a portion (from one-third to as much as two-thirds) of the fermented vinegar from the tap end. Next, the displaced vinegar is replaced with fresh wine or cider, added through the filling device, without disrupting the film. The process takes two to three weeks, or longer, for each completed cycle, depending on the temperature and the rate of volume exchange. Although other methods require significantly less time, the quality of vinegar produced by the Orleans and similar barrel methods is considered to be far superior to those produced by more rapid methods.

Trickling generator processes

In the vinegar fermentation, the rate at which ethanol is oxidized to acetic acid depends on the presence and availability of oxygen and the surface area represented by the air-liquid interface. In other words, the ability of acetic acid bacteria to perform the acetic acid fermentation is limited primarily by the diffusion or transport of oxygen from the atmosphere to the cell surface. It is possible, therefore, to significantly accelerate the oxidation of ethanol by increasing the surface area to which oxygen is exposed. This observation, originally described nearly 300 years ago by the famous Dutch physician-chemist Hermann Boerhaave, forms the basis for the trickling generator processes, which are sometimes referred to as simply quick vinegar processes. The earliest examples of trickling generators used on an industrial scale were the Schutzenbach and Ham processes, introduced in 1823 and 1824, respectively. In these systems, which are still widely used, ethanolic substrates are circulated or trickled through cylindrical fermentation vessels containing inert packing materials, such as curled wood shaving, wood staves, or corn cobs (Figure 12.4). Many other materials can be used, provided they do not contribute off-flavors or are degraded. Their main function is to increase the total surface area within the fermentor, especially at the air-liquid interface.

As the inoculated substrate or feedstock passes from the top to the bottom of the vessel, the total surface area of the liquid material increases as it moves around and between the

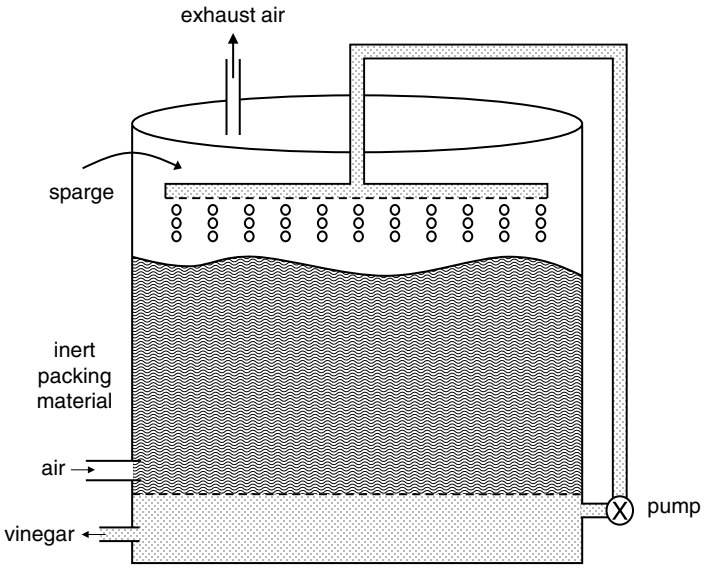


Figure 12.4 Trickling generator system.

particulate packing material. Growth of acetic acid bacteria will then occur at the air-liquid interface, such that the ethanol concentration decreases and the acetic acid concentration increases during the transit of substrate from top to bottom. Holes can be drilled into the side of the vessel to ensure that aeration is adequate. When the liquid effluent reaches the bottom section (the collection chamber) it may be returned to the top until it is sufficiently acidic and can be called vinegar. Alternatively, a second tank can be used to increase the oxidation rate and product throughput. The inoculated mash is fermented first in Tank 1, then passed, with aeration, into Tank 2, and back and forth until all of the ethanol is converted to vinegar. About three days are required to convert a 12% (v/v) ethanol solution to vinegar containing 10% to 12% acetic acid.

Many modern trickling generator-type systems have been developed over the past 70 years based on the same principle as described above, but with more control and operational features. The Frings generator, introduced in the 1930s, and still widely used today in various modified formats, provides a means for incorporating air, in a counter-current direction, directly into the fermentation vessel. These systems can also accommodate a heat-exchange step, so that a constant temperature can be maintained. In addition, the feedstock is ordinarily dispensed via a sparging arm that breaks up the liquid into smaller droplets that are more easily distributed within the interior matrix.

Submerged fermentation

Advances in biotechnology, and in particular, the fermentation industry, have led to the development of modern industrial fermentors capable of rapid, high throughput bioconversion processes. Although submerged fermentation systems are now widely used for many non-food industrial fermentation processes, they were actually developed by the vinegar industry (and the Heinrich Frings Company, in particular) more than fifty years ago. The Acetator (the Frings fermentor), Cavitator, Bubble fermentor, and other similar units are

constructed of stainless steel, can be easily cleaned and sanitized, and can operate in batch, semi-continuous, or continuous mode. Most of the vinegar produced worldwide is now made using submerged fermentation systems.

The most important feature of the submerged fermentation systems is their ability to provide rapid and efficient aeration. For example, the Frings Acetator is equipped with turbines that mix the liquid with air or oxygen and deliver the aerated mixture at very high rates inside the fermentor. The aeration system's ability to break up air bubbles and facilitate transfer of oxygen molecules from the gas phase to the liquid phase is essential, since the success of submerged fermentation systems relies on the transfer of oxygen from the medium to the bacteria. The viability of *Acetobacter* cells can be quickly compromised if aeration is even momentarily lost.

In addition to the aeration system, process controls for propellor speed (up to 1750 rpm), nutrient feed, foam-handling, and temperature further provide for consistent, continuous operation. Temperature control may be of particular concern in large-scale fermentations because ethanol oxidation is an exothermic reaction and heat is released. The medium temperature, if not controlled, can increase from an optimum of 28°C to 30°C to as high as 33°C. Even this modest increase in temperature can result in a serious decrease in fermentation productivity.

The size and design of submerged fermentation systems vary considerably. Production capacities of 2,500 liters per day of 10% acetic acid for very small-scale fermentors, up to 30,000 liters per day for large-scale reactors, are possible. In general, the starting material contains both ethanol and acetic acid (from a prior fermentation). After a single cycle of sixteen to twenty-four hours, the ethanol concentration will be reduced from about 5% to less than 0.5%, and the acetic acid will increase from around 7% to 12%. Up to half of the vinegar is then removed and replaced with fresh ethanol feedstock. Cell growth during a typical fermentation cycle is modest, with only a single doubling of the initial population.

Post-fermentation processing

Depending on the manner in which the fermentation occurs, the vinegar may be relatively clear, in the case of barrel-aged product, or very turbid, in the case of submerged fermentation-produced product. Thus, vinegar obtained by the Orleans method or after aging and subsequent transfer in barrels may require little, if any, filtration. In contrast, removing suspended cells from vinegar produced by submerged fermentation generally requires more elaborate filtration treatments, including the use of inert filtration aids. Vinegar produced by trickling fermentation processes may also require a filtration treatment.

Although it may seem counter-intuitive to pasteurize vinegar (after all, what can grow in a 1 M acetic acid solution?), heat treatments up to 80°C for 30 to 40 seconds are common in the vinegar industry. Usually the targets are acetic acid bacteria, lactic acid bacteria, and wild yeasts and fungi.

Finally, as noted earlier, some vinegar products are aged, usually in wooden barrels. However, this practice is usually reserved for high quality wine vinegars.

SPOILAGE, BACTERIOPHAGES, AND OTHER PROBLEMS

Perhaps the most frequent microbial problem associated with vinegar spoilage is caused by oxidation of acetic acid by *Acetobacter*. As described earlier, these bacteria have the metabolic capacity to oxidize the acetic acid to CO_2 and water. However, the genes that encode for citric acid cycle enzymes necessary for acetic acid oxidation are not induced in

the presence of ethanol. Moreover, acetic acid oxidation is apparently repressed when the acetic acid concentration is below 6% to 7%. Thus, under the ordinary conditions in which the acetic acid fermentation occurs – when the ethanol concentration ranges between 2% and 10% and the acetic acid concentration is above 6% – the oxidation pathway is not active. Only under prolonged fermentations, when the ethanol is completely dissipated and when the acetic acid concentration is low, would over-oxidation occur.

Spoilage of vinegar by other organisms is rare, although acetic acid-tolerant fungi, such as *Moniliella acetoabutens*, have occasionally been known to grow in raw vinegar. In the distant past, it was not uncommon for vinegar to become contaminated with mites and flies. The vinegar eel, in particular, was once known for infecting traditional open vat or trickling fermentation systems, but they are now infrequently present. The eel is actually a small worm (i.e., a nematode classified as *Anguillula aceti*) whose main effect is aesthetic rather than influencing the fermentation of product quality. Vinegar eels are readily removed by filtration and easily killed by heat.

Given that vinegar fermentations are conducted in the presence of ethanol and acetic acid, one might expect that bacteriophages would not be a serious problem. However, the absence of a heating step for the substrate, the repeated use of the same culture, and the generally open manner in which vinegar fermentations are conducted, certainly provides many of the right conditions for phage proliferation. Even submerged fermentations in enclosed stainless steel fermentors do not preclude phage infections, unless pasteurized substrates are used or other phage control measures are applied. Nonetheless, although phage problems have occasionally been reported, the occurrence is apparently low, and there are only a few published accounts of phage infections in vinegar production facilities (although the incidence of unpublished phage problems may be higher).

In general, phages are more commonly associated with trickling generator processes, because those fermentations are usually conducted by a diverse mixture of strains where phages would likely proliferate. In fact, trickling processes are considered to be the main reservoir for *Acetobacter* phages. The diversity of strains, however, also reduces the risk of detrimental infections. In contrast, submerged fermentation systems could be sensitive to phage problems, since they often rely on a single strain whose infection could conceivably result in a slow or failed fermentation.

VINEGAR QUALITY

At least two criteria must be considered to assess the quality of vinegar. In contrast to other fermented foods, where authenticity is rarely disputed, vinegar can be adulterated, either with less expensive vinegar or with other acidic agents. In rare instances, adulteration is caused by even more dubious means. For example, acetic acid produced via chemical synthesis can be diluted and then marketed, illegally, as fermentation-derived vinegar. Thus, the first quality criterion is based on establishing that the vinegar is actually vinegar (and not just diluted acetic acid) and that the type of vinegar indicated on the label accurately represents what is in the product. Satisfying this requirement, however, is no easy matter, and requires rather sophisticated testing. Discriminating analyses that are used to distinguish samples of vinegar involve measuring selected chemical constituents, including minerals, alcohols, acids, phenolics, and other volatile compounds. Using such an approach, it is even possible to differentiate the fermentation process used to produce the vinegar (e.g., traditional versus quick acetification methods).

The other main characteristics on which vinegar quality is based are those that relate to flavor, aroma, and other organoleptic properties. Vinegar flavor is particularly influenced by the raw ethanolic material from which it was made. Thus, wine vinegars contain a mixture of phenolic compounds that are ordinarily present in grapes. And although acetic acid is by far the predominant flavor present in vinegar, other volatile flavor compounds are also present and contribute to the overall flavor profile of vinegar. The enzymes, alcohol dehydrogenase and aldehyde dehydrogenase, that oxidize ethanol and acetaldehyde, respectively, also oxidize other alcohols and aldehydes. Thus, if the must, mash, or other starting material contains these substrates, a diverse array of acidic and non-acidic end-products can be obtained. These products include formic, propionic, and butyric acids, and glycerol. Other volatile compounds commonly found in vinegar include diacetyl, acetoin, and ethyl acetate.

Of course, the ultimate measure of vinegar quality, as for other fermented food products, is based on sensory analysis. Performing these analyses for vinegar, however, is particularly challenging since acetic acid has a strong, overpowering flavor. Therefore, samples can be diluted, mixed with lettuce, or even served in small amounts in wine glasses. In fact, sensory analysis of vinegar is not unlike that used for wine analysis, in that both generally require trained tasters and both rely on similar descriptors (e.g., woody, fruity, etc.).

Finally, there is one particular acetic acid-containing product – called kombucha – that has become one of the most popular "new" fermented beverages (Box 12.4). The starting material include tea leaves, buts its manufacture requires addition of a special culture that initiates a rather complex fermentation. Despite the rapid growth of commercial manufacture of kombucha, home-made versions remain popular.

Box 12.4 Krazy about kombucha

It is a rather peculiar observation, given their very long history, but many fermented foods have seemingly all of a sudden become trendy and hip. From artisan cheeses and sausages, kimchi and kefir, to craft beers and single barrel whiskies, fermented foods and beverages are among the most popular of all food categories.

Now there is kombucha tea. Actually, the kombucha craze began decades ago. A New York Times article from 1994 refers to the fad as having "spread like a new-age chain letter" and being "the pet rock of the 1990s". Commercial kits for home-brewing kombucha became available in the US in the early 1990s, and bottled versions soon appeared on shelves at specialty grocery stores. Kombucha is now produced by several manufacturers and is widely available – it can even be found at Walmart. Annual sales in the US are over $500 million.

History Interestingly, the origins of kombucha go back at least 2000 years to northeast China during the Tsin Dynasty (Stadelmann, 1961). At the time, the beverage was consumed for its putative healing benefits and energizing powers. The drink was brought to Japan by a physician named Kombu in 414 AD to treat the Emperor's digestive problems. It eventually spread to Russia and eventually Eastern Europe and the United States. Along the way, more than 100 different names were assigned to this beverage, including kombucha, tea fungus, Haipao, Manchurian

Mushroom, Kargasok Tea, Fungojapon, Tschambucco, Mo Gu, Champagne of Life, and teakwass. (Tietze, 1996).

Manufacture Traditional kombucha is prepared by boiling black tea leaves and adding an ample amount of sugar. The latter serves as the fermentation substrate (Figure 12.4.1A). After cooling, the sweetened tea is inoculated with a culture containing an undefined mixture of bacteria and yeasts. The traditional kombucha starter culture is referred to a "SCOBY" or symbiotic culture of bacteria and yeasts. The SCOBY itself is a white, gelatinous, cellulose-containing mat that either sinks, floats, or sits sideways in the fermentation vessel (Figure 12.4.1B). It consists of acetic acid bacteria and ethanol-fermenting yeasts (see below). With every fermentation, a new layer of culture will grow on the surface, and as a result SCOBY backslopping is common.

The beverage is fermented at room temperature for 7 days to as long as 30 days, depending on the desired flavor profile. Shorter fermentations result in a sweeter flavor (with residual sugars) compared to longer fermentations that yield products having a vinegary, sour taste. Gas (CO_2) is produced as well, providing effervescence.

The SCOBY and kombucha microbiota The kombucha biofilm or pellicle is a complex microbial community consisting of prokaryotic bacteria and eukaryotic yeasts. Cell populations of about 10^4 –10^6 CFU/mL are reached within 6–14 days of fermentation (Chen and Liu, 2000). Until recently, researchers relied on culture-dependent methods to identify bacterial and yeast species in SCOBY biofilms. The primary strains included *Acetobacter*, *Gluconobacter*, *Brettanomyces*, *Candida*, *Zygosaccharomyces*, *Saccharomyces*, *Pichia*, *Mycoderma, Mycotorula,* and *Torulopsis* (Jankovic and Stojanovic, 1994; Liu et al., 1996).

More recently, SCOBY biofilms have been studied using molecular methods. The most in-depth study to date, using high-throughput DNA sequencing, showed that five bacterial phyla were present across geographically distinct kombucha samples (Marsh

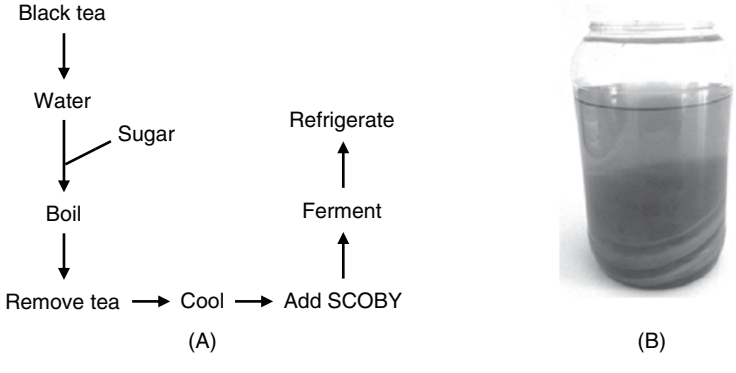


Figure 12.4.1 Kombucha manufacture (A) and kombucha tea with SCOBY (B). Adapted from Mo et al., 2008. Photo courtesy of Heather Hallen-Adams.

et al., 2014). These phyla included Actinobacteria, Bacteroidetes, Deinococcus-Thermus, Firmicutes and Proteobacteria, with Proteobacteria being the most abundant. The most dominant genera were the acetic acid bacteria, *Acetobacter, Gluconacetobacter,* and *Komagataeibacter.* They are reported to be responsible for the cellulose mat (Machado et al., 2016; Nguyen et al., 2008). Lactic acid bacteria (LAB) are also present in kombucha. The most abundant are *Lactobacillus* and *Lactococcus*, with *Leuconostoc* present at lower levels. The fungal population in kombucha is dominated by *Zygosaccharomyces lentus* and *Zygosaccharomyces bisporus*.

The population of microorganisms within the biofilm depends on the culture origin and varies from country to country. The differences in culture likely account for the very different flavor and metabolite profiles (Teoh et al., 2004). The complexity of the biofilm and the successions that occur during fermentation make this fermented food an excellent model to study microbial ecology.

End-products Given the diversity of the SCOBY organisms and the presence of yeasts, lactic acid bacteria, and acetic acid bacteria, a wide range of end-products are formed in kombucha. Nonetheless, the main fermentation products are ethanol, acetic acid, lactic acid, and carbon dioxide. The yeast hydrolyzes the sucrose into glucose and fructose and fermentation yields ethanol and carbon dioxide. Homofermentative lactic acid bacteria produce lactic acid, whereas heterofermentative species produce ethanol, carbon dioxide, and acetic acid. Acetic acid bacteria oxidize the ethanol to produce acetic acid. These bacteria can also oxidize glucose to produce gluconic acid. Collectively, the organic acids give kombucha its characteristic sour, acidic flavor and decrease the pH in the final stages of the fermentation to approximately 2.6. This is much lower than the initial pH 5.5 of the tea infusion (Kallel et al., 2012).

As noted above, kombucha yeast produce ethanol. Importantly, the ethanol concentration can be as high as 0.8–1.15% (alcohol by volume), depending on how much sugar is added into the fermentation, how long the fermentation is allowed to proceed, and the oxidative activity of acetic acid bacteria (Greenwalt et al., 2000; Sievers et al., 1995). However, kombucha is considered a non-alcoholic drink and cannot exceed the 0.5% limit for non-alcoholic beverages set by the Alcohol and Tobacco Tax and Trade Bureau. In addition, the complexity of the fermentation can makes it difficult to predict how much ethanol will be produced during shipping and storage on store shelves (especially if refrigeration is not maintained). Indeed, high ethanol content has led to kombucha recalls. Certainly, more research on how to control ethanol formation during production, transport, and storage is needed.

Health properties Kombucha has gained an internet reputation for being a cure-all beverage in many parts of the world (Chakravorty et al., 2016). Health claims and personal testimonies from kombucha enthusiasts are easy to find. Kombucha is reportedly able to "detoxify" the body, decrease blood pressure, reduce obesity, protect against diabetes and cancer, improve eye sight, eliminate grey hair, promote better sleep, increase libido, and improve mood. Unfortunately, there is no human clinical evidence for any of these health claims.

While kombucha may indeed possess *in vitro* antimicrobial activity against pathogenic bacteria, including *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enteritidis*, and *Listeria monocytogenes* (Sreeramulu et al., 2000), this is likely due to the presence of acetic acid and nothing specific to kombucha. In contrast, the starting material (i.e., black tea) may itself contain flavonoids, antioxidants and other bioactive molecules, whose concentrations are increased during fermentation (Chakravorty et al., 2016). Studies using animal models have shown that kombucha may have hepatoprotective, hypocholesterolaemic, and antioxidative activities (Murugesan et al., 2009, Yang et al., 2009, Vina et al., 2014). However, such benefits have not been reported in human clinical studies.

Safety Kombucha is widely made by home "brewers", and control of the fermentation is easy to maintain. Thus, very low pH levels, near 2.5, can be reached, especially during long fermentations. This makes kombucha a highly acidic drink. Although rare, individuals sensitive to this high level of acid may be at risk. For example, two women who consumed kombucha on a daily basis suffered severe acidosis, resulting in the death of one due to a perforated intestine (Centers for Disease Control and Prevention, 1995). An HIV patient was also reported to have suffered acidosis from kombucha consumption (Kole et al., 2009). There are other reports of dizziness, nausea, allergic reactions, vomiting and lead poisoning after consuming kombucha, and the drink is often contraindicated for pregnant women (Jayabalan et al., 2014). Finally, researchers are also concerned that pathogenic molds within the SCOBY can tolerate low pH and produce mycotoxins.

References

Centers for Disease and Control Prevention. 1995. Unexplained severe illness possibly associated with consumption of kombucha tea – Iowa. MMWR Morb. Mortal. Wkly. Rep. 44:892–900.

Chakravorty, S., S. Bhattacharya, A. Chatzinotas, W. Chakraborty, D. Bhattacharya, R. Gachhui. 2016. Kombucha tea fermentation: Microbial and biochemical dynamics. Int. J. Food Microbiol. 220:63–72.

Chen, C. and B.Y. Liu. 2000. Changes in major components of tea fungus metabolites during prolonged fermentation. J. Appl. Microbiol. 89:834–839.

Greenwalt, C.J., K. H. Steinkraus, and R. A. Ledford. 2000. Kombucha, the fermented tea: microbiology, composition, and claimed health effects. J. Food Prot. 63:976–981.

Jankovic, I. and M. Stojanovic. 1994. Microbial and chemical composition, growth, therapeutical and anti-microbial characteristics of tea fungus. Microbiologija 31:35–43.

Jayabalan, R., R.V. Malbaša, E.S. Lončar, J.S. Vitas, and M. Sathishkumar. 2014. A review on kombucha tea – microbiology, composition, fermentation, beneficial effects, toxicity, and tea fungus. Compr. Rev. Food Sci. Saf. 13:538–550.

Kallel, L., V. Desseaux, M. Hamdi, P. Stocker, and E.H. Ajandouz. 2012. Insights into the fermentation biochemistry of Kombucha teas and potential impacts of Kombucha drinking on starch digestion. Food. Res. Int. 49:226–232.

Kole, A.S., H.D. Jones, R. Christensen, and J. Gladstein. 2009. A case of kombucha tea toxicity. J. Intensive Care Med. 24:205–207.

Liu, C.H., W.H. Hsu, F.L. Lee, and C.C. Liao. 1996. The isolation and identification of microbes from a fermented tea beverage, Haipao, and their interactions during Haipao fermentation. Food Microbiol. 13:407–415.

Machado, R.T.A., J. Gutierrez, A. Tercjak, E. Trovatti, F.G.M. Uahib, G. de Padua Moreno, A.P. Nascimento, A.A. Berreta, S.J.L. Ribeiro, and H.S. Barud. 2016. *Komagataeibacter rhaeticus* as an alternative bacteria for cellulose production. Carbohydr. Polym. 152:841–849.
Marsh, A.J., O. O'Sullivan, C. Hill, R.P. Ross, and P.D. Cotter. 2014. Sequence-based analysis of the bacterial and fungal compositions of multiple kombucha (tea fungus) samples. Food Microbiol. 38:171–178.
Mo, H., Y. Zhu, and Z. Chen. 2008. Microbial fermented tea – a potential source of natural food preservatives. Trends Food Sci. Technol. 19:124–130.
Murugesan, G.S., M. Sathishkumar, R. Jayabalan, A.R. Binupriya, K. Swaminathan, and S.E. Yun. 2009. Hepatoprotective and curative properties of Kombucha tea against carbon tetrachloride-induced toxicity. J. Microbiol. Biotechnol. 19:397–402.
Nguyen, V.T., B. Flanagan, M.J. Gidley, and G.A. Dykes. 2008. Characterization of cellulose production by a *Gluconacetobacter xylinus* strain from kombucha. Curr. Microbiol. 57:449–453.
Reiss, J. 1994. Influence of different sugars on the metabolism of the tea fungus. Zeitschrift fur Lebensmittel-Untersuchung und-Forschung. 198:258–261.
Sievers, M., C. Lanini, A. Weber, U. Schuler-Schmid, and M. Teuber. 1995. Microbiology and fermentation balance in a kombucha beverage obtained from a tea fungus fermentation. System. Appl. Microbiol. 18:590–594.
Sreeramulu, G., Y. Zhu, and W. Knol. 2000. Kombucha fermentation and its antimicrobial activity. J. Agric. Food Chem. 48:2589–2594.
Stadelmann, E. 1961. Der Teepilz und Seine Antibiotische Wirkung. Zentbl. Bakteriol. Parasitenkd. Infektkrankh. Hyg. Parasit. Inf. Hyg. 180:401–435.
Teoh, A.L., G. Heard, and J. Cox. Yeast ecology of kombucha fermentation. 2004. Int. J. Food Microbiol. 95:119–126.
Tietze, H. 1996. Kombucha: The Miracle Fungus, 6th edn. Phree Books, Australia.
Vina, I., P. Semjonovs, R. Linde, and I. Deninxa. 2014. Current evidence on physiological activity and expected health effects of kombucha fermented beverage. J. Med. Food. 17:179–188.
Yang, Z.W., B.P. Ji, F. Zhou, B. Li, Y. Luo, L. Yang, and T. Li. 2009. Hypocholesterolaemic and antioxidant effects of kombucha tea in high-cholesterol fed mice. J. Sci. Food Agric. 89:150–156.

BIBLIOGRAPHY

Adams, M.R. In: Microbiology of Fermented Foods, Volume 1, 1998. B.J.B. Wood, ed. Blackie Academic and Professional (Chapman and Hall), London.
Adams, M.R. and M.O. Moss. 1995. Food Microbiology. Royal Society of Chemistry, Cambridge.
Budak, N.H., E. Aykin, A.C. Seydim, A.K. Greene, and Z.B. Guzel-Seydim. 2014. Functional properties of vinegar. J. Food Sci. 79:757–764.
Cleenwerck, I. and P. de Vos. 2008. Polyphasic taxonomy of acetic acid bacteria: an overview of the currently applied methodology. Int. J. Food Microbiol. 125:2–14.
Giudici, P., F. Lemmetti, and S. Mazza. 2015. Balsamic vinegars: tradition, technology, trade. Springer, Heidelberg.
Gullo, M., E. Verzelloni, and M. Canonico. 2014. Aerobic submerged fermentation by acetic acid bacteria for vinegar production: Process and biotechnological aspects. Process Biochem. 49:1571–1579.
Matsushita, K., H. Toyama, N. Tonouchi, and A. Okamoto-Kainuma. 2016. Acetic Acid Bacteria. Ecology and Physiology. Springer, Heidelberg.
Saichana, N., K. Matsushita, O. Adachi, I. Frébort, and J. Frebortova. 2015. Acetic acid bacteria: a group of bacteria with versatile biotechnological applications. Biotechnol. Adv. 33:1260–1271.
Solieri, L., and P. Giudici. 2009. Vinegars of the World. Springer-Verlag, Italy.
Yamada, Y. 2016. Systematics of acetic acid bacteria. In: Matsushita, K., H. Toyama, N. Tonouchi, and A. Okamoto-Kainuma. Acetic Acid Bacteria Ecology and Physiology. Springer, Heidelberg, pp. 1–50.

13 Distilled spirits

> Best whisky in the world prize won by Japanese single malt … Scottish distilleries are in shock.
> From a news item in The Independent, November 3, 2014

> For relaxing times, make it Suntory time.
> As said by Bill Murray in the film, Lost in Translation (screenplay by Sofia Coppola), referring to the iconic Japanese whisky.

Aye, one knows we are in a global food world (that may even be upside down), when the best Scotch-style single malt whisky is produced in Japan. Yet, this surprising event emphasizes one of the important themes of this book, namely that the production of high quality fermented food products is not determined by border. Nearly all of the fermentations described in this text could, in theory, be produced anywhere in the world, provided the manufacturer had access to the relevant raw materials as well as the necessary knowledge and skill.

It is also true that many food and beverage fermentations could be produced with minimum hardware, cultures, or other inputs. As noted previously, most raw food substrates support growth of microorganisms and if left on their own, will for better or worse, become fermented. In contrast, the manufacture of distilled spirits does require specialized distillation equipment and engineering know-how. Of course, the original still was nothing more than a simple extraction pot, but as described below, eventually more sophisticated devices were developed.

There are several common features among all distilled products. They all start with ethanol-containing starting materials, usually made from a cereal or grain mash or from wine. The separation of ethanol and other volatile compounds from the aqueous mixtures is based on the same distillation principles. The final distillates all contain concentrated ethanol. Despite these common features, however, the finished products and manner of manufacture can be very different. Thus, the specific starting materials can vary widely, as can the nature of the fermentation and the addition of flavoring agents or other ingredients. The distillation equipment and the conditions of distillation also can vary. Some products require a long aging period in specific types of barrels, and when the product is finally bottled it must meet particular regulatory or quality standards. Even the spelling depends on the product (i.e., whisky versus whiskey).

Microbiology and Technology of Fermented Foods, Second Edition. Robert W. Hutkins.

SOME DEFINITIONS

Distilled spirits refer to a wide range of products that includes whisky, gin, vodka, Tequila, brandy, and rum. Many of these products are associated with specific regions or countries. Hence, Scotch whisky, Irish whiskey, Bourbon whiskey, and Canadian whiskey are very similar products distinguished, in part, on the style and method of manufacture, but perhaps more so based on geographical considerations. These distilled spirits are produced and are popular on every continent, although there are also many other distilled products produced on a smaller scale and are less well-known.

Specific and legal definitions exist in the US, Canada, Europe and other countries for distilled spirits. In the US, the definitions for specific distilled alcoholic products are given in Chapter 27 (Alcohol Tobacco and Firearms) of the Code of Federal Regulations. According to this document, detailed standards of identity are given for gin, vodka, whisky, brandy, rum, and Tequila. Another group of products include cordials and liqueurs, made by addition of fruits, plants, or other natural flavoring materials to distilled products. Similar regulations exist (EC No. 110/2008) that define and describe distilled products produced in the European Union.

It is important to note that throughout the world, distilled spirits are not only important for culinary and cultural reasons, their production, marketing, and consumption also have important economic impacts. In particular, these products are heavily taxed, for domestic as well as export markets. Thus, they provide considerable revenues for the governments where they are produced and sold.

PRODUCTION AND CONSUMPTION

As noted above, although distilled spirits are produced around the world, most products are associated with particular geographic locations. Thus, by international agreements, Scotch whisky can only be made in Scotland, and Tequila is made only in Mexico. While production of other products, such as rum and vodka, are associated with the Caribbean or Eastern Europe, these products can actually be made anywhere. Likewise for Bourbon – although most is made in Kentucky, this style of whiskey is made throughout the US. Thus, apart from the labeling, marketing, and regulatory considerations, many of these geographical distinctions have become rather blurry.

Overall per capita consumption for distilled spirits has generally changed relatively little in recent years. In some regions consumption has actually decreased (Europe), whereas others have increased (India, China). Moreover, consumption of specific products appears to follow particular trends, such that sales of some products remain mostly flat (rum, vodka), while others are doing quite well. For example, whiskey consumption continues to increase due to the popularity of several specific products. Bourbon, in particular, has had near double-digit growth in the past decade (according to the Distilled Spirits Council). Likewise, consumption of single malt Scotch tripled from 2002 to 2016, and Irish whiskey increased by even more. Although Europe and the US have traditionally been the leading consumers, other regions around the world are now major consumers of whisky (Figure 13.1).

PRINCIPLES OF DISTILLATION

The principle of distillation is well-known to students who have completed a basic course in chemistry. Molecules dissolved in liquids heated to their boiling point will evaporate or volatilize. In an open system, they will be lost to the atmosphere. If the vapors are trapped and

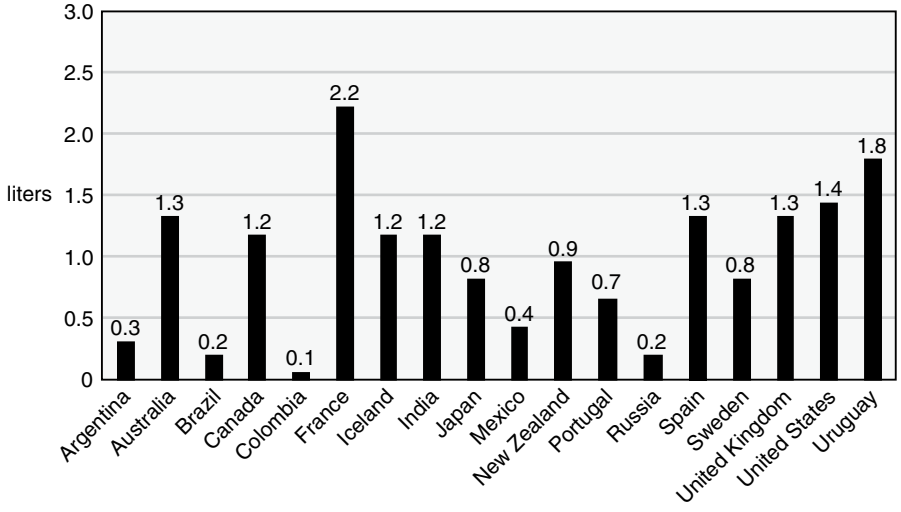


Figure 13.1 Global consumption (liters per person per year) of whiskey (2014). Source: Euromonitor International, 2014.

then cooled (among the main elements of a distillation apparatus), the molecules will condense. If a suitable collection system is in place, the condensed fractions can easily be separated from the original material. Since ethanol has a boiling point of 78.4°C, when an aqueous ethanol solution is heated, the ethanol becomes volatile before the water (boiling point = 100°C) and can be separated from the water phase. The distilled ethanol will be more concentrated, and, depending on the distillation conditions, may contain other volatiles that contribute color, flavor, aroma, and other properties.

Following a general discussion on the history of distilled spirits and an overview of the fermentation conditions and manufacturing methods, specific features of the main distilled products will be discussed.

HISTORY

Based on excavations in Egypt, Iraq, and other Middle East locations, archaeologists have estimated that distillation was probably first used around 5000 years ago. However, the first applications of this "technology" were not for alcohol, per se, but rather were for creating fragrances and perfumes, extracting oils from herbs, or for medicinal concoctions. Indeed, these early efforts at distillation were mostly performed by alchemists, the ancient forerunners of chemistry. The early distillation devices consisted of pots that contained an inner ring that was enclosed by a cover (Figure 13.2). When the liquid material was heated, the evaporated portion would condense on the inside of the cover, forming condensate droplets. The latter would slide down the sides of the pot and collect on the inner ring. Similar apparatuses, dated from about the same period, were also discovered in China.

The art of distillation eventually spread to other Mediterranean regions during the Greek and Roman Empires, as well as throughout Asia. Improvements included an "arm" that allowed the condensed material to exit the pot and collect in a separate vessel. Eventually, cooling water would be used to condense the vapors, and by the Middle Ages, glass distillation stills were developed.

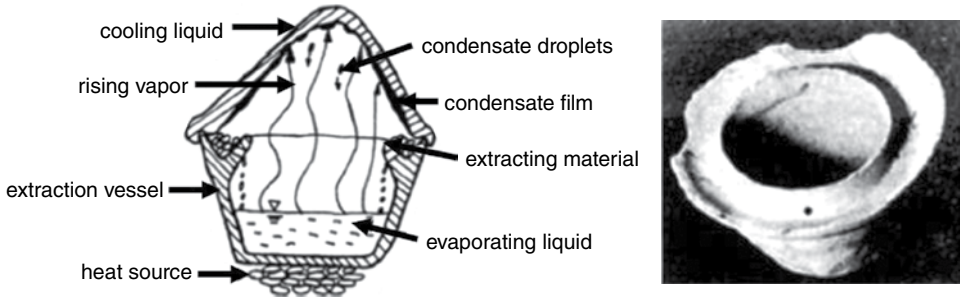


Figure 13.2 Early distillation device. From Kockmann, 2014.

As noted above, distillation was used primarily for materials other than alcohol. In the thirteenth century, the distillation of wine was described, although the alcohol recovered had medicinal value as a solvent and was not necessarily intended for consumption. In the Middle Ages, the manufacture of beer and wine was an "art" practiced by monks. Many of the early distilled spirits were therefore produced in monasteries. The fifteenth and sixteenth centuries marked the beginning of the scientific era and applications of the scientific method. In the seventeenth century, the famous chemist, Robert Boyle, showed that different substances could be separated by distillation, based on the different temperatures at which they boiled or evaporated. More modern distillation systems were soon introduced. Although alcohol was among the products produced by these stills, there was also considerable interest and value in industrial chemicals for non-food applications.

The early alcoholic products produced by distillation were usually made from wine. Since distilled alcohol occupied less volume than the starting material, such products had space and transportation advantages. The aesthetic and quality value was also appreciated. By the eighteenth and nineteenth centuries, distilled alcoholic products were well established in Europe, the UK, and the Americas, with many distilleries, some working in continuous mode, in operation.

Converting wheat, corn, and rye into alcoholic beverages had another advantage – namely this was an economical and efficient way to utilize excess grains. Owing to the high alcohol content, distilled beverages are exceptionally well preserved, more than the mash or wine from which they were made. The latter products, as noted in Chapters 10 and 11, are subject to microbial and chemical spoilage. In addition, small pot stills were widely present on farms so that distilled products could be made and sold, traded, or bartered. Of course, when grains for making bread or for other applications were in short supply, the reverse situation occurred – not enough grains were available to satisfy distillery demands.

The popularity of distilled products among the citizens throughout the world was keenly observed by governments, rulers, and other authorities. Collection of duty, taxes, excise, and other forms of restitution based on distilled spirits were common ways for authorities to raise funds. Revolts, wars, and rebellions occurred often as a result of tax-collecting efforts and other measures imposed on distilleries. In Scotland, Ireland, and the US, taxation and other policies led many individuals and entities to produce illicit spirits (commonly known in the US as "moonshine"). Despite the acknowledged economic impacts made by the distilled spirits industry (as well as wine and beer) in the US and elsewhere, there are still, even in 2017, debates about the appropriate level of taxation on this industry. In large part, taxation policies are driven by the societal costs of alcohol-related problems and to discourage over-consumption.

One specific historical event that had a major influence on the distilled spirits industry was the nineteenth century temperance movement that eventually led to the Prohibition era in the US. Passed in 1920, the 18th Amendment to the US Constitution (along with other ensuing laws) made alcohol production and consumption illegal. Overnight, hundreds of breweries and distilleries closed, and only a few, selling "medicinal" spirits were allowed to remain open. Even abroad, the impact was devastating, as distilleries in Scotland and Ireland were affected due to the loss of the US market. In contrast, other "export" producers managed better, as whiskey production in Canada and rum in the Caribbean continued, with products illegally smuggled into the US. Locally distilled products (e.g., bathtub gin) were also produced, much of it of questionable quality. When the 21st Amendment was passed in 1933, repealing the 18th Amendment, distilleries once again were opened and the industry was revived.

MANUFACTURE OF DISTILLED SPIRITS

The manufacture of distilled spirits involves several steps, some required and others optional (Figure 13.3). First, an ethanol-containing starting material must be prepared. This material is then distilled. The distilled ethanol or spirit can then be aged, flavored, used in other distilled products, or bottled as is. Each of these steps is described below in greater detail.

Starting materials

All distilled spirits start with the manufacture of an ethanol-containing mash. The specific substrates used for the mash are dictated by the product one intends to make. Thus, gin, vodka, and whisky are derived from cereal- and grain-based mashes, Tequila is from the agave plant, rum is made from sugar cane, and brandy starts with fermented grapes or other fruits. The main requirement is that the starting material contains either fermentable sugars or starchy substrates that can be converted to fermentable sugars.

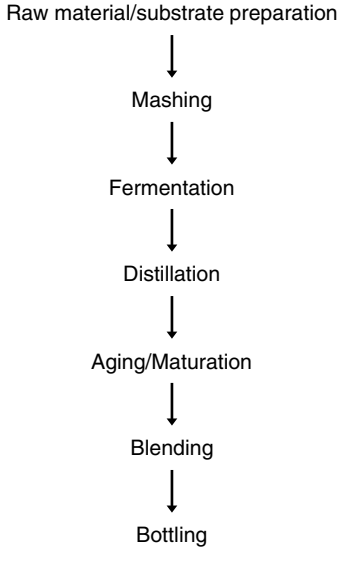


Figure 13.3 Simplified flow chart for manufacture of distilled spirits.

Historically, the choice of substrate was also influenced by what was available in the local area or region. Over time, particular distilled spirit products became associated with those regions. Eventually, the starting materials and methods of manufacture became codified by local or regional jurisdictions. Thus, for many of the cereal- or grain-based products, the specific type of material used is specified by government standards of identity or other regulatory requirements. This is particularly the case for the numerous whisky products produced in various regions. For example, Bourbon (a US-made whiskey) is made from a fermented mash containing at least 51% corn. Malt whisky, such as those produced in Scotland, must contain at least 51% malted barley. Likewise, rye whiskey requires that the mash contains at least 51% rye.

For products that are based on starch conversion, the first step is to form a mash. This process is similar to that described for beer manufacture. The preparation of the malt for some products, in particular, whisky from Scotland, has several unique features related to the kilning process (described below). Ultimately, malt is mixed with water, and the mixture is heated to extract amylolytic and proteolytic enzymes as well as starches and proteins. During mashing, enzymatic hydrolysis occurs and maltose, glucose, and isomaltose are formed from starches, and amino acids are formed from proteins. At the end of mashing, this fermented mash or wort serves as the fermentation substrate. It should be noted that starch conversion to fermentable sugars is also necessary for mashes containing potatoes (e.g., vodka). There is no boiling step for this mash (unlike mashes for beer), providing for the opportunity for additional hydrolysis of starches and oligosaccharides.

For other products not requiring a mashing step, the starting material is prepared according to the specific requirements for the subsequent fermentation. For example, brandy is derived from the distillation of wine (or other fermented fruit). However, the grape cultivars used to make wine for brandy are not necessarily the same ones used to make table wines. The time at which grapes are harvested and how the grapes are treated are also different. Somewhat different considerations exist for other products. For example, sugar cane is the starting material for rum, but the manner in which the sugar is actually used can vary for different types of rum products. The sugar can be in the form of molasses or as cane juice or cane syrup.

Fermentation and cultures

For many distilled sprit products, fermentations are performed in modern stainless steel fermentors, with capacities of over 500,000 L. Although fermentors constructed from wood still exist, they are infrequently used due to cost, maintenance, and sanitation challenges. Nonetheless, they can still be found in smaller whisky and other spirit distilleries.

The wort or starting mash is inoculated or pitched (usually less than 1%) with selected strains of *Saccharomyces cerevisiae.* These yeasts are available from commercial culture suppliers as cream yeasts, compressed yeasts, or dry yeasts. Some distilleries will use their own house cultures or other proprietary strains. Distilleries will also re-use their cultures via backslopping. An exception is for rum, for which naturally-occurring wild yeasts are sometimes used. For the most part, yeasts used for distilled spirits are the same or similar to those used for brewing. For products made from grain or cereal mashes, they should be able to ferment maltose and maltotriose to completion. Similarly, when sugar-containing fermentation medium is used (e.g., rum or brandy), the yeast should ferment glucose, fructose, and sucrose. In addition, yeasts for distilled spirits should be active at the outset, have rapid fermentation rates, and high conversion to ethanol. Tolerance to stresses, including temperature and ethanol is also important (Box 13.1). In general, fermentations should be complete within just a few days.

Box 13.1 Cultures, distilled

In the production of distilled spirits, it is understandable that the fermentation step does not receive as much attention as substrate preparation, distillation, maturation, or other processing steps. In the past, yeast cultures did their job and there was not much more to it than that. Nonetheless, there is now considerable interest in developing and using distiller's strains with specific traits and performance characteristics (Russell and Stewart, 2014; Walker et al., 2012).

For the most part, yeasts used for distilled spirits were historically derived from brewer and bakers strains of *Saccharomyces cerevisiae*. Indeed, for many years, whisky was made using yeasts supplied from local breweries. Currently, there are now several commercial sources (providing yeasts in either cream, compressed, or dry forms). However, some distilleries grow and manage their own strains. In either case, yeasts are chosen on the basis of several necessary attributes (Russell and Stewart, 2014). These include the ability to ferment sugars present in malt with full attenuation (i.e., complete fermentation) and appropriate flocculation. In addition, there are also "aspirational" traits – properties that distillers would like their yeasts to express, including high temperature and ethanol tolerance, growth in high gravity mashes, ability to utilize a arrange of substrates, and production of consistent flavors (Table 13.1.1; Walker et al., 2012). A yeast check-list was prepared by these authors, ranking the priority attributes yeasts should have for whisky and other distilled spirits.

Although few strains possess all of these traits, many of the genes that confer these phenotypes have been identified. In fact, entire genomes have been sequenced, and gene transfer systems are well established. However, consumer concerns and potential objections to genetically modified organisms have precluded potential applications. Nonetheless, traditional techniques for generating hybrid strains have been used. Indeed, most yeast strains are probably naturally occurring hybrids (Stewart et al., 2013).

Table 13.1.1 Aspirational traits for distiller's yeast[1].

Trait	Function
Consistent flavor	Relevant sensory characteristics
Temperature tolerance	Growth up to 40°C
Rapid fermentation rates	Fermentation times < 30h
Increased ethanol tolerance	Tolerant to 15% ethanol
Enhanced osmotolerance	Growth at high gravity (>1080)
Broad substrate range	Consume substrates, leave less behind

[1] Adapted from Walker et al., 2012.

References

Russell, I. and G. Stewart. 2014. Distilling yeast and fermentation. In: Whisky: Technology, Production and Marketing, 2nd edn. I. Russell and G. Stewart, eds. Academic Press/Elsevier, Boston, MA.

Stewart, G.G., A.E. Hill, and I. Russell. 2013. 125th Anniversary Review: Developments in brewing and distilling yeast strains. J. Inst. Brew. 119:202–220.

Walker, G., J. Brosnan, T. Bringhurst, and F. Jack. 2012. Selecting new distilling yeasts for improved fermentation and for sustainability. In: Distilled Spirits: Science and Sustainability. Walker, G.I., I. Goodall, D. Murray, and R. Fotheringham, eds. Nottingham University Press, Nottingham, pp. 1–11.

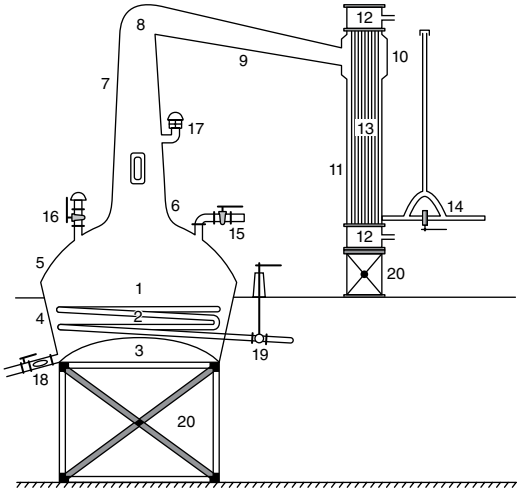
Distillation engineering

Two types of stills are used to distill ethanol in the beverage industry. Pot stills (also referred to an "alembic") operate only in the batch mode, whereas column stills ordinarily operate continuously. The prototype of the pot still was developed 2000 years ago. The more modern version, however, dates back only a few hundred years ago. The continuous still, in contrast, has only been around since the 1830s. It is now used to produce the majority (by volume) of distilled spirits produced around the world.

Pot stills

Although many improvements have been made, the pot stills used today are not much different than those made centuries ago. For example, many are made from copper, the original material used for these stills. Copper is a malleable metal with excellent conductance properties. Copper stills also have the added benefit of reacting with volatile sulfur compounds, limiting their escape into the distilled product. Nonetheless, many modern pot stills are now constructed with stainless steel but either are lined with copper or contain copper only at the upper portion of the still. Pot stills are also widely used, especially in Scotland and Ireland, where their use is required for malt whiskies, and in France for the manufacture of cognac. Pot stills are also used for gin.

At the simplest level, there are five main parts to the pot still – the heating medium, the boiling pot or vessel, the head, the neck, and the condenser (Figure 13.4). Even a basic still,



Key

1. Pot
2. Steam heating coil
3. Crown
4. Flue plate
5. Shoulder
6. Ogee
7. Swan neck
8. Head
9. Lyne arm/lye pipe
10. Vapour chamber
11. Shell and tube condenser
12. Water jacket
13. Tube bundle
14. Tail pipe with siphon
15. Changing line/valve
16. Air valve
17. Anti-collapse valve
18. Discharge line/valve/sight glass
19. Steam line/valve
20. Cradle

Figure 13.4 Components of the traditional pot still. From Nicol, 2014 with permission.

however, will be a bit more involved than this. For example, almost all modern pot stills also contain a rectifier section whose function is to separate some of the undesirable volatile portions in the vapor from the ethanol. There are also variations based on particular product applications. Thus, for the manufacture of cognac and other types of brandy, devices called Charentais(e) stills are used, which are very similar to the traditional pot still except for the unique design of the head. Of course, another difference between stills is size – some are as small as a few thousand liters, whereas others can hold more than 100,000 liters.

Heating occurs by one of several ways. The most common configuration uses coils located at the bottom of the vessel that circulate steam. Older stills use gas-fired heating from just below the still. The latter can result in cooked-on material that affects product quality as well as heat transfer. In either case, the product is heated in the pot or kettle. The heated ethanol volatilizes at 78.3°C (at sea level), and the vapor collects at the top or "head" of the pot and is discharged through the neck. The latter is sometimes referred to as the Lyne tube. The ethanol and other volatiles then reach the condenser and the distillates are collected.

Not all of the volatiles are immediately removed from the pot still. A portion of the vapor condenses on the vessel wall in the upper sections and is returned to the pot. This is called reflux and can affect product composition. The amount of material refluxed depends mainly on the configuration of the still.

The composition of the condensed product that collects over time is not uniform. The first portion, referred to as the "foreshots" or "heads", contains the most volatile compounds (i.e., those with the lowest boiling points). Although ethanol is present in this fraction at high concentrations (about 70–80%), so too are several undesirable compounds, including methanol, acetone, and acetaldehyde. Fusel oils, consisting of higher alcohols like butanol and propanol, are less soluble and also appear in the heads. Collectively, these materials are referred to as "congeners".

Because this first fraction contains a high concentration of objectionable compounds, it is separated as it collects from the condenser and is excluded from the final product. Following this first "cut, however, the second (so-called "heart" or middle) fraction is collected, forming the main product. This material contains less ethanol (about 60–70%), but also less congeners.

Ultimately, after the next "cut" a third fraction, called the "tails", is collected. It initially contains about 60% ethanol and less as distillation continues. The compounds with the highest boiling point will also be present in this fraction. The heads and tails are usually combined, and re-distilled to recover the ethanol. When the distiller actually makes the cut between the heads, heart, and tails has a major influence on the composition and quality of the final product. Likewise, the temperature, reflux conditions and other operating parameters can also affect the concentration of different constituents and overall product quality.

The construction of the basic pot still, as noted above, can also be modified to enhance the purity and quality of the product. For example, the length, shape, angle, and position of the Lyne arm influence the rate at which volatiles are vaporized or returned as reflux. Another still component, the condenser, can not only affect how energy (i.e., heat) is conserved, but can also affect product quality. Two main configurations exist – shell-and-tube and worm condenser; the former is now more common.

Perhaps most importantly, however, is the addition of a second or even third distillation step. Each successive distillation results in an increase in the ethanol concentration and decrease in congeners and other impurities. Thus, Scotch whisky is distilled twice and Irish whiskey is distilled three times.

Continuous stills

In the early 1800s, various continuous stills were developed. One of the first such stills used commercially in Scotland (but not successfully) was patented by Robert Stein in 1828. However, a much improved version was patented and manufactured by Aeneas Coffey in 1830. The Coffey continuous still (or simply the "patent still") revolutionized the whisky industry. Manufacturers of other distilled spirits also adopted this technology.

The continuous still had many advantages, not the least of which was its economical operation. Pot stills are one batch-at-a-time and require cleaning between batches. In contrast, in continuous stills, the fermented, alcohol-containing liquid is fed continuously into the still and product containing the concentrated ethanol is collected without interruption and without having to disassemble equipment. Product throughput can be increased dramatically.

Continuous stills contain several key components and features that are similar, but somewhat different than those that comprise the pot still. Rather than heating the starting material (or wash) directly or via heating coils, heat is supplied in the form of steam. Instead of a pot- or bowl-shaped vessel, continuous stills use long columns. The latter have a unique construction, such that very high concentrations of very pure ethanol can be achieved.

The Coffey continuous still, as were other versions that followed, contained two main components, called the "analyzer" and the "rectifier" (Figure 13.5A). Positioned within both of these columnar structures are horizontal plates. The process starts by the continuous addition of the wash (i.e., the fermented mash) into the top portion of the analyzer component. Perforations in the plates allow the liquid to drip to the plate below. At the same time, steam is introduced at the bottom of the column. As the steam rises, it heats the liquid moving in the opposite or counter-current direction. The liquid at the bottom, closest to the source of the steam, is hottest and the volatiles are vaporized, rising upward. As the vapors reach the cooler upper plates, those that have higher boiling points condense and are refluxed. Those with lower boiling points make it further up the column. Eventually, those volatiles with the lowest boiling points (including ethanol) emerge from the top of the column. High boiling point compounds (including water) collect at the bottom of the column and can be removed along with the stillage material.

The volatile material that exits the analyzer is then introduced into the bottom of the second column, the rectifier. This column operates much the same as the analyzer, providing greater purity of the product that emerges at the top. In the Coffey still, the wash actually passes through the rectifier section first via copper tubing. This provides a basis for preheating the wash (and conserving energy) prior to entering the first analyzer column. The vapor is then condensed and collected.

Single column stills are also common. For these stills, rectification occurs in the upper portion of the column and is enhanced by increasing the number of plates (Figure 13.5B).

PRODUCTS

Hundreds of different distilled spirit products are produced globally. They are grouped in various ways. For example, it is often convenient to classify them simply as brown or clear. According to the US Standards of Identify, distilled spirits are defined broadly by "class" and specifically by "type". Thus, whisky is a class and Scotch whisky is a type. Gin is also a class, with three specific types defined by US standards – distilled, redistilled, and compounded gin (see below). European regulations are similar, but differences exist. For example,

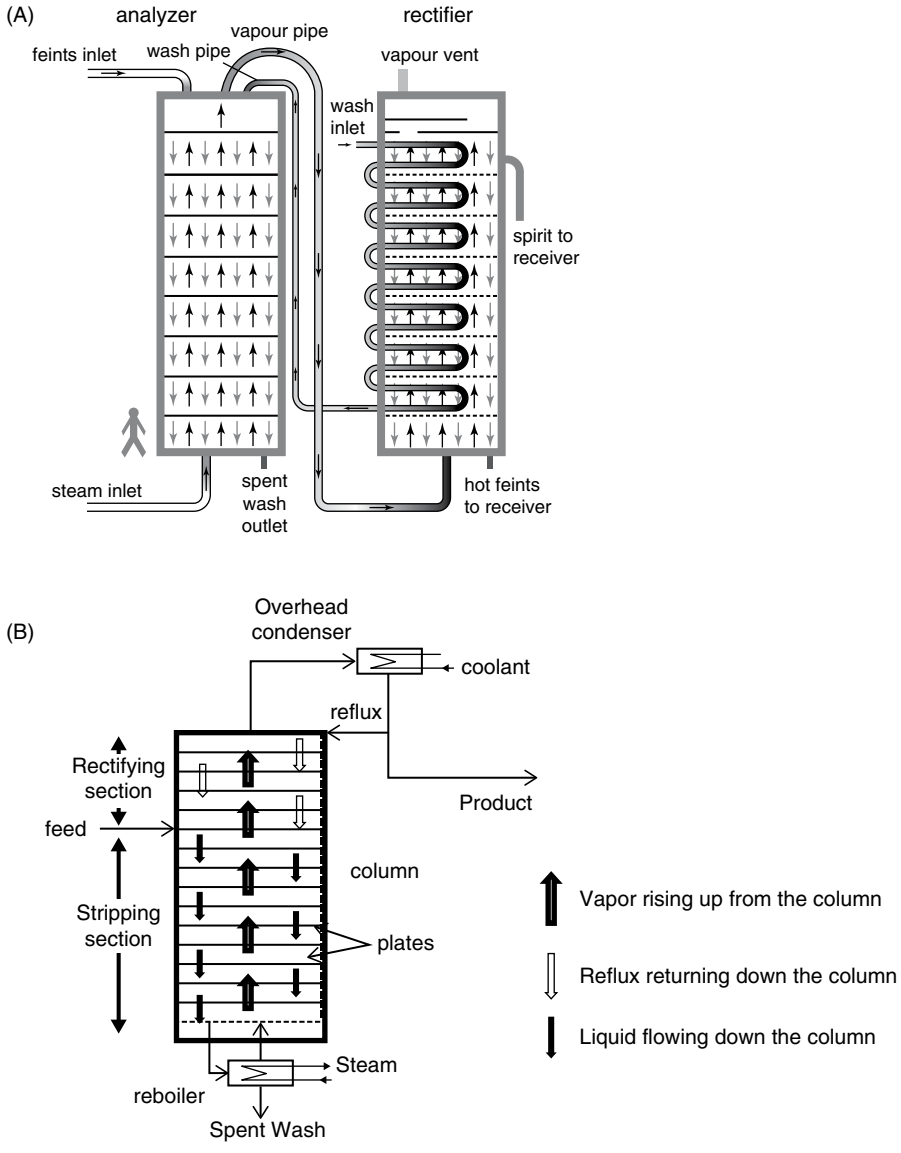


Figure 13.5 Major components of the distillation column. Separate analyzer and rectifier sections are shown in A (courtesy of the Scotch Whisky Association) and a combined column is shown in B. From Murray, 2014 with permission.

while three types of gin are also recognized, the EU refers to them as gin, distilled gin and London gin.

Neutral spirits

The most basic of all distilled spirits are neutral spirits. These can be made from nearly any starting material and are distilled to achieve high ethanol concentrations (>95%). When bottled, they must have at least 40% ethanol (80 proof), meaning that can be diluted with

water to achieve the desired proof. Neutral spirits are colorless and have little flavor. Vodka, described in more detail below, is an example of a neutral spirit.

Whiskey

Of all the fermented products described in this text, perhaps none is as connected to the regions of its production as is whisky. Thus, in Scotland, whisky (spelled sans "e") is as much a part of the national and cultural identity as are castles, lochs, and famous golf courses. Whisky manufacturing is certainly important to the Scottish economy, accounting for 5 billion pounds of economic impact and 80% of all food and drink exports (Scotch Whisky Association Report, 2015). Likewise, whiskey (spelled with "e") has been a major part of the Irish culture and economy for several centuries. Although developed more recently, India and Japan also have substantial whiskey manufacturing capacity and markets.

In North America, whiskey production and consumption are also part of the cultural landscape. Colonists began producing a form of whiskey around the mid-seventeenth century, and by the time of the Revolution, General George Washington organized construction of whiskey stills to provide nourishment for soldiers. A less popular directive was issued a few years later when President George Washington called for a tax on whiskey distilleries. Whiskey was so important to the nation's first president that a distillery he built in his home was once the largest distiller in the entire country.

Whiskey has remained an important part of American culture and commerce. Whiskey production is especially important to the regional economies in Tennessee and Kentucky. Since about 2005, increased domestic and international demand for Bourbon whiskey has been a tremendous economic boon, particularly in Kentucky, which produces 95% of the world supply of Bourbon. Growth in the US Bourbon market has even affected auxiliary industries, such as the cooperage industry that makes the barrels in which Bourbon is aged. Although Bourbon is most often associated with distillers and brands located in Kentucky, Bourbon refers to a type of whiskey made from a particular mash. It can be produced anywhere. Indeed, consumer interest in "local" and "craft" produced products has led to many new Bourbon distilleries across the US.

Apart from the geographical distinctions, whiskies are also grouped according to the nature of the starting material and the means by which distillation occurs. For example, Scotch malt whiskey is made from 100% malted barley and is distilled using batch distillation (see below). In contrast, Scotch grain whiskey is made from a mixture of cereals and malted barley and is distilled using continuous stills.

How the malt used for whiskey is prepared is of particular importance and can impart unique characteristics to the final product. This is because the kilning step used in Scotland traditionally relied on burning peat to provide the heat necessary to dry the malt. This peat step introduces smoky flavors and aromas into the malt which then carry over into the Scotch whisky. Different Scotch whiskies are often described based on the extent to which peat or smokiness is present.

Whiskey manufacture

Whisky (or whiskey) is produced around the world. Each of the main producers, Scotland, Ireland, the US, and Canada are known for their distinctive styles and method of manufacture (Figure 13.6). In the next section, the main general manufacturing steps will be reviewed, with an emphasis on the unique features associated for different whiskies.

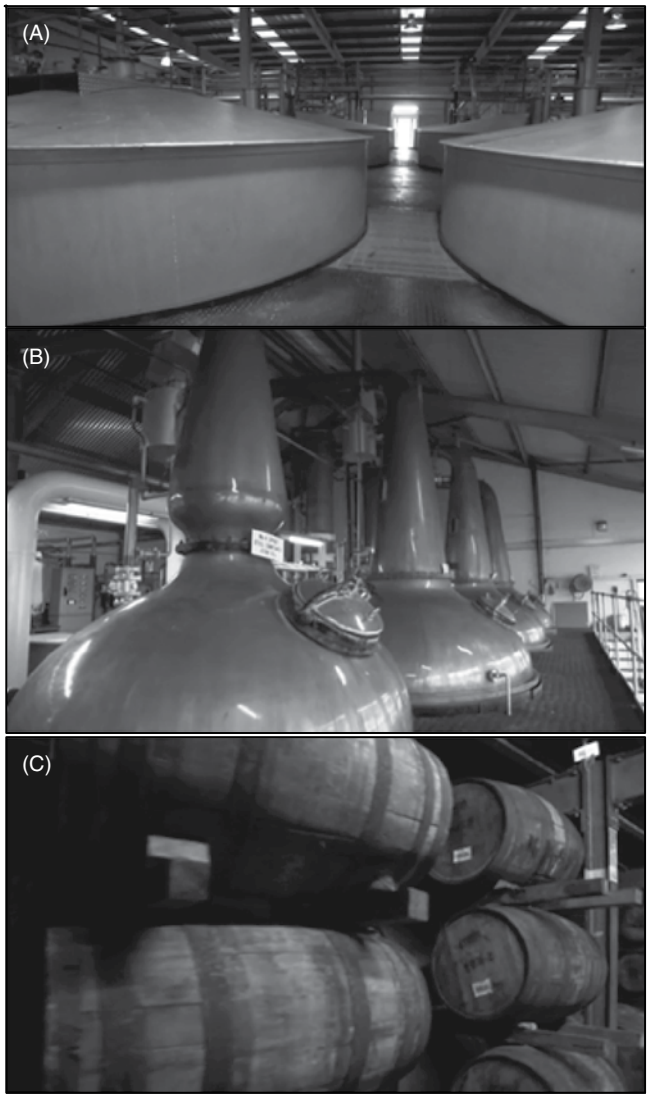


Figure 13.6 Manufacture of Scotch whisky, Mash tun (A); Still (B); and barrel aging (C). Images courtesy of the Scotch Whisky Association, Edinburgh, Scotland.

Mashing and fermentation

Ultimately, all whiskey, whether with or without the "e", or whether produced in batch or continuous stills, refer to a class of distilled spirits made from a fermented grain mash. In the US more than 30 different types of whisky are defined (some of the main ones are described in Box 13.2). Despite the many different types of whiskies, whether from Scotland or elsewhere, they share similar manufacturing steps and common equipment.

As noted above, malt whiskey is made from a mash containing germinated barley malt. In the US, at least 51% malt is required. In Scotland, malt whisky must be made from 100% malted barley. Note that for both, other criteria also exist, as described below. The malt

Box 13.2 Spirit nomenclature, straight up

In the United States, the federal entity responsible for regulating distilled spirits is the Alcohol and Tobacco Tax and Trade Bureau or TTB. The TTB publishes Standards of Identity for the most common distilled spirits in the Beverage Alcohol Manual (BAM).

The Standards of Identity, described below, are from the BAM. Although specific for the US, they are not that different from those used in Europe and other countries. Also, note that the required alcohol and proof for most of these products are not detailed below, they are indeed stated in the BAM.

Distilled spirits. Refers to one of several broad classes or specific types of distilled ethanol products, including whisky, rum, brandy, and gin.

Neutral spirits. A class of spirits distilled from any material at or above 95% alcohol by volume; bottled at not less than 40% alcohol by volume (80 proof). The latter requirement (40% alcohol, 80 proof) is common to most distilled spirits.

Grain spirits are a type of neutral spirits that are distilled from a fermented mash of grain and stored in oak containers.

Vodka is a type of neutral spirit that is distilled after treatment with charcoal or other materials, so that the final product is without distinctive character, aroma, taste, or color.

Gin is a class of distilled spirit whose main characteristic flavor is derived from juniper berries that is produced by distillation or mixing of spirits with juniper berries and other aromatics or extracts derived from these materials. Three types of gins are defined:

- **Distilled gin** is produced by original distillation from mash with or over juniper berries and other aromatics or their extracts, essences or flavors.
- **Redistilled gin** is produced by redistillation of distilled spirits with or over juniper berries and other aromatics or their extracts, essences or flavors.
- **Compounded** gin is produced by mixing neutral spirits with juniper berries and other aromatics or their extracts, essences or flavors.

Note that in Europe, gins are similarly produced, but are named somewhat differently. For example, London gin is analogous to the US redistilled gin.

Brandy is a class of distilled spirit made from the fermented juice, mash or wine of fruit or from its residue and having the taste, aroma and characteristics generally attributed to brandy. Many types of brandy are recognized, including Cognac (brandy distilled in the Cognac region of France in compliance with the laws and regulations of the French Government).

Whisky is a class of distilled spirit made from a fermented mash of grain produced in such a manner that it possesses the taste, aroma, and characteristics generally attributed to whisky. Many different types are defined, some based on raw materials or manufacturing criteria, but geography is also very important. Note that BAM spells all whiskey/whisky products without the "e". The main types include:

- **Bourbon whisky** is produced in the US from a fermented mash of not less than 51% corn and stored in charred new oak containers.
- **Rye whisky** is produced from a fermented mash of not less than 51% rye and stored in charred new oak containers.
- **Straight whiskey** is produced from a fermented mash of less than 51% of any one type of grain and stored in charred new oak containers for 2 years or more.
- **Scotch whisky** is a distinctive product made in Scotland, in compliance with the laws of the United Kingdom.
- **Irish whisky** is a distinctive product of Ireland, manufactured either in the Republic of Ireland or in Northern Ireland, in compliance with their laws regulating the manufacture of Irish whisky.
- **Canadian whisky** is a distinctive product of Canada, manufactured in Canada in compliance with the laws of Canada regulating the manufacture of Canadian whisky.

Rum is a class of spirit distilled from the fermented juice of sugar cane, sugar cane syrup, sugar cane molasses, or other sugar cane by-products that has the taste, aroma, and characteristics generally attributed to rum.

Tequila is a class of spirits distilled from a fermented mash derived principally from the Agave Tequilana Weber, with or without additional fermentable substances, made in compliance with the laws and regulations of the Mexican Government, and having the taste, aroma and characteristics generally attributed to Tequila.

Cordials and liqueurs refer to a class of flavored spirits containing not less than 2.5% by weight sugar, dextrose, levulose or a combination. They are made by mixing or redistilling any class or type of spirits with or over fruits, flowers, plants or pure juices, from other natural flavoring materials, or with extracts derived from infusions, percolation or maceration of such materials.

serves as the source of substrates and as the source of enzymes (which the reader should readily note is analogous to beer). The malt is ground, water is added, and the mixture is heated to about 60–70°C for up to 2 hours. For grain whiskey, other cereals are used in the mash, including corn, wheat, or rye. However, malt must still be added for saccharification. For these mashes, the grain must first be pre-heated (boiling for 1–2 h) to gelatinize the starch prior to the mashing step.

Similar to brewing, the mash at this point (called wort) contains fermentable sugars and free amino acids, as well as spent grains. The latter is separated by filtration, and the wort is transferred to fermentors. One difference between wort for beer and the wort described here is that there is no kettle boil step. Thus, during the fermentation step, hydrolysis of starch and protein continue to occur. Large distilleries will use large fermentors, up to 100,000 L. Selected strains of *S. cerevisiae* are then added (or pitched). Fermentation occurs at 20–30°C for up to 3 days, yielding about 10% ethanol. This liquid is then used as the starting material for the distillation step.

Distillation

Two types of stills are used for distillation of whiskey. Batch stills are the traditional stills used for malt whisky mashes in both Scotland and Ireland. Indeed, for many years, all distilled spirits were made using batch stills. Continuous stills were introduced in the 19th century. They are now used for grain whisky in Scotland and for Bourbon and other whiskies produced in the US and Canada.

As noted from the discussion above, Scotch malt whisky and Irish malt whiskey are distilled two or three times, respectively. For Scotch, in the first distillation, the fermented mash or beer, containing about 8–9%, is fed into the wash still. The material is boiled and the distillate, called the "low wines" is collected. It will contain about 24–26% ethanol, representing a three-fold concentration. The low wines distillate is then fed into the second or spirit still. It is at this point where the critical separation of the heads, the whisky (or heart), and the tails occurs. The heads and tails are combined and returned to the spirits still. The whisky (about 63–70% ethanol) is collected in a "spirit safe", a vessel that is literally under lock and key. In nearly every jurisdiction, this careful handling and accurate measurement of the product is necessary because taxes must be paid based on the ethanol content.

Production of Irish whiskey is similar. The low wines from the first distillation are divided into "strong low wines" and "weak low wines". The latter are redistilled in the "low wines" still yielding "strong feints" and "weak feints". The former are again re-distilled, generating heads, tails, and whiskey. Due to the additional distillation step, Irish whiskey will contain ethanol concentrations of 88–92%.

Aging and maturation

Although the distilled product is drinkable, it has relatively little flavor or character. During aging, flavor and color development occurs and the whisky becomes smoother and more complex. These enhancements, however, do not occur unless the whiskey is aged in suitably prepared oak barrels. This is because the oak provides many of the reactants necessary to generate flavor and color compounds. The extraction of these substances from the wood is enhanced by ethanol. Because the oak barrels are permeable to air, many of the chemical reactions that occur are oxidations or influenced by the presence of oxygen.

Cooperage science

Barrels and casks have been used as vessels to store liquids and foodstuffs for thousands of years. Humans had learned how to bend wood presumably for making boats, baskets, and tools, and the same techniques were likely used for making barrels. As storage vessels, they had many advantages compared to other types of vessels (e.g., pottery or leather). Barrels could be rolled and were easy to move, they could be stacked, they could be sealed, and they were durable. Barrel-making was so important that by the Middle Ages an entire trade comprised of skilled coopers (who shaped the wood) and hoopers (who banded them together) had evolved. Moreover, barrel-making (or "cooperage") was not only practiced throughout Europe and the UK, but also in Asia. Apart from its interesting origins and history, cooperage technology continues to be a very important part of the distilled beverage industry (Box 13.3).

Barrels have long been the vessel of choice for ethanol-containing liquids like whisky, rum, brandy, beer, and wine. For many of these products, humans learned that wooden

Box 13.3 The wood barrel – an essential ingredient in whiskey manufacture

The successful manufacture of whiskey, rum, brandy, and other distilled products requires high quality malt, corn, rye or other raw materials. There is another "raw material", sometimes over-looked, that has a major influence on the quality of the finished product. Specifically, much of the flavor, aroma, color, and overall sensory appeal of these products are formed directly from constituents present in the barrels (Piggott and Conner, 2003). In addition, wooden barrels provide the ideal environment, with the perfect atmospheric conditions, in which most of the relevant biological, chemical, and physical reactions occur (Figure 13.3.1A). Simply stated, without the barrel, whiskey would not exist (Work, 2014). Thus, it is not surprising that an entire industry, devoted to making and preparing barrels, has developed.

History

Barrel-making is an ancient art with a long history (nicely reviewed by Twede, 2005 and Work, 2014). Indeed, long before their flavor-enhancing qualities were recognized, barrels were valued as durable containers or "packages" for a variety of liquid and non-liquid food products. In addition, Work (2014) noted that barrels were also the "container of choice" for gunpowder, nails, animal skins, and a range of other non-food products. Archaeological evidence suggests that wooden barrels were being made in the Middle East 5000 years ago. Eventually, barrels began to be used for wine, and by the Roman era, a barrel-making industry had formed, presumably having adopted tools and technology used for ship-building.

Although there were not many alternatives, barrels still had many advantages compared to pottery or metal. Wood was strong, inexpensive and plentiful. Then there was the shape – on its side, even a full barrel weighing more than 100 kg could easily be moved. They could also be stacked into a compact pile for storage or shipping.

So important was barrel-making that by fifteenth century, a sophisticated "cooperage" industry had emerged. Highly trained craftsmen hand-made the staves and heads, the metal hoops, and then assembled each barrel, one-at-a-time. There were guilds and eventually unions that organized. With the Industrial Revolution, barrel-making became more mechanized, but at about the same time, other ways to store, package and transport food materials were introduced. One of the few industries, however, to stick with barrels were the wine and whiskey industries.

Why barrels?

Tradition, perhaps, is one reason why barrels have continued to be used for these products. In theory and in practice, whiskey and wine could be stored in stainless steel vats, to which wood shavings had been added to contribute flavor and color. However, this type of maturation is not often done. Indeed, most whiskey, rum, and distilled wine products are required (by local statutes) to be held in barrels having a specific composition for a specific time. The barrel is so much a part of the whiskey industry that "single barrel" and "single cask" products are now being marketed. Interestingly, while barrels have long been used for storing whiskey, the deliberate practice of aging

and maturation is rather recent (Conner, 2015). Prior to the 1900s, most whiskey was consumed without aging.

Barrel composition

Barrels for whiskey are almost always made from oak (genus *Quercus*). The most common type is American white oak (*Quercus alba* is the main species) although European oak (*Quercus robur*) is not uncommon. About 1,000,000 whiskey barrels are made each year from American white oak (Work, 2014). For barrels made from either French or American oak, the trees must grow for 100–200 years.

Currently, the barrel type, as well as aging time in the barrel and alcohol strength (or proof), are regulated by Alcohol and Tobacco Tax and Trade Bureau or TTB. Similar regulations exist in other countries. There are major differences, however, between how the barrels are treated. First, according to the TTB, the barrels used for Bourbon and other American whiskies must be newly constructed; thus they can only be used once. Second, the interior must be charred. This means that the inside is exposed to gas-fired flames with temperatures of nearly 1000°C (Figure 13.3.1B). Presumably, the charring step was initially performed to sterilize previously used barrels. However, another major benefit eventually became evident. Specifically, the act of charring also generates hundreds of heat-induced compounds from precursors in the wood (see below). Thus, when neutral spirits were added to these charred barrels and allowed to age, all of the sensory attributes that we now associate with whiskey had developed.

Aging conditions

In contrast to American whiskey, Scotch whisky as well as Canadian, Irish, or Japanese whiskies are aged in previously used barrels (e.g., either from Bourbon or sherry barrels). Although many of the flavor, aroma, and color compounds would have been extracted in the original maturation period, there are still an abundant and diverse range of compounds that will be extracted into the re-filled whiskey.

Barrels are not impermeable to water vapor, so that as evaporation occurs, the contents can become more concentrated. Likewise, alcohol can also evaporate into the atmosphere. The extent to which water and ethanol are lost depends on the ambient

(A) (B)

Figure 13.3.1 Barrels during aging of whisky (A), and the charring step that occurs prior to filling (B). Photos courtesy of the Jack Daniel's Distillery.

temperature and the relative humidity in the maturation room or warehouse (also called rickhouses). These rooms typically are not climate-controlled, so fluctuation is normal. In general, for example, high temperatures increase the rate at which both ethanol and water will evaporate, whereas high humidity increases ethanol evaporation more so than water. Conversely, at low humidity, less ethanol will evaporate, leading to higher ethanol concentrations in the barrel. When the latter occurs, the ethanol level can be higher than permitted by the local regulation, so that a dilution step is required. Alternatively, appropriately-labeled "cask strength" whiskies, that can exceed 100 proof, may be permitted.

References

Piggott, R. and J. Conner. 2003. Whiskies. In: A.G.H. Lea and J.R. Piggott, eds. Fermented Beverage Production, 2nd edn. Springer Science, New York, pp. 239–262.

Twede, D. 2005. The cask age: the technology and history of wooden barrels. Packag. Technol. Sci. 18:253–264.

Work, H. 2014. Wood, Whiskey and Wine: A History of Barrels. Reaktion Books. London.

barrels contributed critical flavor, color, aroma, and other organoleptic attributes. Indeed, for many distilled beverages, maturation and flavor development will not occur unless the product is aged in barrels. After all, as noted above, whisky right out of the still has no color and has a mostly harsh flavor. Indeed, this material would not be much different from a neutral spirit like vodka or gin. Thus, aging in wood makes all the difference.

Whisky barrels are made from oak, either American white oak or French (or European) oak. Since chemical constituents in the wood are extracted into the product and react chemically, the composition of the wood is very important. American oak generally contains higher levels of specific lactones that serve as a source of vanilla flavors. French oak barrels, in contrast, provide more tannic flavors. How the barrels are treated prior to filling is also critical. In particular, whisky barrels are charred such that major changes to the interior wood develop. Specifically, caramelization and Maillard browning reactions occur, and hundreds of new flavors and pigments are formed that eventually end up in the whisky.

Blending

Whisky is only rarely bottled directly from the barrels or casks. This is mainly because the ethanol content is too high. Going into the barrel, whisky is about 70% ethanol, much too high for bottling. Moreover, some moisture is lost during aging, so the ethanol concentration can increase even more. Only very small amounts are bottled straight from the cask, as these products are high in ethanol and have pronounced flavor and aroma characteristics. In contrast, most whisky is diluted with water to give whatever proof is required, usually 40–48%.

As noted above, malt whisky made in Scotland must contain 100% malted barley. When Scotch malt whisky is made from a single distillery, using pot stills, and is aged for a minimum of 3 years in oak casks, the whisky can be called "single malt Scotch whisky". Although these whiskies have been made for hundreds of years, this designation has, in the past 20–30 years, gained an enviable reputation.

The other major way Scotch whisky is produced is as blends. However, in contrast to the relative simplicity of single malt whisky, the nomenclature for other products can be somewhat more complicated. Thus, there is "blended malt Scotch whisky" – whisky that contains single malt from two or more distilleries. Scotch whisky can also be made, as described above, from grains other than barley malt. This so-called grain whisky is also distinguished from malt whisky by virtue of how it is distilled – continuous instead of batch. Grain whisky has less flavor and color and is less expensive to make. Although grain whisky has a very small market, most is used for blending. Specifically, grain whisky can be blended with malt whisky to give "blended Scotch whisky". On a volume basis, this one of the most popular forms of Scotch whisky consumed worldwide.

Ireland, of course, also produces all malt and blended whiskies, however, their manufacture is somewhat different (e.g., they are distilled three times). Although "single malt" versions of Irish whiskey are produced, they are not subject to specific regulations as are single malts from Scotland.

Despite the caché for "single malt Scotch whisky", blending malt and grain whiskies from one or more distilleries serves several important functions. First, adding grain whiskey to malt whiskey can also help to round out or balance the flavor. Blending of different batches provides a basis for producing a product with greater consistency and minimizing batch-to-batch variations. Finally, single malt whisky is more expensive, so blending with grain whisky reduces the cost and makes Scotch whisky more affordable.

Bourbon and other whiskies from North America

In North America, definitions and nomenclature for whiskey are quite different and more complicated (especially in the US). There is no real equivalent for the single malt whisky produced in Scotland. Indeed, malt whiskey in the US need only contain 51% malted barley to be called "malt whiskey". There is also no requirement that US whiskey must come from a single distillery or that it be produced via batch distillation. Thus, in the US, blending and continuous distillation are the norm. However, another importance difference between whiskey produced in the US versus Scotland or Ireland is the use of grains other than barley. In particular, rye and corn are frequently used. Indeed, many of the most well-known American whiskies have gained their reputation on the use of these alternative grains.

The fermentation for Bourbon whiskey is similar to other whiskies in that they all rely on *S. cerevisiae*. However, a technique known as "sour mashing" is often practiced in the US prior to the yeast fermentation. For these whiskies, a portion of the mash is inoculated with suitable lactobacilli and allowed to grow and acidify the mash until the pH decreases to about 3.8. Heat is applied (100°C) to inactivate the bacteria and the sterile sour mash is then inoculated with yeast and allowed to actively ferment before being added to the main mash.

The main type of whiskey produced in the US is Bourbon whiskey. As defined by the US Code of Federal Regulations, "Bourbon whiskey" is whiskey made in the US from a mash containing not less than 51% corn, distilled to an alcohol concentration of not more than 80% (by volume), stored at not more than 62.5% alcohol, and bottled at not less than 40% alcohol. Another requirement is that the whiskey must be aged in charred oak barrels. Importantly, the barrels must be new for each batch. Given the recent global popularity and increased consumption of Bourbon, the new barrel requirement has had considerable impact on the cooperage industry, especially in Kentucky where most Bourbon is produced.

In contrast to Bourbon whiskey and malt whiskey, when the main part (at least 51%) of the mash is rye, the whiskey can be called "rye whiskey". Rye whiskey must also meet the other criteria (i.e., aging in new charred casks). Rye has its own characteristic spicy flavor and appeal. Yet, another type of US whiskey is called "straight Bourbon whiskey". In this context, the descriptor "straight" refers to whiskey (whether Bourbon, rye, or malt) that contains no added flavors or colors or added spirits and is aged for at least two years.

Although Bourbon can be produced anywhere in the US, as much as 95% is produced in one state, Kentucky. The neighboring state of Tennessee is also a major producer of whiskey. Although most Tennessee-produced whiskies satisfy the requirements to be called straight Bourbon whisky, they undergo one additional step during their manufacture. Specifically, Tennessee whiskey is filtered through sugar maple charcoal after distillation and before aging in charred oak barrels. While Tennessee whiskey is recognized by various trade agreements, it does not have its own US standard of identity.

Collectively, Kentucky and Tennessee produce most of the whiskey produced in the US and their domestic and export markets have grown dramatically. Recently, craft manufacturers from across the US have begun to produce and distill whiskey for smaller, local markets. Just like for artisan cheese, beer, wine, and sausage products, this emerging market for micro-distillery whiskies reflects the growing consumer interest in high quality fermented foods and beverages. In addition, many of the major whiskey distilleries in the US have introduced "single barrel" or "small batch" product lines with long aging periods to attract consumers willing to pay premium prices for such products.

Finally, Canadian whiskey also has its own unique properties and method of manufacture. Although Canada has a reputation for rye whiskey, these products are different from the typical American rye. In particular, Canadian whiskey is usually made from a mash containing less than the 51% rye required for US rye whiskey. Instead, most of the mash is made from corn. Because rye grown in Canada is described as being more "spicy" than rye grown in the US, even with less rye in the mash, Canadian whiskey may still have good rye flavor and a lighter character. Other criteria include aging requirements (three years) and alcohol levels (not less than 40%). According to Canada's Food and Drug Regulations, there are few other requirements, and no distinction is made for Canadian Whiskey, Canadian Rye Whiskey, and Rye Whiskey. However, it is common practice for Canadian whiskey producers to mash the different grain components separately and to combine them later as blends. Canadian whiskey is also aged in both new and used barrels that account for the lower wood-flavored character.

Gin

Gin refers to a class of distilled neutral spirits having flavors derived from juniper berries. It is essentially flavored ethanol. Although several types of gin are produced around the world and the nomenclature for the European and American versions is slightly different, gin products are generally very similar.

Historically, the practice of using distillation to extract flavors and aromas from plant material paralleled that of distillation to produce spirits from wine and fermented grain-based beverages. The latter were often harsh, but could be made more palatable by the addition of these flavor extracts. Thus, adding flavors was an alternative to long aging in barrels. Thus, the term, "gin", is derived from the European spelling of the juniper plant – genièvre in French, ginepro in Italian, and jenever in Dutch.

Gin was first produced in Holland about 500 years ago and was introduced in England in the early 1600s. Thanks in part to its relatively low price and minimal regulation, by the end

of the century, gin had become one of the most widely-consumed alcoholic products in England. So popular was gin that much of the product was of poor quality and there were worries about the effect of so much gin consumption on public welfare. These concerns led to implementation of taxation and regulatory policies to reduce consumption. As quality improved, gin became a more respectable spirit, a reputation enhanced by the popularity of "cocktails" (like gin and tonic) in the nineteenth and twentieth centuries.

Gin manufacture and distillation

The mash can be from any of several sources, including grain, molasses, or potato. Distillation is by pot stills, or in the case of re-distilled gin, the first distillation can be performed by continuous stills. According to US standards, gin can be produced one of several ways. Gin that is distilled from an ethanol-containing mash with or over juniper berries or other aromatics (or their extracts) is simply "distilled gin". Gin can also be redistilled from distilled spirits, again with juniper berries or other aromatics (or their extracts). This product is called "redistilled gin". Finally, "compounded gin" is made by adding juniper berries or other aromatics (or their extracts) to neutral spirits. In addition to juniper, the other common aromatics used for gin include coriander, Angelica, and orange peel, and cardamom. All of these products must contain at least 40% ethanol (by volume) or 80 proof.

European versions are similar. Thus, according to these standards, "gin" is derived from an ethanol mash having an "agricultural origin", to which natural juniper flavors are added. The product, "distilled gin" is made by re-distilling neutral spirits in the presence of juniper or other botanical flavors. A third type, "London dry gin", is also re-distilled, but to higher purity.

For those gin types made by re-distillation, the product from the first distillation is diluted with water to about 45% alcohol (by volume). The juniper and other flavoring agents ingredients are added either directly or on trays or in pouches. The second distillation occurs in pot stills, with the head and tails separated and saved. The heart or middle run material, diluted to the required minimum, is bottled as gin.

Vodka

Vodka is perhaps one of the simplest distilled spirits. By US standards, vodka is a type of neutral spirit that is distinguished by its lack of taste, color, aroma, and character. These "attributes" are achieved in part by continuous distillation and also by treatment with filtering agents, mainly charcoal. The lack of flavor or aroma is also characteristic of how the EU defines vodka. Specifically, vodka, according to EU regulations, is a spirit that is (1) made from "ethyl alcohol of agricultural origin" from "potatoes and/or cereals or other agricultural materials"; and (2) "distilled and/or rectified so that the organoleptic characteristics of the raw material used or by-products formed in fermentation are selectively reduced". The only real differences are based on ethanol content – a minimum of 40% in the US and 37.5% in Europe. Flavoring and other agents (to promote a desirable mouthfeel) are also allowed.

The starting material can be from one of many agricultural substrates, including corn, wheat, potato, molasses, and sugar. The choice depends on cost, availability, and geography. Potatoes are common in Russia and Eastern Europe, whereas sugar beet and molasses are

common in the US and Canada. Fermentation conditions are similar to that of other alcoholic beverages. The mash is inoculated with *S. cerevisiae*, and after 2–3 days, under conditions similar to that of other alcoholic beverages, about 6–10% ethanol will be produced. The product is then distilled by continuous stills to rectify and purify the product. Usually two or more stills will be used to obtain high purity vodka.

Additional purification and removal of residual congeners and other compounds is achieved, as noted above, by one of several ways. The distilled spirit can be added to large columns containing granulated charcoal. Alternatively, activated charcoal can be added to tanks containing the product, followed by filtration to separate the solids.

Rum

Rum is quite different, in many respects, from the other distilled products described above. Historically, rum has its origins not in Europe or Asia, but rather in the New World, mainly the Caribbean (see below). Rum production then spread back to the Old World, where it became popular in England, France, and Spain. In particular, rum production occurred wherever there were sugar plantations. Currently, rum and rum-like products are produced all over the world.

In addition to differences in its origins, rum also differs microbiologically and technologically from whiskey, gin, vodka, brandy, and other spirits. For example, as described below, the rum fermentation is performed not simply by *S. cerevisiae*, but rather by a range of microorganisms including yeast and bacteria. In addition, the means by which the product is produced and distilled is also different, with specific manufacturing details varying considerably from country-to-country and producer-to-producer. Finally, a wide variety of products are produced commercially, ranging from clear rums with essentially no aging and little flavor to very dark, well-aged rums with strong flavors. Many of the latter types of rum are not even consumed directly, but rather are used as flavoring agents.

By US standards, rum belongs to a class of distilled spirit made from fermented juice of sugar cane, sugar cane syrup, molasses, or sugar cane by-product. It is distilled at not less than 95% alcohol and bottled at not less than 40% alcohol (80 proof). Again, EU regulations are very similar, except for the final alcohol levels (37.5% minimum). However, because rum is produced mainly outside the US and the EU, and is more often subject to local standards, many very different varieties of rum exist.

Manufacture and distillation

The main starting materials for rum include sugar cane juice and molasses. The latter is more commonly used based on availability, storage stability, high fermentable sugar content, and especially cost. Molasses is produced as a by-product from refinement of sugar cane, and it is generally inexpensive compared to sugar cane juice. In addition, some of the flavor and aroma components of molasses can make it all the way into the finished product. Nonetheless, for some traditional rum products, cane sugar is preferred.

Because molasses contains as much as 50% sugars (glucose, fructose, and sucrose), it is first diluted 3 to 4-fold, such that it contains about 15% sugars. The diluted molasses is pH-adjusted to about 5.0 and fermentation is initiated by one of several ways. For many years, rum was made by natural or spontaneous fermentation, relying on the wild microorganisms present in the raw molasses. Although molasses is a nutrient-rich medium, it is obtained via

a heat process (i.e., during sugar refinement and crystallization, the sugar-containing juice is boiled). Thus, the number of bacteria and yeasts present in this material is low, and natural fermentations can be undependable.

Rum manufacturers, therefore, began to use the stillage from a previous batch to supplement the molasses. This stillage material (called vinasse) also contained microorganisms, especially yeasts belonging to the genera *Schizosaccharomyces* and *Saccharomyces.* These yeasts are the dominant organisms involved in naturally fermented rum. The main species of *Schizosaccharomyces* isolated from rum fermentations, *Schizosaccharomyces pombe*, is osmotolerant and fermentative, but grows slowly. In contrast, *S. cerevisiae*, grows faster but produces fewer flavor and aroma compounds. Lactic acid bacteria, as well as clostridia and corynebacteria, may also be present in the vinasse, but their contribution, whether for the better or worse, has not been established. Despite the apparent need for rum starter cultures, few commercial cultures are available. This may be because of tradition and the successful use of backslopping techniques.

Rum can be distilled by either pot stills or continuous stills. In general, pot stills are used for darker rums that are intended for long aging (up to 12 years) in barrels. Continuous stills are used for white rums that are either not aged or aged for shorter times. Amber rums are usually continuously distilled and aged for intermediate times; they may also contain post still rum. Aging is performed in charred oak barrels, resulting in the formation of flavors and aromas derived from the wood.

Tequila

Tequila is unique among other distilled spirits in that its production is associated within one geographical location, namely Mexico. According to the US standards, Tequila must be distilled in Mexico, comply with Mexican laws and regulations, and must have the taste, aroma, and characteristics associated with tequila. Thus, while products similar to Tequila may be produced outside of Mexico, they cannot use these names. Tequila also differs from the other distilled spirits discussed so far in another important aspect. In particular, the substrate for the fermentation is used specifically for Tequila and related products.

The Tequila starting material is the agave plant, a succulent from the family Asparagaceae (of which the popular vegetable asparagus is also a member!). *Agave tequilana* or "blue agave" produces large shoots and leaves, and perhaps more importantly, a large pineapple-shaped "heart" or pina. The latter (sometimes also called "heads") typically weigh around 40 kg, but can range from 20–100 kg. The heads are rich in inulin, a polysaccharide containing fructose residues linked β-2,6. When the heads are heated, either by baking in ovens or steamed in autoclaves, thermal hydrolysis of the inulin occurs, releasing free fructose molecules. The cooked heads are then milled or crushed, in the presence of water, releasing a fructose-rich juice or sap. This material, called wort, serves as the fermentation substrate. It contains about 4–10% fermentable sugar. Although most Tequila is made from a wort containing 100% juice from the agave, it is permissible to add adjuncts, usually sugar cane, to as much as 49% of the total sugar. Products made from 100% blue agave will declare this on the label, whereas adjunct-containing tequila (known as "mixtos") will state the product was "made with agave". The latter can even be shipped and bottled outside of Mexico.

Traditionally, the fermentation was started by natural fermentation. Both *Saccharomyces* and non-*Saccharomyces* yeasts (mainly *Kluyveromyces*) are involved, but ultimately the fermentation is dominated by *S. cerevisiae.* The fermentation can also

be initiated by addition of commercial baker's yeast. More recently, strains of *S. cerevisiae* isolated from agave or from Tequila have been isolated and propagated and then used as Tequila starter cultures.

In addition to ethanol, other end-products are produced during the Tequila fermentation. In particular, methanol is produced, not by fermentation, but rather is released from agave pectins by enzymatic hydrolysis. Various organic acids, esters, and aldehydes are also produced either by chemical or microbial reactions.

After fermentation, for traditional Tequila the wash is distilled by a one or two pot still similar to that of other batch distillations. Distillation with a single pot still will result in a product containing more congeners. When two pot stills are used, the heads and tails are separated and the heart or middle material, containing about 20% ethanol, is collected from the first still and re-distilled in a second pot still. The final distilled product should have an ethanol concentration above 50%. Continuous stills are also used for Tequila, and the product can be used as is or mixed with Tequila produced from pot stills.

After distillation, the Tequila can be bottled or aged. The non-aged or "white" Tequila is clear or lightly colored, with minimal complexity and flavors derived mainly from the agave. In contrast, more complex flavors are formed when Tequila is aged. American or French oak casks or barrels are used for aged Tequila. Aging can be for as few as two months to as long as three years. The barrels are charred, but unlike for Bourbon whiskey, the Tequila barrels can be re-used. Thus, casks used previously for aging whiskey or sherry are common. Aged Tequila develops many unique flavors as well as color from the wood, similar to other aged spirits. Note, that it is permissible to add caramel color to make so-called gold Tequila, so color by itself is not a suitable indicator of aging.

Tequila is not the only product made from agave. A related product, mescal, is made from the maguey plant, *Agave potatorum,* which is similar to the blue agave used for tequila.

Brandy

Brandy, by virtue of being made from wine, is perhaps the original distilled spirit. Any fruit substrate can be used for brandy, not just grapes. Therefore, many types of brandy have become popular, including those made from pear, apple, and cherry. Wine brandies are often associated with region. Thus, the well-known brandies, Cognac and Armagnac, are produced in specific regions in southwest France. However, brandy is produced throughout Europe, as well as South America and South Africa. In the United States, brandy must conform to a standard of identity that describes the starting fruit or juice, the ethanol concentration, the duration of aging, and other compositional and manufacturing details.

In general, the base wine production follows that for other white wines. Skins and seeds are removed immediately after crushing and pressing to minimize pigment extraction. Distillation can occur as soon as the wine is fermented. Two types of distillers or stills are used, pot stills and continuous stills. The pot still, used for Cognac, consist of a single pot or boiler that is directly heated. A portion of the vapor is condensed in a reflux condenser and returned to the boiler, and the remaining vapor condenses and is collected.

After distillation, brandy is aged in oak barrels, which results in extraction of oak flavor, aroma, and color compounds. The base wine and perhaps even the distilled wine may be rather nondescript. Thus, it is during the aging and blending steps where brandy and Cognac develop their main quality attributes. Most American brandies are aged for two to three years; however, some Cognacs and other premium brandies are aged for more than 20 years.

OTHER DISTILLED PRODUCTS

Several other products, including cordials and liquors, are made by adding a wide range of flavoring agents to distilled spirits. These include coffee, cocoa, sugar, cream, honey, and various nut-derived flavors. In addition, aromatic herbs and botanicals can be added to spirits or spirit-fortified wine, yielding bitters, vermouth, and other similar products (Box 13.4).

Box 13.4 Flavored spirits

For nearly as long as humans have produced and consumed wine and other ethanol-containing beverages, botanicals and other flavorings have also been added. Initially, these were purportedly used for medicinal purposes (both oral and topical), and for many centuries, they remained popular as health-promoting elixirs. Evidence that specific botanical extracts have therapeutic properties has even been reported in the scientific press (McGovern et al., 2010). Although Hippocrates, the famous Greek "father of Western medicine" is often credited for "prescribing" ethanol extracts containing plant components, this practice had probably originated many centuries earlier. That wine or beer or spirits were used for extraction was no accident – ethanol was undoubtedly the best solvent around. Certainly it was much more effective than water for extracting volatiles, aromatics, oils, and other organic compounds.

It probably did not take long to realize that extracts derived from spices, herbs, and other plants imparted pleasant flavors and aromas. There is evidence that roots and herbs were added to wine in Asia as flavoring agents at least 3000 years ago (McGovern et al., 2010). Even bitter-tasting materials were found to be desirable. As noted in Chapter 10, brewers have been adding hops and other bitter flavorings to beer for at least a thousand years. Other flavors would have conferred spicy, aromatic, and anise-type flavors.

By the 1500s, distillation technology began to be used for making distilled alcoholic beverages. Sometime early in the seventeenth century, juniper berries were added to the distillate and the product was redistilled. In French, juniper is genièvre; in Dutch jenever. Whatever the etymology, the product became known as gin. In addition to juniper, gins are now commonly flavored with other botanicals, including coriander, citrus peel, caraway seed, and cardamom.

Many other new and distinctively flavored distilled alcohol-containing products were soon introduced and quickly became established. Several of these products share common flavor characteristics but differ mainly in geography. Others are unique to particular regions. For many products, the flavoring agents are added after distillation, for others, the distilled product is redistilled in the presence of the flavoring agent. For the most part, however, these products are grouped according to their predominant flavors. The most common flavors include anise or licorice, aromatic or flowery, and spicy (Tonutti and Liddle, 2010). A few of the more popular products are described below.

Vermouth Sometimes referred to as an "aromatized wine", vermouth was originally produced in Germany. It was made by addition of wormwood, a perennial plant,

to wine. Wormwood (*Artemisia absinthium*) is wermut in German, hence the product became known as vermouth. Wormwood has a pronounced bitter flavor, and vermouth is now flavored with other less bitter and more aromatic roots and herbs (Tonutti and Liddle, 2010). Distilled ethanol is also added to about 20%, thus vermouth may be considered as a fortified spirit. It is most often consumed as an aperitif and also as an ingredient in martinis.

Anise-flavored liquors There are dozens of distilled spirits and liquors that are characterized by an anise flavor. These products are often associated by the country or region of their manufacture. Although they differ in how they are produced, they share several common features. Apart from the anise flavor, many are also known for their cloudy or almost milk-like appearance when diluted with water. Some products are also sweetened and licorice is added to others to enhance flavor. Popular products (and the regions for which they are known) include absinthe (France), anisette (Mediterranean), pastis (France), ouzo (Greece), raki (Turkey), and Sambuca (Italy).

Bitters These flavored spirits are made, as indicated by their name, by adding bitter herbs, roots, bark or other plant materials to distilled spirits. Bitters are often used in cocktail recipes or are consumed straight as digestives (an after meal beverage). They are chemically diverse, comprised of more than 100 distinct volatiles (Johnson et al., 2015).

Other flavored spirits Among the more highly flavored spirits is chartreuse, which takes its name based on the French monasteries from where they were originally made in the 1700s. These are known for their yellow or green color and complex flavor.

References

Johnson, A.J., H. Heymann, and S.E. Ebeler. 2015. Volatile and sensory profiling of cocktail bitters. Food Chem. 179:343–354.

McGovern, P.E., M. Chirstofidou-Solomidou, W. Wang, F. Dukes, T. Davidson, and W.S. El-Deiry. 2010. Anticancer activity of botanical compounds in ancient fermented beverages (Review) Int. J. Oncol. 37:5–14.

Tonutti, I. and P. Liddle. 2010. Aromatic plants in alcoholic beverages. A review. Flavour Fragr. J. 25:341–350.

BIBLIOGRAPHY

Berger, R.G. 2007. Flavours and Fragrances. Chemistry, Bioprocessing and Sustainability. Springer, Berlin.

Kockmann, N. 2014. History of distillation. In: Górak, A. and E. Sorensen, eds. Distillation: Fundamentals and Principles. Academic Press, New York.

Lea, A.G.H. and J.R. Piggott, eds. 2003. Fermented Beverages Production, 2nd edn. Kluwer, New York.

Murray, D. Grain whisky distillation. In: Russell and Stewart. 2014. Whisky: Technology, Production and Marketing, 2nd edn. Elsevier Ltd, Oxford, pp. 179–198.

Musto, D.F. 1996. Alcohol in American history. Sci. Am. 274:78–83.

Nava-Cruz, N.Y., M.A. Medina-Morales, J.L. Martinez, R. Rodriguez, and C.N. Aguilar. 2015. Agave biotechnology: an overview. Crit. Rev. Biotechnol. 35:546–559.

Nichol, D.A. 2014. Batch distillation. In: Russell and Stewart. Whisky: Technology, Production and Marketing, 2nd edn. Elsevier, Oxford. pp. 155–178.

Russell, I., and G.G. Stewart, eds. 2014. Whisky: Technology, Production and Marketing, 2nd edn. Academic Press/Elsevier. Boston, MA.

Tonutti, I. and P. Liddle. 2010. Aromatic plants in alcoholic beverages. A review. Flavour Frag. J. 25:341–350.

Walker, G.M. and P.S. Hughes, eds. 2010. Distilled Spirits, Volume 3: New Horizons: Energy, Environment and Enlightenment. Nottingham University Press, Nottingham.

Walker, G.I., I. Goodall, D. Murray, and R. Fotheringham, eds. 2012. Distilled Spirits: Science and Sustainability. Nottingham University Press, Nottingham.

14 Fermented foods from the Far East

> Soy sauce makes me excited just thinking about it. Every food is recreated by soy sauce. Soy beans, salt and water, in harmony, through time. It is the basis of seasonings, the foundation. There are sauces aged five years, ten years, aged for one hundred years. These kinds of soy sauces are passed down for generations. They are heirlooms.
>
> Jeong Kwan, Buddhist nun and philosopher chef (from Chef's Table)

HISTORY

Most of the fermented foods described in the previous chapters (cheese, yogurt, bread, beer, wine, sausage, and vinegar) had their origins thousands of years ago. Not surprisingly, Asian-type fermented foods have been produced and consumed just as long. In addition, these Asian fermented foods had many of the same general characteristics and properties as those that developed in Middle Eastern and Western cultures. Accordingly, these products were comprised of ingredients native to their geography, they had enhanced functional and nutritional properties, and they were well preserved. Likewise, as for fermented foods from the West, these Asian fermented foods were subject to similar cultural, social, and religious influences. Finally, they had important economic impacts.

Despite these similarities, there are many prominent differences between Western and Asian fermented foods. Not only are the starting materials and the specific types of products produced in the Far East different, but the actual means by which fermentation occurs are also dramatically different from products that evolved in the West. Whereas the fermented foods industry in Westernized countries, and especially in the United States, have become large, mechanized, and technologically well defined, production methods for fermented foods in the Orient vary greatly, with many small manufacturers still in operation. The trend toward large-scale production, however, has now become evident in China, Japan, Malaysia, Thailand, Korea, and many other Far East countries. Many of the factories that produce soy sauce, for example, are as large and modern as any factory in the west.

Another main difference between the fermented foods that evolved in West and those that evolved in the Far East concern is that the latter relied far less on animal products. Several reasons likely accounted for this difference. First, religions practiced in the Far East often excluded meat products and instead promoted diets based on plant proteins. Buddhism, for

Microbiology and Technology of Fermented Foods, Second Edition. Robert W. Hutkins.

example, which developed in the sixth century BCE, prohibited animal foods in the diet. Second, that the Far East was generally more densely populated meant that less space could be devoted to animal agriculture and pastoral grazing practices. Instead, greater emphasis was placed on growing plant foods, such as rice and soybeans, for human consumption. In addition, economic pressures led to greater reliance on inexpensive food commodities, such as cereals, grains, and legumes. Finally, the ready availability of fish and seafood provided an abundant source of inexpensive, high-quality protein.

Other major differences between fermented foods of the West and East relate more directly to the fermentation substrates, the manner in which the fermentations occur, and the types of microorganisms that are involved. The substrates used for the fermented foods of the West, for example, are usually simple sugars, whereas in many of the Asian-type fermentations, the substrates consist largely of complex carbohydrates in the form of starch and other polysaccharides. There are few free fermentable sugars present in rice and soybeans, certainly not enough to support growth of the organisms involved in these fermentations. Likewise, the availability of amino acids necessary to satisfy the nitrogen requirements of fermentative organisms is also limited. Therefore, the manufacture of the Asian fermented products must start with a step that converts the starch to sugars and proteins to amino acids.

There is, of course, other fermented products popular in the West that share this starch-to-sugar conversion requirement – namely, beer and distilled beverages from grains. As described in Chapter 9, the conversion of starch to free sugars is catalyzed by various amylolytic enzymes present in malt during a step called mashing. The free sugars, mainly maltose and glucose, are then readily fermented to ethanol and carbon dioxide by yeasts. In the production of Asian fermented products, a very similar mashing process occurs, with one significant difference. The enzymes necessary for saccharification (i.e., starch hydrolysis) are supplied not by malted cereals, but rather by fungal organisms, previously grown on cereal matter. Like malt, the production of this enzyme-laden fungal material is itself an important part of the overall manufacturing process.

Another important difference between fermented foods of the East and West, as noted above, concerns the very nature of the fermentation. For the most part, dairy, meat, vegetable, and ethanolic fermentations are conducted under predominantly anaerobic conditions. In contrast, aerobic conditions are necessary (at least, for certain critical parts of the process) to produce many of the Asian fermented products. Moreover, whereas pure starter cultures, containing defined strains, are now well accepted and widely used for most fermented food products in the West, defined cultures are infrequently used (except by large manufacturers) in the production of Asian fermented foods. Finally, although fungi are used in the manufacture of a few Western-type fermented foods, such as some cheeses and sausages, otherwise fungi are not essential. In contrast, fungi are indispensable in most of the Asian fermented foods. In fact, the food products described in this chapter are often referred to simply as fungal-fermented foods.

It should be noted that the consumption of Asian fermented or fungal-derived foods is no longer confined to Asia or Asian populations. Chinese foods have long been part of the Western cuisine, but in the past two decades, Japanese, Thai, Vietnamese, Indonesian, Korean, and other cuisines from the Far East are now widely consumed throughout the world. Asian foods, flavors, and ingredients continue to appear on lists of most popular culinary trends. It is remarkable, however, that few consumers realize that so many of these foods contain, as primary ingredients or constituents, fermented food products. Perhaps just as surprising is how far Asian fermented foods have penetrated into the US ethnic foods market, such that many of these products are available in American grocery and specialty food stores. Thus, tempeh, miso, kimchi, fish sauce, and a large array of soy sauce varieties

are now widely available. Due to the savory, meat-like flavor (referred to as umami) some of the products impart, they have also become popular as vegetable-based meat substitutes (as "faux" meats). So popular are these products outside of Asia that several large manufacturing facilities are now operating in the United States and Europe.

TYPES OF ASIAN FERMENTED FOODS

There are hundreds of different types of fermented foods produced in China, Japan, the Philippines, and throughout Asia and Southeast Asia. In general, however, these products can be grouped into two broad types of fermented foods: those that are plant-based and those that are fish-based. The former are made using primarily soy and rice as substrates, but other grains and legumes are also common. For the most part, these soy-based fermentations have been industrialized and the products are now produced on a large scale. In addition, many of the microbiological and biochemical events that occur during the fermentation are now well-established. However, in contrast to the soy- or plant-based fermentations, the fish fermentations are generally not well defined, nor has there been, until recently, very much published research (at least in English-language journals) on the microorganisms and biochemical reactions involved in their manufacture.

PLANT-BASED FERMENTATIONS

Soy and rice are the most frequent substrates for Asian fermented foods. Wheat flour is also often included as an ingredient in many of these products. With few exceptions, however, there is one common starting material – called koji – that is essential for most Asian fermented foods. As will be described in more detail below, koji is simply a moldy mass of grain, and is derived from the Japanese word meaning "moldy grain". In some cases, the koji mold is added to a portion of the raw material which is later added to the remaining substrate (analogous, in a way, to a bulk culture or bread sponge). In other applications, the koji mold is added to the entire raw material. Koji is used not only for production of the many soy sauce-type products that will be described later, but also for sake and related rice wines. Because of the important role koji plays in so many of the products discussed in this chapter, its manufacture and microbiological and enzymatic properties will be described separately.

KOJI MANUFACTURE

First, it is necessary to recognize there are many types of koji used in the Far East, and that each fermented food requires a specific type of koji. Thus, Japanese soy sauces generally use a koji that is different from the one used to make Chinese-style soy sauces, and both are different from the koji used for sake manufacture. In general, koji is often referred to by its intended product (e.g., sake koji or shoyu koji) or by the substrate from which the koji is prepared (e.g., rice koji, wheat koji, barley koji, or soybean koji).

Despite the type of koji or how it is made, the purpose and function are always the same. Namely, koji provides a source of enzymes necessary to convert solid, raw materials containing complex and non-fermentable substrates into soluble, metabolizable products that can be easily fermented by suitable microorganisms. Importantly, koji contains not only the amylolytic and proteolytic enzymes for hydrolysis of starch and protein, respectively, but an array

of enzymes capable of hydrolyzing peptides, lipids, cellulose, pectin, and other complex substrates. The koji also serves as a source (often the main source) of substrates for the enzymes produced by the koji fungi.

Raw materials preparation

The manufacture of koji starts with the appropriate processing of the raw materials. When soybeans are used, they are first soaked for about twelve hours in several changes of water. For traditional koji-making, they are then cooked, usually with steam under pressure, for one hour – a process called puffing. For some products, soybean koji also contains wheat, which is prepared by roasting wheat kernels (or wheat flour) at a high temperature, followed by a crushing step. Rice koji for sake manufacture is prepared from polished rice (rice minus the bran) that is steeped and steamed, similar to soybean koji.

Despite the seemingly simple process involved in preparing the koji substrate, many innovative steps have been designed to enhance the digestibility of the raw soybeans and ultimately improve yield and product quality. For soy sauce, digestibility or yield is based, in large part, on the amount of total protein nitrogen that is converted to amino nitrogen during the mashing step. However, how the soybeans are initially heat processed influences the rate and extent that the soy proteins are hydrolyzed. This is because only denatured soy proteins are hydrolyzed by fungal proteases, whereas native proteins are not. Thus, a cooking step is essential. Yields of about 82% can be achieved when a traditional cooking process (e.g., 118°C at 0.9 kg/cm^2 for 45 minutes) is used. In contrast, high-temperature, short-time cooking not only enhances protein extraction, but also can provide amino acid yields greater than 90%. It is now possible, for example, to perform the cooking step in less than two minutes, using temperatures and pressures above 150°C and 5 kg/cm^2, respectively.

Microorganisms

Once the koji substrates are prepared, they are inoculated with koji fungi. There are essentially two means by which koji fungi can be introduced. One is simply to use a pure culture containing spores of *Aspergillus oryzae* and/or *Aspergillus sojae*. Alternatively, the mixture can be inoculated with 0.1% to 0.2% of a seed culture called tane koji. Tane koji is made by inoculating soaked, steamed, and cooled rice (usually brown rice) with spores of *A. oryzae* or *A. sojae*. The inoculated rice is incubated overnight, then transferred and distributed evenly into shallow trays and incubated at 30°C for five to seven days. Intermittent mixing provides aeration necessary for fungi growth. Eventually, the moldy rice mixture is dried and packaged. This tane koji material is then used to inoculate other koji mixtures.

Ordinarily, multiple strains are used to make tane koji, depending on the final product that is manufactured. Similar to other fermented foods, soy sauce quality depends on performance of the culture, i.e., koji strains. In general, koji mold strains are selected on the basis of their ability to produce proteolytic, peptidolytic, and amylolytic enzymes with high activity. These strains should also sporulate and grow well on the substrate. Some species of *Aspergillus* are known to produce aflatoxin and other mycotoxins. Obviously, strains used in the manufacture of koji (or any food) should not be capable of producing these toxins. Indeed, none of the koji strains that have been isolated and studied produce mycotoxins under ordinary production conditions (see below). Finally, koji strains should be genetically stable and produce products having consistent flavor and color properties. The importance of these fungi in these fermentations has led to several genome sequencing projects (Box 14.1).

Box 14.1 Koji genomics: *Aspergillus oryzae* and *Aspergillus sojae*

Students and other readers of this text might get the impression that the fermented foods world revolves around lactic acid bacteria and *Saccharomyces cerevisiae*. Certainly, the role of molds in the manufacture of fermented foods was noted in previous chapters on cheese and sausage. However, perhaps it is now time to elevate the status of the main koji mold species, *Aspergillus oryzae* and *Aspergillus sojae*, to that of the other organisms mentioned above (Figure 14.1.1). Not only are these organisms required for several widely consumed products, they are also used on a very large volume. In addition, the enzymes produced by these fungi are industrially important for a variety of food and non-food applications.

***Aspergillus* genomes** Like lactic acid bacteria, yeasts, and other organisms used in fermented foods, the genomes of koji fungi have also been sequenced, revealing valuable practical information. Indeed, one of the early fungal genomes to be sequenced (2005) was for *Aspergillus oryzae* RIB40, a strain used for koji manufacture (Machida et al., 2005). Not surprisingly therefore, among the most abundant groups of enzymes encoded by this genome were secreted hydrolases and membrane transporters used for metabolism of starches and proteins (Cerqueira et al., 2013). Thus, this finding was consistent with the ability of this organism to grow on food substrates like rice, wheat, and soybeans. That this organism is more amylolytic than *A. sojae* was explained when the genome of the latter was sequenced, revealing a lower copy number for a specific amylase gene (Sato et al., 2011). Moreover, despite the genetic similarity between *A. oryzae* and toxigenic *Aspergillus flavus*, neither *A. oryzae* (nor *A. sojae*) express functionally active toxins, such as cyclopiazonic acid or aflatoxins.

There are now ten sequenced koji genomes in the NCBI genome database – nine for *A. oryzae* and one for *A. sojae*. The genome information has not only provided a basis for comparing these species to other *Aspergillus*, but has also had practical implications (Takahashi, 2014). In part, this is because the proteases, amylases, and other enzymes secreted by *A. oryzae* and *A. sojae* are critical to the success of fungal fermentations. Thus, strains that naturally over-express (or can be modified to do so) these enzymes would be valuable. In soy sauce, for example, the enzyme, glutaminase, is necessary for formation of the umami flavor component, L-glutamate, via deamidation of L-glutamine. From the *A. oryzae* and *A. sojae* genomes, more than 20 glutaminase genes have been identified. Given the industrial importance of these glutaminases, researchers have begun to characterize these enzymes and determine which ones are involved in soy sauce production (Ito et al., 2012).

In addition to enzyme discovery, the genomes have also been mined for regulatory functions that may be involved in expression of functionally important genes. Specifically, transcription factors, those genes that bind to promotor regions and control transcription of genes or operons, may be modified such that expression of multi-enzyme pathways is stimulated or repressed (Takahashi, 2014). Similarly, in another recent study, researchers showed that starch metabolism in *A. oryzae* was influenced by two transcription factors, AmyR and MalR (Suzuki

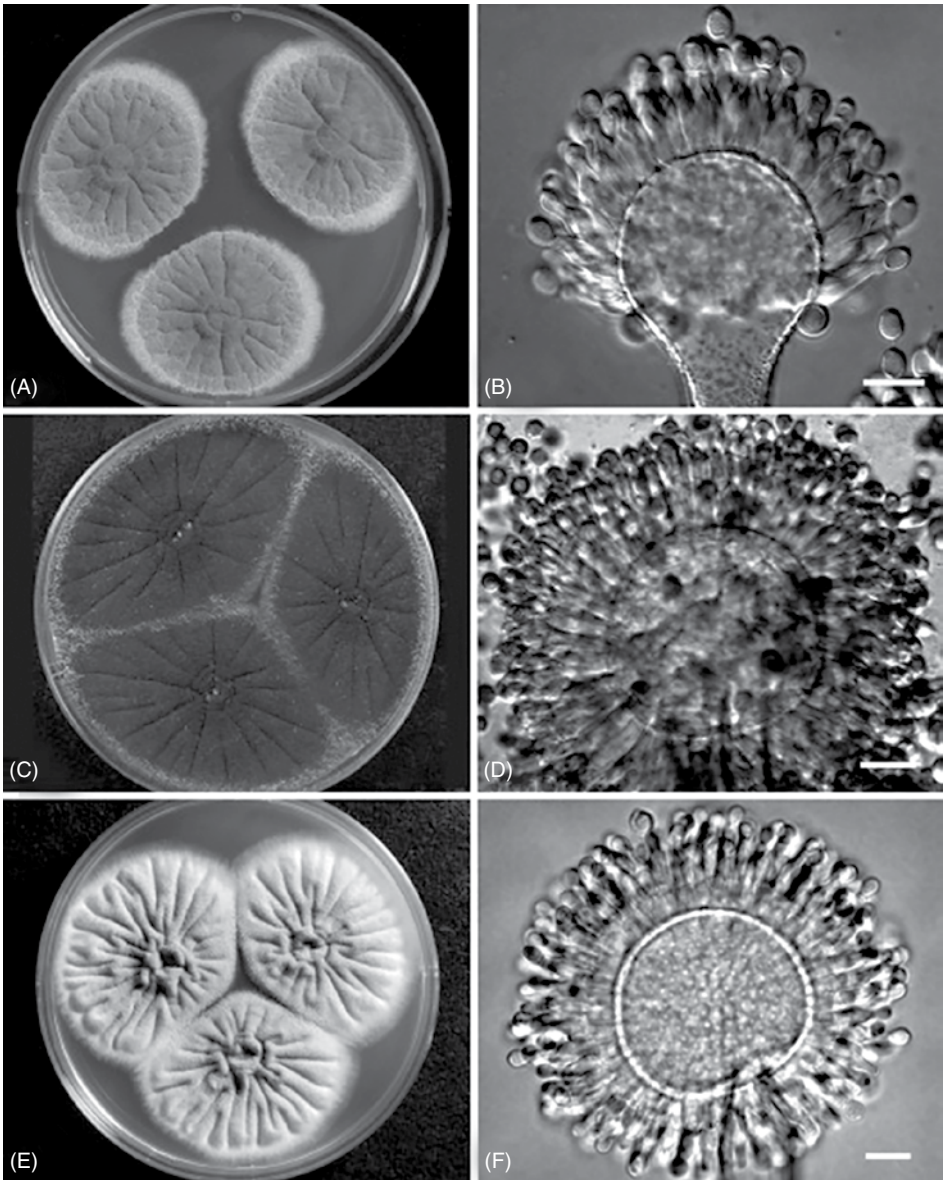


Figure 14.1.1 Colony (A, C, E) and microscopic (B, D, F) morphologies of koji molds. Shown are *Aspergillus oryzae* (upper panel), *Aspergillus luchuensis* (middle panel), and *Aspergillusl luchuensis* var. *kawachii* (lower panel). Scale bars = 20 μm. From Park et al., 2017, with permission.

et al., 2015). Finally, while genomes tell which genes are present they do not predict which genes are expressed. Strains, for example, having very similar genomes may nonetheless have different phenotypes. Thus, transcriptome analyses have been conducted to determine differences between seemingly similar strains (Zhao et al., 2015).

References

Cerqueira, G.C., M.B. Arnaud, D.O. Inglis, M.S. Skrzypek, G. Binkley, M. Simison, S.R. Miyasato, J. Binkley, J. Orvis, P. Shah, F. Wymore, G. Sherlock, and J.R. Wortman. 2013. The *Aspergillus* Genome Database: multispecies curation and incorporation of RNA-Seq data to improve structural gene annotations. Nucleic Acids Res. 42(Database issue):D705–D710.

Ichinose, S., M. Tanaka, T. Shintani, and K. Gomi. 2014. Improved α-amylase production by *Aspergillus oryzae* after a double deletion of genes involved in carbon catabolite repression. Appl. Microbiol. Biotechnol. 98:335–343.

Ito, K., K. Matsushima, and Y. Koyama. 2012. Gene cloning, purification, and characterization of a novel peptidoglutaminase-asparaginase from *Aspergillus sojae*. Appl. Environ. Micobiol. 78: 5182–5188.

Machida, M., K. Asai, M. Sano, T. Tanaka, T. Kumagai, et al., 2005. Genome sequencing and analysis of *Aspergillus oryzae*. Nature 438:1157–1161.

Machida, M., O. Yamada, and K. Gomi. 2008. Genomics of *Aspergillus oryzae*: learning from the history of koji mold and exploration of its future. DNA Res. 15:173–183.

Park, H.-S., S.-C. Jun, K.-H. Han, S.-B. Hong, and J.-H. Yu. 2017. Diversity, application, and synthetic biology of industrially important aspergillus fungi. Adv. Appl. Microbiol. 100.

Sato, A., K. Oshima, H. Noguchi, M. Ogawa, T. Takahashi, T. Oguma, Y. Koyama, T. Itoh, M. Hattori, and Y. Hanya. 2011. Draft genome sequencing and comparative analysis of *Aspergillus sojae* NBRC4239. DNA Res. 18:165–176.

Suzuki, K., M. Tanaka, Y. Konno, T. Ichikawa, S. Ichinose, S. Hasegawa-Shiro, T. Shintani, and K. Gomi. 2015. Distinct mechanism of activation of two transcription factors, AmyR and MalR, involved in amylolytic enzyme production in *Aspergillus oryzae*. Appl. Microbiol. Biotechnol. 99:1805–1815.

Takahashi, T. 2014. Application of genomics in molecular breeding of the Koji molds *Aspergillus oryzae* and *Aspergillus sojae*. In: H. Anazawa and S. Shimizu, eds. Microbial Production: From Genome Design to Cell Engineering. Springer, Japan.

Zhao, G., Y. Yao, C. Wang, F. Tian, X. Liu, L. Hou, Z. Yang, J. Zhao, H. Zhang, and X. Cao. 2015. Transcriptome and proteome expression analysis of the metabolism of amino acids by the fungus *Aspergillus oryzae* in fermented soy sauce. BioMed Res. Int. Article ID 456802.

After the koji substrate (rice, soybeans, wheat, or barley) has been inoculated, it is mixed and incubated in large rectangular trays or boxes (5 × 12 meters) with a depth of about 30 to 40 cm. Very large producers may use vats that can accommodate even greater quantities. Perforations in the trays enhance air and moisture circulation. Temperature control is particularly important because some proteolytic enzymes tend to be produced at lower temperatures (25°C to 30°C), and amylases are produced at higher temperatures (>30°C to 35°C). Modern manufacturers now rely on temperature programs to maximize enzymatic activities. In fact, the incorporation of continuous cookers, automated mixers, and other modern devices has made it possible to automate the entire koji-making operation so that as much as 3000 kg of koji can be produced per hour. After two to four days at 30°C, the mass should be covered throughout with mold growth.

MANUFACTURE OF SOY SAUCE AND RELATED PRODUCTS

Soy sauce is one of the most widely consumed products in Asia. In Japan, per capita consumption is about 6 liters per person per year, or around 20 g per day. Dozens of different types of soy sauces are manufactured in Asia. In fact, even within the same country, there

Table 14.1 Types of soy sauces.

Product	Country	Description
shoyu	Japan	Five types based on soybean and wheat content
chiang-yiu	China	Similar to shoyu tamari (mostly soybeans)
kecap	Indonesia	Two main types, manis (sweetened) and asin (salty)
kicap	Malaysia	Tamari type
kanjang	Korea	Tamari type
see-iew	Thailand	Two main types, sweetened and salty
toyu mansi	Philippines	Lemon-like flavored

Table 14.2 Compositions of different types of shoyu[1].

Product	Total N[2] (g/100 ml)	Reducing sugars[3] (g/100 ml)	Ethanol (v/v)	pH	Color
Koikuchi	1.6–2.0	2.8–5.5	2.1–2.5	4.7–4.9	Dark brown
Usukuchi	1.2	4.0–5.0	2.1–2.6	4.8	Light brown
Tamari	1.8–2.6	2.4–5.3	0.1–2.4	4.8–5.0	Dark red-brown
Shiro	0.5	14–20	0–0.1	4.5–4.6	Yellow/tan
Saishikomi	2.0–2.4	7.5–9.0	0–2.2	4.6 -.8	Dark brown

[1] Adapted from Yokotsuka and Sasaki, 1998 and Fukushima, 1989.
[2] Comprised of amino acids, small peptides, and ammonium salts.
[3] Consisting of about 85% glucose, 10% galactose + mannose, and 5% pentoses.

may be several distinct products, each having their own particular qualities and each made according to specific manufacturing procedures (Table 14.1).

Moreover, quality standards further distinguish one product type from another. For example, in Japan (where the name for soy sauce is shoyu), three shoyu production methods and five types of shoyu products are recognized by the Japan Agricultural Standard (Table 14.2). In addition, there are three quality grades for each Japanese shoyu – Special, Upper, and Standard, that are assigned based on manufacturing methods, composition, flavor, and color. The most widely consumed variety of shoyu in Japan is koikuchi, which contains 50% wheat. In contrast, tamari, another Japanese shoyu, contains little or no wheat. Similar versions of these soy sauce products, containing either part wheat or no wheat, exist in China, Taiwan, and other Asian countries. The popularity of gluten-free products now extends across many product categories, including soy sauce. Thus, tamari, which is traditionally made without wheat, has been re-branded in some markets, as "gluten-free" soy sauce.

Koji

The manufacture of traditional soy sauce or shoyu starts with preparation of the raw materials and addition of the koji (Figure 14.1). The soy fraction consists of whole beans, soy meals, or soy flakes. It is now common to use de-fatted flakes rather than whole beans or flakes to improve yield, reduce fermentation times, and minimize issues related to the oil phase appearing in the soy sauce. When beans are used, they are washed, sorted, and soaked overnight, then cooked under pressure and cooled rapidly. Flakes are simply soaked and cooked (as for whole beans). At the same time, whole wheat kernels, if included, are roasted

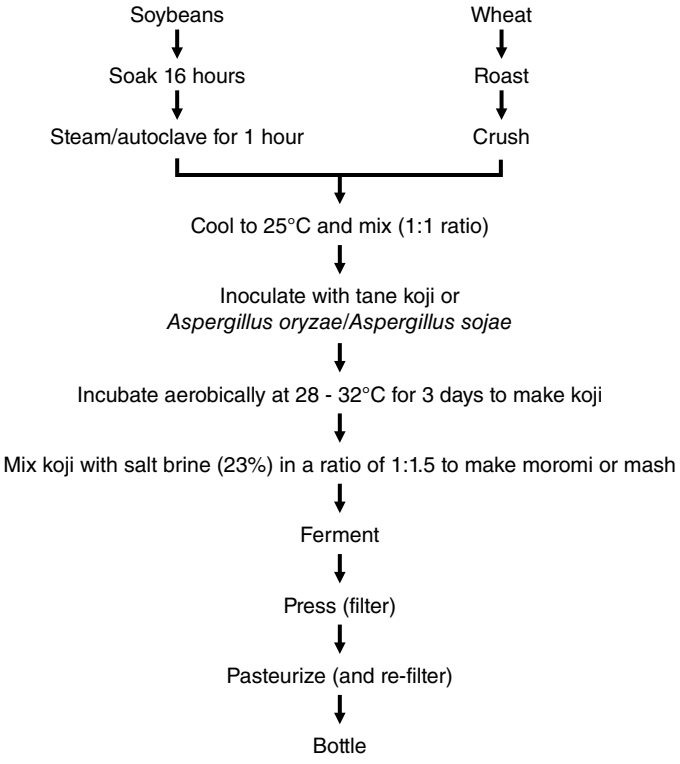


Figure 14.1 Manufacture of shoyu. Adapted from Yoneya, 2004.

and crushed. The wheat adds flavor and color, reduces the moisture so that growth of undesirable bacteria is minimized, enhances mold growth, and contributes glutamic acid-rich proteins. When wheat is used, the soy-wheat ratio depends on the manufacturer's preference and can range from 50:50 to 67:33.

The soybean-wheat mixture is then inoculated with either tane koji or a pure culture of suitable fungal strains of *A. oryzae* or *A. sojae* to start the koji fermentation. As described above, the inoculated material is incubated in large trays, boxes, or vats that are perforated to allow air and moisture to circulate throughout the material and to enhance fungal growth. The temperature is maintained at about 30°C, which means that the incubation rooms or vats must have cooling capacity, since fungal growth generates heat. Once the koji is mature (i.e., covered completely with mold), it is fully developed and ready to be used for the fermentation.

Mashing

It is during the next step, mashing, where the koji enzymes begin to hydrolyze proteins, polysaccharides, and other substrates, and where microorganisms begin to use the products of these reactions. First, however, a high salt brine containing 20% to 25% sodium chloride is added to the solid material. The volume added may vary, depending on the manufacturer's specifications, but a ratio of about 1:1.2 to 1:1.5 (solid to brine) is normal. The high salt concentration in the mash (ranging from 16% to 19%) restricts growth of

microorganisms to only those that are especially halo- or osmotolerant. The mash material, referred to as "moromi" or the "moromi mash", is allowed to ferment in large (300,000 L) tanks for up to a year. Whereas wooden tanks were commonly used, these were first replaced by concrete. Now resin-coated iron or fiberglass tanks are common. Obviously, tanks constructed from stainless steel, which are typically used for cheese, sausage, and other fermented foods, would not be suitable for soy sauce, given the corrosiveness of the salty and acidic moromi.

The moromi can be held at temperatures ranging from 15–35°C. Moromi held at lower temperatures are thought to produce a better quality product, but take longer. In contrast, higher temperatures can be used to complete the fermentation in as little as three to four months. Agitation and forced aeration generally enhances enzymatic activity and microbial growth during mashing.

Moromi enzymology

The koji molds, *A. oryzae* and *A. sojae*, produce a wide assortment of enzymes. They are especially prolific at secreting proteases, peptidases, cellulases, and amylases as they grow in the koji. During the mashing step, these enzymes degrade soy (and wheat) proteins, starches, and other macromolecules. The end products formed from these enzymatic reactions are either necessary as nutrients or can be fermented directly by the mash microbiota. As noted above, the actual amounts and specific types of enzymes produced by koji *Aspergillus* can vary, depending on the incubation temperature, strain, and substrate used during koji manufacture. In general, the lower the temperature, the greater will be the enzyme activity (within a range of 20°C to 35°C).

Protein hydrolysis occurs via one of several different fungal proteases and peptidases, each having specific pH optima and substrate preferences. In terms of enzyme activity, alkaline protease has more than ten times the activity of other proteases. This enzyme is active between pH 6.0 and 11.0, although its pH optima is about pH 10.0. A semi-alkaline protease (pH optima 8.3), as well as at least two neutral-proteases and three acid-proteases (pH optima around 7.0 and 3.0, respectively), all contribute to hydrolysis of soy proteins. The peptides that are generated are further hydrolyzed by peptidases that also have individual pH optima and are specific for particular amino or carboxy amino acid residues.

Enzymes that release glutamic acid or that convert glutamine to glutamic acid are especially important, because this amino acid is responsible for much of the flavor-potentiating characteristics of soy sauces. Salt-tolerance is another important property of koji proteases, since they will still retain activity in the presence of high salt concentrations. A nearly complete hydrolysis of soy proteins is desirable, since the subsequent lactic acid and yeast fermentation, color and flavor development, and overall organoleptic quality of the finished soy sauce depend on amino acid formation during the mashing step.

Most of the amylolytic activity present during mashing is due to the concerted action of fungal α- and β-amylases that convert starch to simple sugars. Although some sugars are used to support mold growth, most remain in the mash where they will later serve as substrates during the fermentation stage of the process. This simple sugar fraction is comprised of several reducing sugars, including glucose and maltose, as well as xylose, galactose, and arabinose that are released from other polysaccharides present in the soy or wheat mixture (discussed below). The reducing sugars play an especially important role in color and flavor development, since they react with free amines during non-enzymatic browning reactions.

The other main groups of enzymes important during mashing are the cellulases, pectinases, hemicellulases, and other tissue-degrading enzymes. They enhance extraction of substrates from the soybean and wheat tissues, thereby increasing yield and nutrient availability. Formation of pentoses and other sugars also provide additional substrates for microorganisms and for browning reactions.

Fermentation

Immediately after the addition of the salt brine to the koji, the mash will become populated by bacteria and yeasts that were present as part of the natural koji microbiota (Table 14.3). The mold from the koji, however, are salt-sensitive and are quickly depleted (Figure 14.2). Organisms initially present include both lactic and non-lactic acid bacteria, as well as various yeasts.

Although the initial pH of the mash (6.5 to 7.0) is conducive for all of these organisms, the high salt concentration poses a substantial barrier. A mash containing 18% salt will have an osmolality of more than 6 Osm and a water activity (a_w) of about 0.88. This is lower than what most of the moromi organisms can tolerate. Thus, the salt-sensitive organisms, including wild yeasts, *Bacillus,* and Gram-negative bacteria, will not survive for long and will be undetectable in the mash within a month or two. In contrast, the restrictive conditions will select for those organisms capable of tolerating a high salt, high osmotic pressure, low a_w environment. Among the lactic acid bacteria that can tolerate these conditions and predominate the early stages of the mash fermentation are species of *Lactobacillus, Weissella, Pediococcus,* and *Tetragenococcus*. Although they may be present initially at low levels ($<10^3$/ml) in the mash, they can reach cell densities of 10^7 cells/ml or higher after six to eight weeks. It is now possible to add selected strains of these organisms to the moromi, rather than rely on their natural occurrence.

Salt-tolerant yeasts, *Zygosaccharomyces rouxii* and *Candida versitalis,* may also be present in the mash. These yeasts are even more salt- and acid-tolerant than the lactic acid bacteria. When the mash pH falls below 5.0, growth of *Lactobacillus delbrueckii* and *Tetragenococcus halophilus* (in the presence of more than 18% salt) will be inhibited, and yeasts will dominate the rest of the fermentation. Eventually, when the pH reaches 4.0, even *Z. rouxii* will stop growing. The manner in which the lactic acid bacteria and yeasts grow during the soy sauce fermentation, therefore, resembles that of a succession-type fermentation as described for other fermentations.

A wide variety of fermentation end products accumulates in the mash and is responsible for the complex flavor of the finished soy sauce (Table 14.4). Growth of the homofermentative *T. halophilus* on glucose results primarily in lactic acid formation. Fermentation of other sugars, particularly pentoses, however, may yield heterofermentative products

Table 14.3 Microorganisms important in soy sauce.

Fungi	Bacteria	Yeast
Aspergillus oryzae	*Tetragenococcus halophilus*	*Zygosaccharomyces rouxii*
Aspergillus sojae	*Lactobacillus delbrueckii*	*Zygosaccharomyces soya*
Mucor spp.	*Lactobacillus fermentum*	*Candida versitalis*
Rhizopus spp.	*Leuconostoc mesenteroides*	*Torulopsis* spp.
	Weissella spp.	*Hansenula* spp.

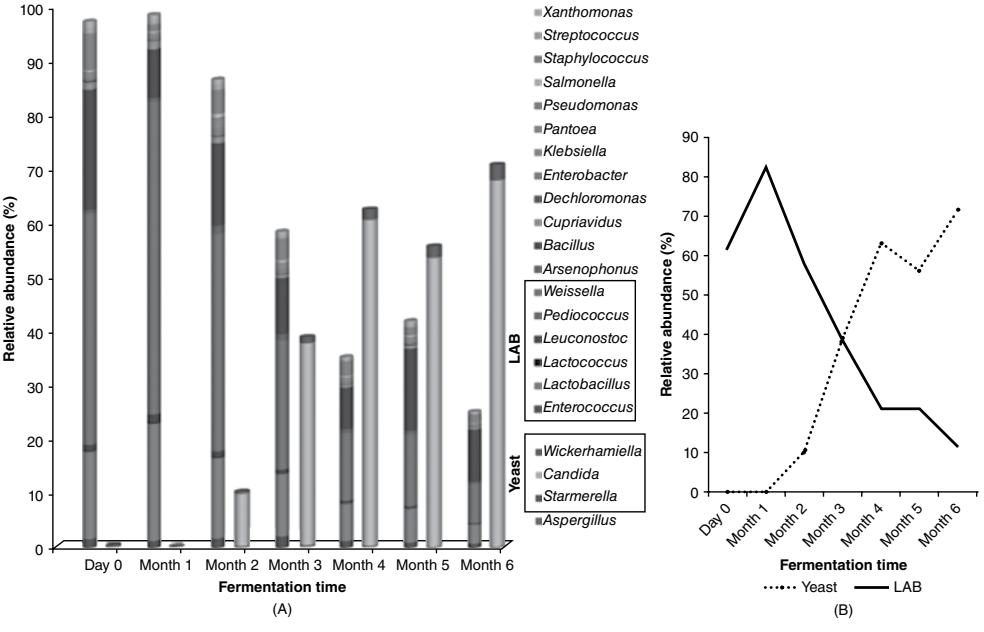


Figure 14.2 Changes in abundance of total microorganisms in a traditional Chinese soy sauce fermentation (A); and yeasts and lactic acid bacteria in the mash (B). From Sulaiman et al., 2014, with permission.

Table 14.4 Concentration of important flavor compounds in shoyu[1].

Compound	Concentration (ppm)	Compound	Concentration (ppm)
Alcohols		Aldehydes	
ethanol	21700–31,500	isovaleraldehyde	233
methanol	0.3–62	isobutyraldehyde	15
2-phenolethanol	4–6	acetaldehyde	5
n-propanol	4	Furanones	
isobutyl alcohol	12	HMF[2]	256
isoamyl alcohol	10	HEMF[2]	15–230
furfuryl alcohol	12		1–5
Acids		Polyols	
lactic acid	14,347	glycerol	10,209
acetic acid	1050–2100	acetoin	10
Esters		2,3-butanediol	238
ethyl lactate	24	Phenols	
ethyl acetate	0.1–15	4-EG[2]	3
methyl acetate	14	4-EP[2]	0.3

[1] Adapted from Feng et al., 2015; Kaneko et al., 2012; Nunomora and Sasaki, 1992; and Steinhaus and Schieberle, 2007.
[2] See text for chemical names.

including ethanol, acetate, and CO_2, as well as other organic acids and alcohols. Acids, amines, ammonia, and other products may be produced from amino acid metabolism. The subsequent sugar fermentation by the yeasts also generates ethanol and CO_2 via the ethanolic pathway, as well as many other higher alcohols, esters, furanones, and other flavor volatiles.

Pressing and refining

After the fermentation is complete and the moromi is adequately aged, the soluble soy sauce must be separated from the solid residue. In the traditional process, the moromi is passed through large vertically-arranged multi-layered sheets of cloth or nylon filters. No pumps are used; instead the moromi trickles through the filters simply by gravity. The manner in which the filters are layered or folded over one another also adds pressure. Alternatively, separation can be accomplished using hydraulic filter presses that force the moromi through sheets of cloth or nylon filters. Pressing can last for several days, with pressures increasing incrementally, up to 100 kg per cm^2. About 90 liters or more of liquid material can be obtained from 100 liters of moromi. The solid filter cake byproduct that is recovered is ordinarily used as animal feed. Greater filtration efficiency and overall shoyu yield can be achieved by enhancing protein hydrolysis and pectinase activities during mashing.

Refining is an important step, since the liquid material that is collected after pressing still contains components that need to be removed. In particular, an oil layer that appears in the upper phase, and a sediment material that collects in the bottom phase, are both undesirable and are separated. The middle phase, containing the shoyu, can be re-filtered and standardized to a desired composition (based on salt and nitrogen concentrations and color and flavor).

Pasteurization and packaging

Although raw soy sauce products are commercially produced, most soy sauce is heat pasteurized. Pasteurization can be performed either in bulk or, more commonly, in a plate-type heat exchange system at 70°C to 80°C. Flash pasteurization, at 115°C or higher for three to five seconds, may also be used. Aside from inactivating enzymes and microorganisms and thereby increasing shelf-life and stability, pasteurization contributes other important properties (Table 14.5). In particular, heating promotes color and flavor development by accelerating the non-enzymatic browning reaction. In addition, heat induces precipitation of insoluble proteins and other components that can then be removed by filtration to give greater product clarity. Finally, the heat step concentrates acids, phenols, and other compounds that either contribute desirable flavors or that have antimicrobial activity. Heating, however, may cause loss of volatile components.

Most soy sauces are packaged in glass bottles varying in size from 0.5 liters to 2 liters. Plastic bottles and 20 liter pails (for institutional use) are also common. Various chemical preservatives, including benzoic acid, benzoate salts, and ethanol, are often added.

Product characteristics

The final chemical composition of soy sauces depends on the specific type being produced. In general, the greater the proportion of soybeans, relative to wheat, the greater will be the total nitrogen concentration. Conversely, products made using higher levels of wheat will contain less nitrogenous material, but more reducing sugars. Thus, shoyu tamari, which is produced mainly from soybeans, contains nearly four times more total nitrogen, but six times less reducing sugars, than shiro, a type of shoyu made mostly from wheat with a small amount of soybeans. The amount of nitrogenous material and reducing sugars, as reflected by the wheat-to-soybean ratio, has a major influence on color. Soy sauce products containing greater levels of wheat are generally more light-colored and products containing more soybeans are dark-colored. Shiro, therefore, has a more tan appearance whereas tamari has a dark red-brown color.

The final composition of soy sauce depends on the specific type and the manufacturer's specifications. In general, soy sauce contains (on a weight/volume basis) about 1.5% total nitrogen, 1% sugars, 1% lactic acid, 2% to 2.5% ethanol, and 14% to 18% salt. The pH is usually between 4.5 and 4.8. Due to the association of high salt diets with hypertension and other human health problems, there has been a considerable effort to reduce the salt concentration in soy sauce products. Among the strategies that have been considered are the use of salt substitutes, salt removal systems, and manufacturing modifications (Box 14.2).

Table 14.5 Beneficial effects of pasteurization on soy sauce.

- Enhances color formation via the Maillard non-enzymatic browning reaction
- Enhances aroma development
- Inactivates enzymes
- Kills microorganisms
- Accelerates precipitation of protein complexes
- Concentrates anti-fungal agents
- Concentrates flavor and aroma compounds

Box 14.2 Taking salt out of soy sauce and other soy-fermented foods

Salt is an essential ingredient in many of the Asian-type fermented foods. Except for tempeh, high levels of salt are used in the manufacture of most soybean-based fermented food products. For example, shoyu and other soy sauces contain between 14% and 18% salt. Likewise, miso contains as much as 13% to 14% salt. Even when used in moderation, these high-salt foods may still contribute a large amount of sodium. A single 1 tablespoon serving (or about 15 ml) of soy sauce contains around 950 mg of sodium or 2.4 grams of salt. Likewise, a cup (about 250 ml) of miso soup contains about the same amount (800–1000 mg of sodium).

Salt consumption Not surprisingly, populations that regularly consume soy sauce, miso, and fish sauce have high salt (and sodium) consumption rates. For example, in Japan, per capita salt consumption during the 1990s averaged around 13 g per person per day. Although salt consumption has decreased in the past decade to about 11 g, this is still considered too high (Miura et al., 2013). Salt consumption in the United States is somewhat less, about 8.5 g per person per day, but still nearly twice that recommended by health authorities (Kotchen et al., 2013).

The problem with high-salt diets is that there is a strong association between sodium or salt consumption and various disease states, including hypertension, stroke, and gastric cancer (Kotchen et al., 2013). Although the health risk of salt over-consumption has been disputed, most public health agencies still recommend that salt intake be reduced, especially for at-risk individuals (Okuda et al., 2014; Toda et al., 2015). Since soy sauce, miso, and fish sauce account for as much as 10–30% of the total salt consumed in Japan, China, and other Asian countries (Brown et al., 2009), decreasing the salt content of these products could contribute to a significant overall reduction in salt consumption.

Salt reduction approaches There are a number of possible ways to reduce the sodium content in soy sauce and related products. The most common means is to manufacture the product in the usual manner (i.e., adding the same amount of salt to the mash as would be done for a conventional fermentation), followed by removal of a portion of the salt. Removing the salt can then be accomplished via one of several desalination technologies. Currently, these methods include ion exchange chromatography using de-salting resins, ion-exchange membrane processing, nanofiltration, and electrolysis (Fidaleo et al., 2012; Luo et al., 2012). Potassium chloride can then be added to satisfy many of the functions played by sodium chloride (e.g., preservation and flavor).

An alternative way to make low-salt soy products is to replace some of the sodium chloride with potassium chloride prior to the fermentation. The potassium salt will still perform the functions of salt, including controlling the microbiota by selecting for salt-tolerant lactic acid bacteria and yeast. There is one downside to both of these approaches – mainly due to undesirable bitter or metallic flavor that is imparted by potassium chloride.

A quite different route to reduce the salt content relies on the addition of novel compounds that contribute "saltiness". These so-called salt mimics have been used for

many as flavoring agents, but are now recognized as suitable salt-substitutes. Examples include several amino acid derivatives and peptides, such as ornithyltaurine, glycine ethyl ester hydrochloride, and lysine hydrochloride (Kuramitsu, 2004).

Obviously, salt performs many functions in fermented foods, in addition to flavor. In particular, reducing the salt content of soy sauce and other fermented soy products may have profound effects on the microbiological quality and stability of these products (Song et al., 2015). Nonetheless, pathogen challenge studies on low-salt miso revealed that even when salt levels were reduced to less than 3%, *Staphylococcus aureus*, *Clostridium botulinum*, *Salmonella typhimurium*, and *Yersinia enterocolitica* were unable to grow or survive, and toxin production was absent (Tanaka et al., 1985). Still, as an additional barrier, some manufacturers add ethanol to the product to control spoilage or pathogenic organisms. Recently, Song et al. (2015) identified autochthonous ethanol-producing yeasts (*Torulaspora delbrueckii* and *Pichia guilliermondii*) that could be added to preserve low-salt soy sauce.

References

Brown, I.J., I. Tzoulaki, V. Candeias, and P. Elliott. 2009. Salt intakes around the world: implications for public health. Int. J. Epidemiol. 38:791–813.

Fidaleo, M., M. Moresi, A. Cammaroto, N. Ladrange, and R. Nardi. 2012. Soy sauce desalting by electrodialysis. J. Food Eng. 110:175–181.

Kotchen et al. 2013. Salt in health and disease - a delicate balance. N. Engl. J. Med. 368:13.

Kuramitsu, R., 2004. Quality assessment of a low-salt soy sauce made of a salty peptide or its related compounds. Adv. Exp. Med. Biol. 542:227–238.

Luo, J.Q., W.B. Huang, W.J. Song, X.F. Hang, L.H. Ding, and Y.H. Wan. 2012. A pilot-plant test on desalination of soy sauce by nanofiltration. Sep. Purif. Technol. 89:217–224.

Miura, K, K. Ando, T. Tsuchihashi, K. Yoshita, Y. Watanabe, H. Kawarazaki, H. Matsuura, M. Kusaka, H. Kai, M. Kawamura, Y. Kawano. 2013. [Scientific statement] Report of the Salt Reduction Committee of the Japanese Society of Hypertension (2) Goal and strategies of dietary salt reduction in the management of hypertension. Hypertens. Res. 36:1020–1025.

Okuda, N., J. Stamler, I.J. Brown, H. Ueshima, K. Miura, A. Okayama, S. Saitoh, H. Nakagawa, K. Sakata, K. Yoshita. L. Zhao, and P. Elliot. 2014. Individual efforts to reduce salt intake in China, Japan, UK, USA: what did people achieve? The INTERMAP Population Study. J. Hypertension. 32:2385–2392.

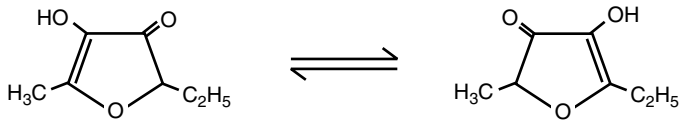
Song, Y.-R., D.-Y. Jeong, and S.-H. Baik. 2015. Effects of indigenous yeasts on physicochemical and microbial properties of Korean soy sauce prepared by low-salt fermentation. Food Microbiol. 51:171–178.

Tanaka, N., S.K. Kovats, J.A. Guggisberg, L.M. Meske, and M.P. Doyle. 1985. Evaluation of the bacteriological safety of low-salt miso. J. Food Prot. 48:435–437.

Toda, A., Y. Ishizaka, M. Tani, and M. Yamakado. 2015. Current dietary salt intake of Japanese individuals assessed during health check-up. Hypertens. Res. 38:163–168.

The flavor of soy sauce

To say that soy sauce flavor is complex would be quite the understatement. Not only are there hundreds of fermentation end-products in soy sauce, but there are also constituents of the raw material as well as compounds generated by heat and chemical reactions that also



4-hydroxy-2-ethyl-5-methyl-3-(2H) furanone

4-hydroxy-5-ethyl-2-methyl-3-(2H) furanone

Figure 14.3 Chemical structure of HEMF. Adapted from Nunomura and Sasaki, 1992.

contribute to the flavor of these products. Moreover, most soy sauces contain up to 18% NaCl. Thus, perhaps the most immediate and obvious flavor one detects is saltiness. However, the flavor of soy sauce is far more complex than simply saltiness. In fact, nearly 200 volatile flavor components have been identified in shoyu, using GC or GC/MS analysis. Several of these, in particular, various furanones and phenolic compounds, are considered to be the most important, based on their relatively high concentrations and their characteristic soy sauce-like flavor.

Furanones refer to a sub-class of heterocyclic organic molecules that exist as ring structures containing 4 carbons and 1 oxygen. The main soy sauce furanones include 4-hydroxy-2-(or 5) ethyl-5 (or 2) methyl-3- (2H) furanone (HEMF) and 4-hydroxy-5-methyl-3 (2H) furanone (HMF). In general, HEMF and HMF confer sweet or roasted flavor notes, respectively. HEMF, in particular, has been considered to be the main flavor responsible for "character-impact" of most Japanese-type soy sauce (Figure 14.3). However, although it has been suggested that furanones are derived from fermentation, their presence and concentration in shoyu depends mostly on the composition of the raw material. For example, HEMF occurs in koikuchi, but not in tamari, indicating that wheat is necessary for its synthesis. Among the phenolic compounds that contribute to soy sauce or shoyu flavor are 4-ethylguaiacol (4-EG) and 4-ethylphenol (4-EP). They are also produced during fermentation by yeasts, but are derived from precursors formed by koji molds grown on wheat. They confer typical soy sauce-like flavors.

In addition to the furanone and phenols, a large number of acids, aldehydes, alcohols, and esters are produced during the shoyu fermentation. Although the concentrations of some of these compounds, i.e., ethanol and lactic and acetic acids, and 2,3-butanediol, can reach very high levels, most are in the order of parts per million. They are generally produced by lactic acid bacteria and yeasts. Other important flavors are generated, along with color, via the Maillard reaction. Although the formation of Maillard reaction products is accelerated by heating (i.e., pasteurization conditions), many end products are also formed during the mashing and moromi aging steps.

Another group of flavor constituents exists that is very important in soy sauce products, but whose flavor is not so easily described. Included in this group are several nitrogenous compounds, especially the amino acid glutamic acid, and the nucleotides inosine monophosphate (IMP) and guanosine monophosphate (GMP). The flavor or taste of these compounds (and their sodium salts) falls outside what are ordinarily considered to be the four basic flavors – sweet, sour, bitter, and salty. Rather, sodium glutamate, sodium inosinate, and sodium guanylate impart a totally unique flavor known as umami, a Japanese term meaning "deliciousness." Umami has been described as conferring a meaty, savory, brothy flavor to foods. The glutamate salt, monosodium glutamate, is also considered to be a flavor enhancer. And although umami is usually associated with Asian cuisines, it is present in a wide array of foods, including cured ham, Parmesan cheese, and shitake mushrooms. Most sensory authorities now consider umami to be the fifth basic flavor.

Non-fermented soy sauce

Given the many steps involved in preparing the raw materials, making the koji, and performing the fermentation, it is not surprising that an alternative, non-biological process for making soy sauce exists. This process is based on an acid hydrolysis of soybeans. Although the finished product is less expensive to make and takes much less time, it lacks the flavor and complexity of fermented soy sauce.

Spoilage and defects

Although the low pH and high salt concentration is inhibitory to most spoilage organisms, benzoate is sometimes added as a preservative, mainly against fungi. Ethanol may also be added for preservation. As noted above, excessive browning during mashing or aging may also be considered a defect, especially when the soy sauce is destined for use as a flavor ingredient in light-colored products (Box 14.3). In addition, some undesirable flavors, such as isobutyric acid and isovaleric acid, may form during prolonged storage.

Box 14.3 Reducing the dark color in soy sauce

The dark brown color of shoyu and other types of soy sauce is formed during fermentation, aging, and pasteurization steps (Miyagi et al., 2013). Color formation is expected and contributes to the overall appearance of many of these products. However, lighter-colored products may be preferred by some consumers, and for some applications, i.e., when soy sauce is used as an ingredient in processed foods, a lighter, amber-like color is more desirable. Although it is certainly possible to produce such products by adding more wheat to the formulation (e.g., shiro has a lighter color than tamari), there is much interest in developing manufacturing processes that result in traditional soy sauce products having a lighter, less brown appearance.

Soy sauce and the Maillard reaction Two general strategies have been devised to "lighten" the color of soy sauce. One approach is biological and is based on controlling the Maillard non-enzymatic browning reaction responsible for brown pigment formation. This reaction requires reducing sugars and primary amino acids. Due to the extensive hydrolysis of soy protein and wheat starches and polysaccharides, both are present at very high concentrations in the moromi mash. Xylose, in particular, is very reactive in the Maillard reaction. Thus, adding xylose-consuming strains of lactic acid bacteria to the mash could decrease the xylose concentration and reduce brown pigment formation. Although such strains do exist, metabolism of xylose does not occur in the mash, due to catabolite repression by glucose (Abe and Uchida, 1989).

A second altogether different approach to de-colorize soy sauce is based on physical separation of the Maillard reaction products that account for brown pigments. Researchers first tried to remove these compounds using various adsorbents, including activated carbon, activated clay, diatomaceous earth, magnesium oxide, and silica gel (Miyagi et al., 2013a). Although reductions in color were observed for

some treatments (without affecting quality), adsorption methods had several technical disadvantages, including leeching and cost.

An alternative method was based on filtration. Although ultrafiltration through 20,000 MW cut off membranes was ineffective at reducing color, various nanofilturation (NF) configurations removed color-forming compounds and retained flavor and acidity (Miyagi et al., 2013b). The color and quality depended on the MW cut off as well as charge of the membranes. Ultimately, by blending NF-processed soy sauce with non-NF soy sauce, the investigators were able to produce a range of products with varying color intensities.

References

Abe, K. and K. Uchida. 1989. Correlation between depression of catabolite control of xylose metabolism and a defect in the phosphoenolpyruvate: mannose phosphotransferase system in *Pediococcus halophilus*. J. Bacteriol. 171:793–800.

Miyagi, A., H. Nabetani, and M. Nakajima. 2013a. Decolorization of Japanese soy sauce (shoyu) using adsorption. J. Food Eng. 116:749–757.

Miyagi, A., T. Suzuki, H. Nabetani, and M. Nakajima. 2013b. Color control of Japanese soy sauce (shoyu) using membrane technology. Food Bioprod. Process. 91:507–514.

MISO

Another very popular fermented soy product produced and consumed thorough the Far East is miso. Although miso originated in China and Korea more than a thousand years ago, Japan is the leading producer and consumer, with per capita consumption at about 5 kg per person per year or 14 g/day. Miso has a flavor somewhat similar to soy sauce, except instead of being a liquid, it is paste-like, with a texture similar to thick peanut butter. However, because miso is more concentrated, the flavor is more intense that soy sauce. It can be used like soy sauce as a seasoning or flavoring agent, but is more commonly used, at least in Japan, to make soups and broths. Similar products are produced in Korea (doenjang), China (jiang), Indonesia (taoco), and the Philippines (taosi).

In Japan, there are three general types of miso. Each is based mainly on composition, specifically, the raw ingredients used to make the miso koji (Table 14.6). Accordingly, rice miso is made using a rice koji, barley miso is made using a barley koji, and soybean miso is made using a soybean-only koji. There are, however, many variations of these three miso types, depending on the salt content, color, and flavor. Color is particularly important and its development occurs mostly as a function of how much grain (rice or barley) is added and how long the product is aged. Thus, lighter colored misos typically contain more rice or barley and are aged for only a few months. They also have a milder flavor, due to shorter fermentation times. For example, white or shiromiso is made using a rice koji, contains soybeans and salt, and is usually light in appearance. This type of miso has a noticeably mild, sweet flavor. Red miso or "akamiso" is made from a barley koji and has a stronger, more robust flavor. Finally, hatcho miso contains only soybeans, is aged for as long as three years, and has a very dark brown color and a strong, complex flavor.

Table 14.6 Types and properties of different types of miso[1].

Product	Moisture (%)	Protein (%)	Reducing sugar (%)	Fat (%)	pH	Color
Rice miso						
Sweet	43–49	10–11	15	3–5	5.4	White or red
Salty	45	10–13	12	6	5.3	Yellow or red
Barley miso						
Sweet	44–47	10	17	4–5	5.2	Yellow
Salty	46–48	13	11	5–6	5.1	Red
Soybean miso	45–46	17–20	4	11	5.0	Red or brown

[1] Adapted from Ebine, 1986 and Baens-Arcega et al., 1996.

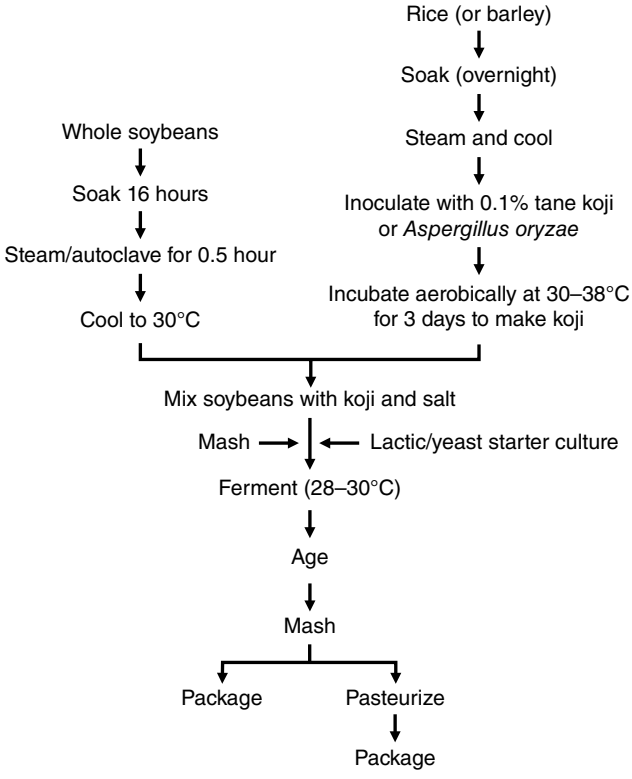


Figure 14.4 Manufacture of miso flow chart. Adapted from Yoneya, 2004.

Manufacture of miso

Miso and related products are manufactured much like soy sauce, except for one major difference (Figure 14.4). In miso manufacture, dry salt, rather than brine, is added directly to the koji-soybean mixture. Therefore, the resulting product has approximately twice the total solids of soy sauce (50% to 60% versus 24% to 28%), with a very high salt content. The process starts with the manufacture of a koji. As noted above, the koji substrate can be rice,

barley, or soybeans. The rice (usually polished rice is used) or barley is soaked in water overnight at 15°C, then steamed in a batch or continuous cooker. After cooling, a tane koji (or a spore culture), containing strains of *A. oryzae* and *A. sojae* with defined properties, is used as the inoculum (at 0.1%). The koji is then incubated at 30°C to 40°C for 40 to 48 hours in fermentation chambers equipped with aerating devices.

The soybeans (usually yellow soybeans are used) are similarly prepared, first by sorting and soaking and then by cooking under pressure (0.5 kg/cm^2 to 1.0 kg/cm^2) for about fifteen to forty-five minutes. At this point in the process, the cooked soybeans are ground or extruded and then mixed with the koji and salt in automated mixing machines. Alternatively, the whole cooked soybeans, koji, and salt can be mixed, and then the entire mixture is mashed. This mashing step is performed using an extrusion-like device that grinds the mixture to a chunky homogenous paste. The mixture is transferred to large tanks or fermentors (stainless steel or epoxy resin-lined steel) with capacities of 1000 kg to more than 100,000 kg. Depending on the type of miso being made, the salt concentration may range from 6% to 13%.

Fermentation

The miso fermentation occurs in a manner similar to that of soy sauce, in that the hydrolysis of complex macromolecules to form simple nutrients and the subsequent fermentation and metabolism of those nutrients occur essentially at the same time. As it does with soy sauce, the koji serves both as the source of proteolytic and amylolytic enzymes, as well as a substrate source for those enzymes. About 50% of the total protein and 75% of the polysaccharides are completely hydrolyzed to amino acids and monosaccharides, respectively. Because miso is made from whole soybeans, the lipid portion (about 3% to 10%) is also present in the miso mash. Fungal lipases (from the koji) hydrolyze triglycerides to di- and monoglycerides, glycerol, and free fatty acids. The latter may accumulate to high levels (as much as 1% to 3% in the miso). Collectively, the amino acids, sugars, and fatty acids provide a rich source of nutrients for fermentative organisms.

In traditional miso manufacture, a miso seed culture, obtained from previous raw miso product, may be used to initiate the fermentation. Although many small manufacturers continue this practice, miso starter cultures are now often used, especially by large modern manufacturers. These cultures ordinarily contain yeast strains of *Z. rouxii* and *C. versatilis,* along with *T. halophilus*, *P. acidilactici, L. delbrueckii*, and other lactic acid bacteria. As these organisms ferment and consume glucose and other free sugars present in miso, several organic acids, including lactic, acetic, and succinic acids are formed. The longer the miso ferments, the lower the sugar concentration. However, the total acid concentration in the finished miso is generally less than 1% and the pH is usually not less than 5.0. About 0.1% to 1% ethanol and 1% to 2% glycerol may be formed during the yeast fermentation.

Miso fermentations are conducted in tanks at 25°C to 30°C. Temperature control and mechanical stirring are common features in many factories. Fermentation can be as short as one month or less to more than two years. Transferring the miso from one tank to another is often done to promote a homogenous fermentation. When the fermentation and aging period is complete, the miso is re-mashed and packaged into small plastic tubs or bags (about 400 g to 500 g capacity). The miso is usually left in its raw state. However, some miso is subjected to a short-time heat treatment to enhance shelf-life by preventing package swelling (due to gas formation). For most miso consumers, raw miso is much preferred. In part, this is because of tradition, but this preference is also based on the putative health properties conferred by

the live microorganisms. In this respect, miso is viewed much like yogurt, in that any heat step that inactivates beneficial microorganisms may be considered tantamount to sacrilege.

Spoilage and defects

Despite the high salt concentration and relatively low pH, growth of spoilage organisms in miso can occur, resulting in gas, off-odors, over-acidification, and surface slime. Yeasts and bacteria responsible for these defects include *Hansenula* and *Z. rouxii*, *Pediococcus acidilactici*, and *Bacillus* sp. Spoilage is more likely to occur when salt levels are reduced (<12%) or when the koji molds are inhibited. Pasteurization, either before or after packaging, inactivates these organisms. However, as indicated above, many consumers prefer raw, non-heat-treated miso.

NATTO

Natto is another soybean-fermented product consumed mainly in Japan, but similar products are also produced in China, Thailand, and the Philippines. Per capita consumption in Japan is about 1.2 kg per person per year or 3 g per day. Natto is used as a condiment or flavoring agent, usually for rice and vegetables or as an ingredient in sushi. Nutritionally, natto is comparable to other fermented soybean products. It contains 16% to 18% protein (45% on a dry basis), with good digestibility and biological value, compared to cooked soybeans. It is considered to be an excellent source of isoflavones and readily absorbed Vitamin K. The latter is thought to contribute to bone-strength and reduced rates of fractures among natto-consuming populations.

Manufacture of natto

The manufacturing procedure for natto begins like that for miso; however, the organisms involved in the fermentation are different and the final product bears little resemblance to miso. Natto is made from whole, somewhat small-sized soybeans that are cleaned, soaked for 12 to 20 hours at ambient temperature, and steamed at 121°C for 20 to 40 minutes. The thoroughly cooked and cooled beans are then inoculated with about 10^6 to 10^7 spores per kg of *Bacillus subtilis* var. *natto* (formerly *Bacillus natto*), and the material is well mixed. The incubated beans are then divided into 100 g portions and placed into packages. According to traditional practices, bundled rice straw was used as the container (some manufactures still use straw). However, polyethylene bags are now more common. In either case, the beans are moved into aerobic incubators at 40°C at 85% relative humidity for 16 to 20 hours. Growth of *B. subtilis* var. *natto* occurs primarily at the surface and is accompanied by a change in color (from yellow to white) and synthesis of a highly viscous material (see below). After incubation, the natto is held at 2°C to 4°C to minimize further growth. The final product is quite different from miso and shoyu. In particular, it has a unique sweet flavor. However, the most striking feature of natto is the texture.

The material produced by *B. subtilis* var. *natto* is unlike other viscous end-products produced by lactic acid bacteria. The latter synthesize exopolysaccharides, whereas the natto bacilli produce γ-poly-glutamic acid (γ-PGA), a polymer of the amino acid, glutamate. Indeed, so much of this material is produced that it can completely cover the entire bean surface, accounting for nearly 1% of the total dry weight of natto. It is so viscous (and sticky and gooey!) that it may influence whether the natto actually gets tasted.

TEMPEH

Tempeh is another mold-fermented soybean product that originated many centuries ago in the Far East. Specifically, tempeh was first produced in Indonesia, where it remains a major food staple and an inexpensive source of dietary protein. For many years, tempeh production had spread only to Malaysia and a few other neighboring countries. Interestingly, tempeh has long been popular in the Netherlands, as Indonesia was once under Dutch rule. More recently, for reasons discussed below, Canada and the United States have become markets for tempeh. Nonetheless, Indonesia is by far the main producer and consumer of tempeh. Current per capita consumption in Indonesia is about 15 grams per person per day. Although tempeh production has been industrialized, a substantial amount of the more than 500 million kg of tempeh produced in Indonesia per year is still made in the home or in small "cottage-sized" production facilities.

It is remarkable, given the fact that tempeh was only "discovered" by American consumers in the last 40 years that it has become quite popular (relatively speaking). Undoubtedly, this sudden popularity is due, in part, to interest in vegetarian cuisine and non-meat alternative food products (so called "faux" meats). However, the popularity of tempeh in the United States may also be due to its nutritional properties. In particular, tempeh is a rich source of high quality protein (as much as 19%); a 100 g serving provides about 25% of the daily value of protein. In addition, tempeh is one of only a few plant-based foods that contain vitamin B_{12} (discussed below).

Another reason, perhaps, why tempeh has attracted interest among US consumers relates to its versatile applications and organoleptic properties. In its raw state, tempeh has a bland, beany, mushroom-like flavor. However, cooking transforms this plain-tasting material into a pleasant, nutty, flavorful product. If one can get past the fact that tempeh consists entirely of moldy beans, its flavor, especially when it is sautéed or fried, resembles that of cooked or roasted meat (sort of). After all, tempeh and muscle protein (i.e., meat) both derive much of their flavor from Maillard reaction products that form when amino acids and reducing sugars are heated at high temperature. The lipid component of soybeans may also serve as a precursor for meaty flavor and aroma development. Finally, it is fair to say that the development of a tempeh "industry" in the United States and consumer awareness of tempeh as a food were both driven by academic interest. In particular, researchers at Cornell University (led by K. Steinkraus) and the USDA (C.W. Hesseltine and H.L. Wang), played key roles in understanding many of the microbiological and technical issues related to tempeh manufacture.

Manufacture of tempeh

The industrial manufacture of tempeh is quite simple, although numerous variations exist, depending largely on the scale of production, geographical and climatic considerations, and manufacturer preferences. The only raw material is soybeans, the fermentation time is short, and there is no aging or ripening period involved. In fact, the entire start-to-finish process is less than forty-eight hours. Tempeh can be considered as solid-state fermentation in that it consists of soybeans that are essentially held together by the mold mycelium that grows throughout and in between the individual beans. It should be noted that although other legumes, cereal grains, vegetables, and even seafood can be incorporated into tempeh (and are commercially available), soybeans are by far the most common starting material.

Substrate preparation

The process starts by sorting the soybeans to remove damaged or moldy beans (Figure 14.5). The beans are soaked for 30 minutes or longer, and the hulls are removed either manually or mechanically. The former method, practiced by very small traditional manufacturers, involves rubbing the beans by hand (or according to some traditions, by feet!) and then separating the hulls by floatation. Manual de-hulling, however, has been largely replaced by the use of various types of mills, which can be used on dry or wet beans. Some manufacturers omit the first boiling step, although this is considered by some tempeh experts to be an essential part of the process, facilitating hydration and hull removal.

The de-hulled beans are then soaked in water. Although this is a seemingly simple step, it has very important implications for tempeh quality. It is during this steeping period (16 to 24 hours at warm ambient temperature), that endogenous lactic acid bacteria grow and produce organic acids. This causes the pH to decrease, and the low pH restricts growth of undesirable spoilage bacteria, as well as potential pathogens. It should be noted, however, that some of these undesirable bacteria can survive during the soaking step even if acid conditions are established. To promote acidification, therefore, the steep water can be acidified directly with lactic or acetic acid.

After the soaking step, the remaining hulls are removed from the beans and the de-hulled beans are heated, either by steaming or boiling (for anywhere from thirty minutes to two hours). This heat treatment enhances extraction of soluble nutrients and inactivates microorganisms that might otherwise interfere with the subsequent fermentation. This step is also necessary to denature trypsin inhibitor, a native soy protein that acts as an anti-nutritional factor.

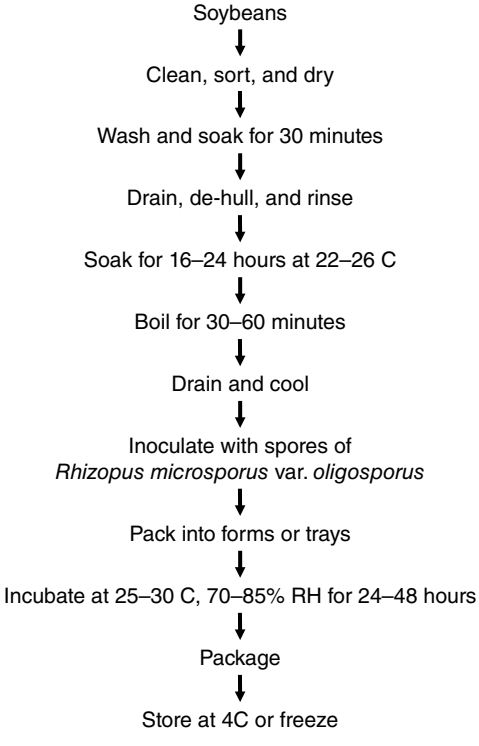


Figure 14.5 Manufacture of tempeh. Adapted from Babu et al., 2009 and Yoneya, 2004.

Inoculation

After cooking, the beans are drained and dried. Next, they are inoculated with either a portion from a previous batch of fully developed tempeh, a wild, mixed strain culture called "usar", or, as is more common in modern manufacture, with a spore culture of *Rhizopus microsporus* var. *oligosporus* (Figure 14.6). Typical culture inocula levels range from 10^7 to 10^8 spores (about 1 g) per kg of beans. Pure *R. oligosporus* cultures can be added directly to the beans, or the spores can be inoculated onto steamed rice, incubated until well-grown, and then used to inoculate the soybeans.

Fermentation

According to traditional Indonesian manufacturing practices, the inoculated beans are shaped into cakes and wrapped in banana leaves. For readers unfamiliar with banana leaves, they can be very large, about 40 cm by 1.5 m. The use of banana leaves as the packing chamber is not merely for natural aesthetics; they provide a moist, microaerophilic environment that supports rapid growth of *R. oligosporus*. However, for large-scale, industrial manufacture of tempeh (and as conducted in the United States), banana leaves are not used. Instead, the inoculated beans are distributed on trays 1 cm to 3 cm deep and ranging in length and width from 1 meter to several meters. After one to two days of incubation in a warm room (ranging from 25°C to 37°C), the beans are covered with white mycelium, and the fermentation is considered complete. The mycelia also will have grown in between the individual beans such that solid soybean cakes will have formed. Detailed analyses have revealed that the fungal hyphae actually penetrate nearly 1 mm into the bean, or about 25% of the diameter of the cotyledon. It is important that the fermentation ends promptly, however, before the mold begins to sporulate. Sporulation is accompanied by the appearance of the dark-colored black or grey sporangia that is generally unattractive to consumers (see below).

Tempeh microbiology

The surface of raw soybeans contains an assortment of Gram-positive bacteria, including *Lactobacillus casei* and other lactic acid bacteria, enterococci, staphylococci, streptococci, and bacilli. Gram-negative bacteria, including *Enterobacter*, *Klebsiella*, and other coliforms

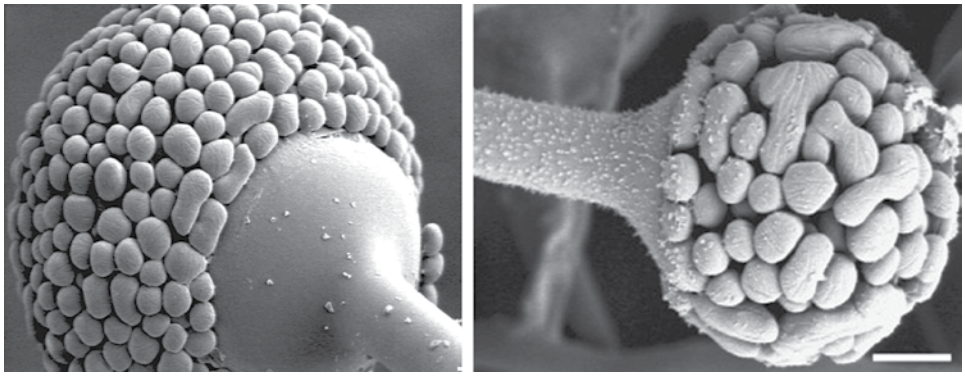


Figure 14.6 Electron micrograph of two strains of *Rhizopus microsporus* var. *oligosporus* isolated from tempeh. From Jennessen et al., 2008, with permission.

are also represented. Finally, yeasts, such as *Pichia*, *Saccharomyces*, and *Candida*, may also be present. In the dry beans, these organisms are generally present at low numbers and are not able to grow. However, during the soaking step, sucrose, stachyose, and raffinose diffuse out of the beans and into the water. Their subsequent hydrolysis by invertases and glucosidases releases glucose and fructose, which can then be used to support growth of the resident microbiota. Consequently, at the end of the soaking period (20 to 24 hours at 20°C), the total microbiota may reach levels of 10^9 cfu per ml or higher. Most of the organisms isolated after soaking are lactobacilli, enterococci, and streptococci. However, the specific species that predominate appears to depend on the temperature and the pH of the soak water (i.e., in those applications where lactic or acetic acid is added to the water). Importantly, the pH values of the soak water, whether acidified or not, generally will decrease to 4.5 to 5.0 by virtue of lactic acid and mixed acid fermentations.

Although the manufacture of tempeh clearly depends on the growth of *R. oligosporus* (discussed below), the fermentation that occurs during the soaking of the soybeans is also essential. This is because the formation of organic acids and the decrease in pH are necessary to control pathogens, including *Salmonella typhimurium*, *Yersinia enterocolitica*, *Staphylococcus aureus*, and *Clostridium botulinum,* that might otherwise grow in non-acidified soybeans. Low pH also inhibits *Bacillus, Enterobacter*, and other microorganisms capable of causing spoilage effects. It is important to note that even if the beans are heated prior to soaking, the lactic fermentation will still occur, although the rate and extent of the fermentation may be affected.

Tempeh cultures

As noted above, the primary fermentation is mediated by growth of *R. oligosporus.* The inoculum can be prepared and used in one of several different forms. First, they can be added to the soybeans directly as a pure spore culture. Recommended strains include NRRL 2710 and DSM 1964 – both were isolated from Indonesian tempeh and both are available from public culture collections. Like the commercial strains used for other fungal-fermented products, tempeh starter cultures should be selected based on specific phenotypic traits (Table 14.7). Alternatively, a backslop material consisting of a dried tempeh culture can be used. Finally, a third form, used in traditional tempeh manufacture, is called usar, and is made by inoculating wild *Rhizopus* spores onto the surface of leaves obtained from the indigenous Indonesian *Hibiscus* plant. After two to three days of incubation, the leaves contain a dense spore crop that can be dried and used to inoculate the soybeans. Species other than *R. oligosporus* may be present when wild cultures are used. Other species isolated from tempeh include *Rhizopus oryzae*, *Rhizopus stolonifer*, and *Rhizopus microsporus* var. *chinensis.*

Regardless of source or strain, tempeh cultures have a limited shelf life, as little as three to four months. This is because the *Rhizopus* spores enter into a dormancy phase during storage that reduces viability and germability (the ability of dormant spores to become

Table 14.7 Properties of *Rhizopus microsporus* var. *oligosporus.*

- Unable to metabolize major soy carbohydrates (sucrose, stachyose, or raffinose)
- Aerobic
- Rapid growth and mycelia production at 30–42°C
- Proteolytic and lipolytic
- Uses free fatty acids, derived from lipids, as an energy source

activated and produce biomass). Moreover, even if the soybeans are inoculated with a pure spore culture of *R. oligosporus*, the tempeh is unlikely to be maintained in a pure state for too long, since the substrate itself will likely be contaminated with an array of different organisms. In fact, as discussed below, other microorganisms may play important nutritional and organoleptic roles during the tempeh fermentation.

Tempeh biochemistry

The tempeh mold, *R. oligosporus* performs several important functions beyond the production of the mycelia mass that literally holds the soybeans together. Importantly, *R. oligosporus* is responsible for causing major biochemical changes in the composition of the soybean substrate (Table 14.8). In particular, soybean lipids and proteins serve as substrates for fungi-excreted lipases and proteases, respectively. During the incubation period, about a third of the lipid and a fourth of the protein fractions are degraded. Lipid hydrolysis results mainly in mono- and diglycerides, free fatty acids, and a small amount of free glycerol. Most of the free fatty acids are subsequently oxidized by *R. oligosporus*, resulting in a 10% decrease in the total dry matter in the finished tempeh. In fact, the relative inability *R. oligosporus* to metabolize the available soy carbohydrates (mainly stachyose, raffinose, and sucrose) means that fatty acids serve as the primary energy and carbon source.

In contrast, only about 10% of the released amino acids and peptides are consumed by *R. oligosporus*. Of the remaining portion, about 25% are assimilated into biomass, and the rest is left in the tempeh. The soluble nitrogen concentration, therefore, increases four-fold, from about 0.5% to 2%. Although *R. oligosporus* has limited ability to metabolize these amino acids, enough ammonia is still formed during the tempeh fermentation to cause the pH to rise from 5.0 to above 7.0. This increase in pH during the fungal fermentation stage underscores the importance of acidification during bean soaking, since the latter step is responsible for inactivating potential pathogens that may have been present in the starting material. Once the low pH barrier no longer exists, pathogens could theoretically grow and cause problems.

Polysaccharide-hydrolyzing enzymes are also produced by *R. oligosporus*. These enzymes attack pectin, cellulose, and other fiber constituents, releasing various pentoses (xylose, arabinose) and hexoses (glucose, galactose). However, the activity of these enzymes during the tempeh fermentation is modest, and only minor amounts of free sugars are found in the final product.

Table 14.8 Composition of soybeans and tempeh[1].

Constituent	Soybeans[2] (g/100 g)	Tempeh[3] (g/100 g)
Moisture	65–70	60
Protein	10–15	20
Soluble nitrogen	0.1	1.2
Carbohydrate	10–12	7
Fat	5–7	11
pH	6–7	6–7

[1] Adapted from Handoyo and Morita, 2006 and the USDA National Nutrient Database (Release 28).
[2] Wet basis, cooked and drained.
[3] Wet basis, fresh.

Tempeh nutrition and safety

Among the most important changes that occur during the tempeh fermentation are those that affect the nutritional quality of tempeh. As noted above, the concentration of the major macronutrients (i.e., protein, fat, and carbohydrates) decreases as the soybeans are converted to tempeh, due to enzymatic hydrolysis. These changes may account, in part, for an improvement in nutritional quality. For example, it has been suggested that protein hydrolysis makes tempeh more digestible, compared to soybeans, although the protein efficiency ratio (used as a measure of protein quality) of tempeh is no higher than an equivalent amount of cooked soybeans. There is also a decrease in the amount of soy oligosaccharides (mainly stachyose and raffinose) during the conversion of soybeans into tempeh. These sugars, which are sometimes considered undesirable due to their ability cause flatulence, are removed from soybeans not by fermentation, but rather by diffusion during the soaking and cooking steps.

Probably the most important nutritional improvement that occurs during tempeh manufacture is the suggested increase in vitamin content. Of particular interest is vitamin B_{12}, whose concentration in cooked tempeh generally ranges from 0.1 μg to 0.2 μg per 100 g. Since soybeans contain negligible levels of this vitamin, its presence in tempeh must occur as a result of biosynthesis by microorganisms in the tempeh. What is surprising, however, is that vitamin B_{12} is made not by *R. oligosporus* nor by lactic acid bacteria, but rather by bacteria that are essentially chance contaminants in the tempeh-making process (Box 14.4). Other vitamins, including riboflavin (B_2), niacin, pyroxidin (B_6), biotin, pantothenic acid, and folic acid, also increase in concentration by up to five-fold during tempeh production. The concentration of thiamine (B_1), however, decreases, and there is no effect on the level of fat-soluble vitamins.

In addition to the increase in vitamin content, the processing and conversion of soybeans into tempeh also results in the degradation of several anti-nutritional factors ordinarily present in soybeans. These compounds are considered anti-nutritional because they either interfere with digestion (trypsin inhibitor), reduce protein quality (tannins), reduce mineral adsorption (phytic acid), cause blood to form clumps (hemagglutenins), or cause metabolic disturbances (goitrogens). Some of the water-soluble, low-molecular-weight compounds, such as phytic acid and polyphenolic tannins, are removed during soaking and washing. In contrast, proteinaceous compounds, including trypsin inhibitor and other protease inhibitors, and hemagglutenins are inactivated by heating steps. It is also possible that some of these compounds are degraded by enzymes produced during fermentation. For example, phytic acid can be hydrolyzed by phytases produced by *R. oligosporus*.

Finally, there has long been concern about the microbiological safety and the possible presence of mycotoxins in tempeh. As is the case with other mold-fermented foods (e.g., soy sauce and miso), the wild or pure culture strains used for tempeh production do not produce mycotoxins. The possibility that tempeh fungi could harbor toxin- producing bacteria as endosymbionts has been suggested, but there are no reports of this phenomenon occurring in tempeh.

Spoilage and defects

In Indonesia, where tempeh is consumed on a near-daily basis, spoilage is not much of an issue, provided the product is eaten within a day or two of manufacture. However, the shelf-life of tempeh held at room temperature is very short, owing to the continued growth of the mold and bacteria. Once *R. oligosporus* begins to sporulate and produce colored sporangia,

Box 14.4 Is tempeh a source of vitamin B_{12}?

Tempeh has long been touted as one of the few vegetable-based foods that contain vitamin B_{12}. Nonetheless, whether tempeh can indeed be considered as a reliable source of vitamin B_{12} has been questioned. This has important nutritional implications since B_{12} is mostly found in animal products (e.g., meat, fish and shellfish, eggs, and dairy products), and individuals who eat vegan or strict vegetarian diets might not otherwise obtain this vitamin.

Interestingly, vitamin B_{12} is not synthesized by plants or animals like other vitamins – indeed, it the only vitamin made exclusively by microorganisms (LeBlanc et al., 2013). Therefore, fermented foods, and tempeh in particular, could theoretically serve as a source of this vitamin. While some of the early studies reported that tempeh contained as little as 0.002 μg/100 g, other studies reported levels as high as 0.8 μg/100 g (Nout and Rombouts, 1990). According to the most recent USDA National Nutrient Database for Standard Reference (Release 28), the vitamin B_{12} content is 0.08 μg/100 for raw tempeh and 0.14 μg/100 grams for cooked tempeh. The Recommended Dietary Amount (RDA) for adults is 2.4 μg (2.6 μg for pregnant females) so even at the higher estimate, it would take a lot of cooked tempeh (more than 1.7 kg!) to satisfy the RDA requirement.

There is no vitamin B_{12} in soybeans (the main ingredient in tempeh). Thus, its presence in tempeh means that it must be synthesized *de novo* by microorganisms present during the fermentation. However, the tempeh mold, *Rhizopus microsporus* var. *oligosporus*, does not produce B_{12}. In fact, the vitamin B_{12} biosynthetic pathway is absent in the Eukaryota (Helliwell et al., 2013). In contrast, vitamin B_{12} is produced by several bacteria and archae, including aerobes (e.g., *Pseudomonas*), anaerobes (e.g., *Propionibacterium* and *Clostridium*), and facultative anaerobes (*Citrobacter*, *Lactobacillus*) (Taranto et al., 2003; Leblanc et al., 2013). Furthermore, a genomic analysis has revealed that the biosynthetic machinery for making B_{12} is widely distributed in bacteria (Fang et al., 2017).

Several bacteria have been suggested to be responsible for B_{12} synthesis in tempeh, including *Klebsiella pneumoniae* and *Citrobacter freundii* (Keuth and Bisping, 1994). However, these organisms are not added to the starting material (only *R. oligosporus* spores are added). Therefore, they must somehow gain access to the soybeans, either via equipment, soaking water, or other means. The conditions during the tempeh fermentation would also have to be appropriate for these organisms to grow and synthesize B_{12}. In a tempeh fermentation that is well-controlled so that these contaminating organisms are absent, B_{12} is unlikely to be present. This explains why vitamin B_{12} production in tempeh is inconsistent at best, and also why nutritionists recommend that vegans include B_{12}-supplemented food in their diets.

References

Fang, H., J. Kang, and D. Zhang. 2017. Microbial production of vitamin B_{12}: a review and future perspectives. Microb. Cell Fact. 16:15.

Helliwell, K.E., G.L. Wheeler, and A.G. Smith. 2013. Widespread decay of vitamin-related pathways. Trends Genet. 29:469–478.

Keuth, S., and B. Bisping. 1994. Vitamin B_{12} production by *Citrobacter freundii* or *Klebsiella pneumoniae* during tempeh fermentation and proof of enterotoxin absence by PCR. Appl. Environ. Microbiol. 60:1495–1499.
Keuth, S. and B. Bisping. 1993. Formation of vitamins by pure cultures of tempe molds and bacteria during the tempe solid substrate fermentation. J. Appl. Bacteriol. 75:427–434.
LeBlanc, J.G., C. Milani, G.S. de Giori, F. Sesma, D. van Sinderen, and M. Ventura. 2013. Bacteria as vitamin suppliers to their host: a gut microbiota perspective. Curr. Opin. Biotechnol. 24:160–168.
Nout, M.J.R. and F.M. Rombouts. 1990. Recent developments in tempe research. J. Appl. Bacteriol. 69:609–633.
Taranto, M., J.L. Vera, J. Hugenholtz, G.F. De Valdez, and F. Sesma. 2003. *Lactobacillus reuteri* CRL1098 produces cobalamin. J. Bacteriol. 158:5643–5647.

the product's appeal also begins to diminish. By the time the tempeh surface has turned from creamy white to black or gray, the shelf-life is essentially finished. Even when stored at refrigeration temperatures, mold growth is slowed but not stopped. Therefore, some form of preservation is necessary. In the United States, tempeh is most often vacuum packaged in oxygen impermeable plastic to restrict growth of aerobic fungi and bacteria. Another effective way to preserve tempeh is freezing, which halts fungal growth. Finally, tempeh can be dehydrated or cooked prior to packaging or made into various processed products, such as vegetarian meat-like foods.

SAKE AND RICE WINES

As noted in Chapter 10, most wines produced throughout the world rely on grapes as the starting raw material. Grapes used for wine-making contain an ample amount of glucose and fructose, which are readily fermented by the endogenous yeasts or added pure yeast cultures. In contrast, when starchy substrates, such as rice, are used as the raw material, the complex polysaccharides (mainly amylose and amylopectin) must first be hydrolyzed to produce fermentable sugars. When rice wines were first developed, this hydrolysis step was done by chewing and masticating the rice. As will be discussed below, saccharification is now performed by a rice koji, not unlike the koji used in the soy sauce and miso fermentations.

The most well-known rice wine is sake (rhymes with hockey), an Asian rice wine that likely originated in China, probably several millennia ago. Sake-manufacture was established in Japan about 2000 years ago. In Japan, sake literally refers to any alcoholic product, but is now generally considered to be derived from rice. Although sake is the most well-known example, many other rice-derived wines and alcoholic beverages exist throughout the Far East. Examples include shaoxing (Chinese rice wine), awamori (a Japanese product distilled from rice), makgeolli (Korean rice wine), and ruou (Vietnamese rice wine). Historically, many of these wines have long been associated with Shintoism and Buddhism religious rites.

For many centuries, rice wines were the most popular alcoholic products consumed in Japan, China, and other Asian countries. However, over the past 50 years, consumption has steadily decreased as consumers have switched to grape wine, beer, and distilled alcoholic products. Per capita consumption of sake in Japan in 2008 was at 6.3 L per person per year (data from the Japan Sake Brewers Association). This is down from the 11.5 L consumed in 1997 (a 33% decrease) and 50% less than in 1973. The number of sake breweries has also decreased by nearly 50%. Per capita consumption of rice wine in China has also decreased

(2012 data from Euromonitor) to about 1 L per person per year. Of course, that is still a lot rice wine, considering there are nearly 1.4 billion people living in China. The decrease in sake popularity in Japan and other Far East countries is due mainly to increased competition. Consumption of beer and wine, as well as distilled spirits, has been increasing dramatically throughout Asia. In China, there has also been an intentional shift to use rice for food rather than alcohol. Safety concerns due to the 2011 nuclear accident in Japan and possible contamination of rice have also contributed to the lower consumption data. In contrast, in the United States, sake and rice wines are on the upswing, perhaps due to the popularity of sushi and Japanese cuisine. Although the total US market is still very small, several modern sake manufacturing facilities are now operating in the US.

Manufacture of sake and other rice wines

Like other fermentations described in previous chapters, sake requires only four simple ingredients – rice, koji, water, and yeast. Although considered a wine, sake is different from grape wine fermentations in at least two main respects. First, as noted above, fermentable sugars are absent in rice. Thus, it is necessary to provide exogenous enzymes, in the form of a koji that can hydrolyze starch to simple sugars that the yeasts can ferment. This part of the sake manufacturing process, therefore, shares similarity with the beer-brewing process, in which malt is used to convert the starch (in barley) to simple sugars. The other major difference that distinguishes sake production from wine is that the saccharification step just described and the actual ethanolic fermentation step occur simultaneously or in parallel. In other words, nearly as soon as sugars are made available by action of koji enzymes, they are quickly fermented by sake yeasts. The implications of these parallel processes will be discussed below.

The sake-making process (Figure 14.7) starts by preparing a rice koji. There are actually several different types of sake produced in Japan, based in part on whether alcohol is added,

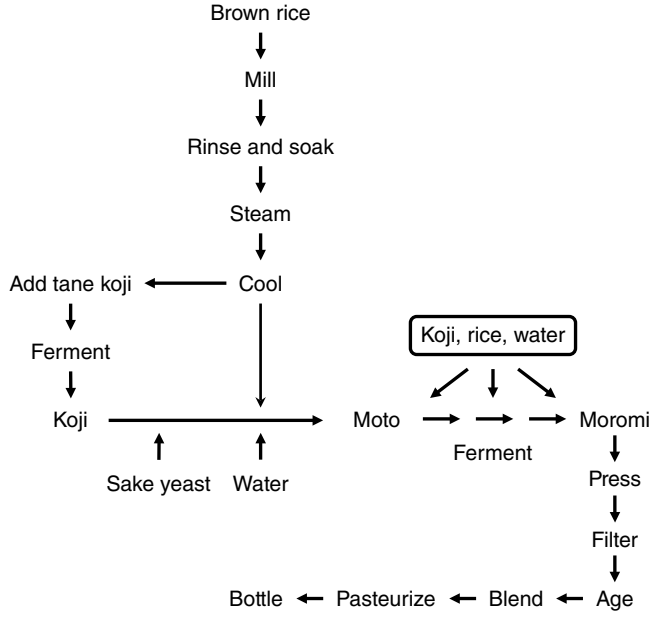


Figure 14.7 Manufacture of sake. Adapted from Yoshiwawa and Ishikawa, 1989.

but mainly on how the rice used to make the koji is milled (or polished). In general, the whole or brown rice is milled to remove 25% to 50% of the surface material (containing the germ and bran), which is necessary since the fat and protein components are undesirable. Next, the rice is rinsed and soaked for several hours to achieve about 30% moisture. The moist rice is then briefly steamed (<1 hour) and cooled to 30°C to 35°C. About three-fourths of this rice is removed and cooled further to 5°C to 10°C for later use (see below). The remaining fourth is used for making koji.

To make the koji, the rice is inoculated with about 0.01% of a tane koji (see above) or an *A. oryzae* spore culture. The material is incubated for forty to 48 hours at 30°C to 35°C under high humidity. The koji is mixed at intervals to re-distribute the growing fungi, maintain aerobiosis, and prevent excessive heat build-up. When finished, the rice should be covered with fungal mycelia and should contain high amylolytic and proteolytic activities.

Next, the koji is moved to a tank, and the steamed rice (from above) and water are added. This material, called moto, is essentially a pre-culture whose purpose is to increase the population of endogenous yeast and lactic acid bacteria (see below) and to initiate fermentation. According to traditional sake brewing practices, this moto seed culture incubates for about two weeks at 15°C, and then defined sake yeast strains are added. It is now more common, however, to add sake yeast at the outset, rather than rely on wild yeast for the initial fermentation. As the moto incubates, enzymatic hydrolysis of rice starch and proteins occur, releasing sugars and nutrients that support growth of the yeast. The moto is eventually transferred to large tanks (capable of accommodating more than 10,000 kg of rice) containing more koji, steamed rice, and water for combined mashing and fermentation.

The fermenting mixture, now called moromi, is then diluted several times with an equal amount of koji-rice-water, at two-to-three day intervals, such that the thick moromi mash increases in volume three- to four-fold. This process ensures that the yeast population remains high throughout the fermentation and provides, on a step-wise basis, adequate amounts of substrates to support an extended, semi-continuous fermentation. This unique fermentation process also serves to maintain high lactic acid levels and high solids content, such that contaminating organisms are inhibited. Thus, even though the sugar concentration never reaches much more than 6% to 8%, the ethanol concentration in the finished product may be 18% or higher. To achieve such a high yield of ethanol by a simple batch fermentation (i.e., where all of the fermentable sugar was initially present) would require a mash containing as much as 30% glucose. This is far more sugar than could be osmotically tolerated by the yeast and would result in a stuck fermentation.

The *S. cerevisiae* strains used for sake manufacture are different from typical wine and beer strains, in that they generally have higher osmotic, acid, and ethanol tolerance. They also produce copious amounts of foam, which occupies about a third of the volume in the fermentation tank, reducing the efficiency of the fermentation. Strains that are defective in making foam (i.e., foamless mutants) could have industrial advantages. In addition to yeast, other organisms are also involved in the sake fermentation. In particular, endogenous lactic acid bacteria, including *Lactobacillus sakei* and other *Lactobacillus* and *Leuconostoc* spp., grow and produce acid early in the fermentation. This early acidification evidently helps to control other adventitious organisms. Ultimately, these lactic acid bacteria will be inhibited by the high ethanol, high acid, and high osmotic pressure that accumulate in the moromi. Lactic acid can be added directly to promote acidification, a now-common practice in modern sake facilities.

After about three weeks, the fermentation is ended, and the moromi is separated by settling and filtration, yielding a sake cake and a very clear, light yellow liquid, which is then

called sake. The sake can be aged (usually only a few months), adjusted to a desirable ethanol content, pasteurized by heat (65°C) or ultrafiltration, and bottled. Like grape-derived wine, the finished sake can be sweet or dry. However, this classification is not entirely based on the amount of residual sugar that is present, but rather on a combination of the sugar, acid, and alcohol content. Depending on the type of sake and consumer preference, sake can be served warmed (35°C to 40°C) or slightly chilled. In general, higher quality sake is usually served at the lower temperature to retain more of the aroma and flavor volatiles.

FERMENTED FISH-TYPE FOODS

Until recently, fish and shrimp sauces and pastes were mostly unknown to Western consumers (the exception being garum and liquamen that originated in the Greco-Roman era). However, these products have long been staple items in much of Southeast Asia. In particular, Thailand, Vietnam, Malaysia, and the Philippines (where they were thought to have evolved) are among the major producers of these products (Box 14.5). Although fresh fish has long been widely available throughout this region, refrigeration has not. Thus, fresh fish would be subject to rapid spoilage if it were not soon consumed. In contrast, fish-type sauces and pastes not only have a long shelf-life, they also serve as an inexpensive source of high quality protein and other nutrients. In addition, these products significantly enhance the flavor of rice, noodles, and other bland-tasting foods. Per capita consumption ranges from 10 ml to 15 ml per day in Vietnam and Thailand to about 1 ml per day in the Philippines.

The manufacture of fish sauces in Southeast Asia is a major industry; over 40 million liters of fish sauce are produced annually in Thailand alone. The popularity of cuisines from those countries in the United States (in food service as well as in processed foods) has undoubtedly led to consumption of fish sauces by US consumers, even if they aren't aware of what actually contributes to the unique flavors of those foods. In regions where these products are produced and consumed, they are considered indispensable flavoring agents, much like salt in the United States or shoyu in Japan.

There are many types of fermented fish products, ranging from light- or dark-colored pourable sauces to very thick pastes (Table 14.9). In some cases, the same manufacturing process is used to make both a liquid and a paste. For example, in the Philippines, a salted and fermented fish mixture, when allowed to settle, yields a supernatant liquid called patis and a sediment material that, when dried, gives a paste called bagoong. Just as with soy sauce and other fermented foods from Asia and the Far East, there are countless versions of fish sauces and pastes, many of which are made in the home or on a very small cottage scale using traditional techniques. Thus, only rather generic descriptions will be given (see below).

Although fish sauces are considered to be fermented foods, this is true only in a rather broad sense. Microorganisms do, indeed, grow and produce end products during the manufacture of these products (see below). However, most of the reactions that are responsible for flavor and aroma development occur as a result of endogenous fish enzymes, released during autolysis of fish tissue, rather than from microbial activities.

Manufacture of fish sauces and pastes

The general procedure for the production of fish sauces is not complicated (Figure 14.8). The starting material can be small (<15 cm in diameter) fish, such as sardines (that otherwise have minimal commercial value), small shrimp, squid, or oysters. Fish is usually used whole

Box 14.5 The archaeology of salt and salted fish in Southeast Asia

Fermented and salted fish products were born out of necessity. In the case of fish sauces, the fish used in these products had few other uses. They were too small to either filet or otherwise use as food. However, they were also nutritious and plentiful. Thus, like other fermented foods made from highly perishable raw foods – milk, meat, produce – some form of preservation was necessary to take advantage of this nutrient-rich material.

Of course, one of the oldest methods used to preserve foods, and fish in particular, is by addition of salt. Throughout Southeast Asia, salt has a long history. Salt making "facilities" have been discovered in Thailand, Vietnam and China, dating back several thousand years (Yankowski et al., 2015). As these authors note, rice, salt, and fish were the main resources used to sustain inhabitants of these regions. Not surprisingly, salt mining, production, and trade all played a key role in the cultural and culinary history.

Modern industrial, as well as traditional salt making is still practiced in Northeast Thailand (a region called Isan). Salt is naturally present in the local sediments, presumably from a time when the area was covered by seawater. The traditional process starts by filling underground, clay-lined basins with sediments and water. After the solids settle, the crude brines are passed through grasses and husks to remove impurities. The filtered brines are then collected and boiled, forming salt crystals. As noted by Yankowski et al. (2015), except for the use of burlap bags and plastic buckets, this leaching and boiling process is still practiced today.

The historical application of this salt for making fermented fish has also been described by the same authors. Fermented fish is still made in much the same way as it has been for centuries. Briefly, cleaned fish is mixed with salt in ratios ranging from 4: 1 to 10:1. Roasted rice is frequently added. The mixtures are fermented in jars for up to a year at ambient temperatures. There are many regional differences, however, and salt levels can even be higher. Of course, another widely consumed product made from fish fermentations is fish sauce, a staple flavoring agent consumed on a daily basis throughout Southeast Asia.

Reference

Yankowski, A., P. Kerdsap, and N. Chang. 2015. "Please pass the salt" – an ethnoarchaeological study of salt and salt fermented fish production, use and trade in northeast Thailand. J. Indo-Pacific Arachaeol. 37:4–13.

and uneviscerated, although de-headed, eviscerated, ground, or cut-up pieces can also be used. The only other ingredient necessary to make these products is salt. The fish-to-salt ratio varies, depending on the product, but usually ranges from 3:1 to 5:1. Fish sauces do not undergo a lactic fermentation, per se, and are preserved mainly by salt and low water activity, rather than by pH. Thus, high salt concentrations are necessary.

After the salt is added to the fish (on wooden or concrete floors), the mixture is moved into tanks (often built into the ground) and covered. The material is held for about six months (or longer) at ambient temperature. At various times, the mixture may be uncovered, stirred,

Table 14.9 Types of fish sauces and pastes.

Product	Country	Description
Nam-pla	Thailand	Fish sauce
Budu	Malaysia	Fish sauce
Patis	Philippines	Fish or shrimp sauce
Ishiru	Japan	Fish sauce
Nouc-mam	Vietnam	Fish sauce
Nam-pa	Laos	Fish sauce
Bagoong	Philippines	Fish or shrimp paste
Mam	Vietnam	Fish paste
Trassi	Indonesia	Shrimp or fish paste
Belachan	Malaysia	Shrimp paste

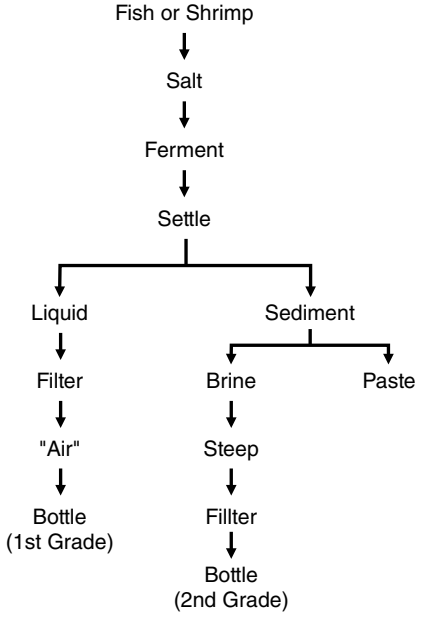


Figure 14.8 Manufacture of fish sauce. Adapted from Lopetcharat et al., 2001.

and exposed to air and sunlight, all of which are thought to improve flavor and color and accelerate enzyme activity. During this "incubation" period, the solid fish material is transformed, or more precisely, liquefied by the action of endogenous fish enzymes. These enzymes, primarily trypsin-like acid-proteases and various endo- and exo-peptidases, are ordinarily present within the intact cells of various fish tissues. However, in the non-living animal, the cells soon autolyze and those enzymes are released, resulting in extensive hydrolysis of muscle tissue. In fresh fish, autolysis and proteolysis result in tissue softening and spoilage; in fish sauce production, the result is liquefaction.

In addition to the physical transformation from solid to liquid, proteolytic digestion of the fish substrates results in formation of free amino acids and peptides. In intact tissue, for example, the soluble nitrogen concentration is essentially nil, but in nam-pla and nuoc-mam

(Thai and Vietnamese fish sauce, respectively) there is more than 2% soluble nitrogen (mostly amino nitrogen). Glutamic acid, which, like in soy sauce products, is responsible for flavor enhancement, is among the amino acids that accumulate in fish sauce. Likewise, nucleotides may also be formed, providing a source of umami or meaty-like flavors (as described above).

Further hydrolysis of peptides and amino acids by enzymes that are either endogenous or microbial in origin (see below) eventually results in a large number of volatile aroma and flavor products. Among those that are most prominent and that confer "fish sauce flavor" are ammonia, triethylamine, and various alcohols, aldehydes, ketones, and lactones. Lipolysis also occurs during fish sauce manufacture, resulting in formation of volatile fatty acids, including acetic, butanoic, and propanoic acids. These compounds are particularly characteristic of fish sauce flavor, which is sometimes described as "cheesy".

After several months of enzymolysis and fermentation, the liquid is separated from the sedimented material by decanting or filtering the liquid directly through the fish solids. This "first run" product has the highest quality. Additional brine can be added to the solid material, the mixture aged for several more weeks (or simply boiled), and then a second, lower quality, liquid is obtained. The remaining solids can then be recovered and used as a paste. Some fish sauces and pastes are aged in the open (and exposed to the sun) for several more weeks to allow partial dissipation of the strong fish aroma. The sauce or paste is then bottled. The final composition of fish sauces is usually about 60% moisture, 30% salt, 10% protein (including amino nitrogen), with a final pH of about 6.5. Pastes, in contrast, contain about 30% moisture, 20% salt, 30% protein, and 20% ash.

Fish sauce microbiology

While it is evident that microorganisms are present during the production of fermented fish sauces, it is not clear to what extent these organisms contribute to the finished product. The microbial population in raw, unsalted fish and shellfish is high in number and rich in diversity. Considering that whole uneviscerated fish (guts and all) are usually used to make fish sauces, the initial load of organisms is significant. In addition, the manufacturing environment is not aseptic, and even the salt (usually obtained by solar drying of sea water) may contribute microorganisms. However, the high salt concentration established early on in the fermentation provides strong selective pressure for halotolerant organisms. Not surprisingly, therefore, there is a shift in the initial bacterial population, from a wide variety of aerobic and anaerobic organisms to a narrower microbiota consisting mainly of salt-tolerant species of *Bacillus*, *Halobacillus*, *Staphylococcus*, and *Micrococcus.* Lactic acid bacteria are also widely present in fish sauce (Table 14.10), including some strains that are capable of growing in media containing 12% (2.1 M) salt.

Establishing the function of the microbiota during the fish sauce fermentation is not easy. It has been suggested that there is a succession that occurs during the fermentation, leading to the eventual dominance of the more salt- and acid-tolerant organisms. While this may be true for some products, in which considerable microbial growth is evident, for other products, microbial growth hardly occurs. In either case, the population eventually decreases, such that at the end of the fermentation (six to 12 months), there are usually less than 10^3 cells per ml. Many of the organisms isolated from fermented fish products have proteolytic activity and likely contribute, at least in part, to the overall proteolysis of fish protein. Lactic

Table 14.10 Lactic acid bacteria associated with fish sauces[1].

Lactic acid bacteria	Non-lactic acid bacteria
Lactobacillus plantarum	*Clostridium*
Lactobacillus farciminis	*Bacillus*
Lactobacillus acidipiscis	*Staphylococcus*
Lactobacillus pentosus	*Kocuria*
Weissella thailandensis	*Virgibacillus*
Leuconsotoc citreum	*Micrococcus*
Lactococcus lactic subsp. *lactis*	*Psychrobacter*
Tetragenococcus muriaticus	*Lentibacillus*
Tetragenococcus halophilus	*Lysinibacillus*

[1] From Chuon et al., 2014; Kuda et al., 2017; Lee et al., 2015; and Tanasupawat and Visessanguan, 2014.

acid bacteria and other anaerobes also can metabolize amino acids, producing volatile fatty acids, amines, ammonia, and other volatile end-products.

SAFETY OF ASIAN FERMENTED FOODS

There are two main reasons why the safety of Asian, fungal-fermented foods has been questioned. First, the *Aspergillus* sp. used in the production of soy sauce, miso, sake, and related products are taxonomically similar to the mycotoxigenic aspergilli that produce aflatoxins, ochratoxin, and other toxins. Despite these similarities, however, surveys in which these foods have been analyzed for the presence of mycotoxins indicate that mycotoxins are not present. Recent studies have shown that very subtle genetic differences exist between industrial strains and mycotoxin-producing strains that would account for these findings (Box 14.6).

A second reason for the concern is due to the suggested epidemiological relationship between consumption of indigenous foods from the Far East and the development of certain cancers, including gastric cancer and esophageal cancer. Indeed, although some cancer rates are higher in China, Japan, and other Far East countries, the evidence suggests that dietary habits other than consumption of fungal-fermented foods may be more likely to be responsible. For example, the Asian diet is high in salt, which may predispose individuals to these cancers. In specific provinces in China, where cancer rates are especially high, consumption of vegetables is very low.

Finally, it is also noteworthy that positive nutritional factors have been associated with fungal fermented foods. In particular, consumption of soybeans (and fermented soy products) have been promoted for contributing many health benefits, including reduced rates of coronary heart disease and mortality due to heart disease. Soybeans contain several flavones, isoflavones, flavanols, and other flavonoids that may have anticarcinogenic activity and whose consumption may lower the risk of certain cancers, including brain cancer and prostate cancer. Although not all of these substances are present in fermented soy products, several specific compounds have been shown to have anti-cancer activity. For example, the flavor furanones HEMF and HMF (discussed previously) that are found in soy sauce inhibit carcinogenesis in laboratory animals.

Box 14.6 Are there mycotoxins in fungal fermented products?

It is well established that growth of fungi is often accompanied by production of mycotoxins. Produced ordinarily as secondary metabolites, some mycotoxins can be extremely carcinogenic or mutagenic. One particular group of mycotoxins, the aflatoxins produced by species of *Aspergillus,* are among the most toxic of all naturally occurring compounds found on the planet.

Aflatoxin genetics As of 2017, there are as many as 18 chemically distinct types of aflatoxins (Xie et al., 2016). Aflatoxin B1 is the most common (and most toxic) aflatoxin produced in foods, but B1, B2, G1, and G2 are also found in human and animal foods (Amaike et al., 2013; Sheikh-Ali et al., 2014). They are produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*, members of the *Aspergillus* section *Flavi* (or the *A. flavus* group). Interestingly, this taxonomic group also includes *Aspergillus sojae* and *Aspergillus oryzae*, the same species used in the manufacture of koji, soy sauce, miso, sake, and other Asian fermented products.

Given the morphological, physiological, and genetic similarities between these different species, questions have been raised regarding the potential ability of *A. sojae* and *A. oryzae* to produce mycotoxins in foods. In the early 2000s, several studies reported that genes (or homologs) that encode for proteins involved in the aflatoxin biosynthesis pathway were present in food production strains (see below). Despite these studies that indicated the metabolic "potential" of *A. sojae* and *A. oryzae* to produce aflatoxins, there had not been any reports of aflatoxins being produced by these fungi or for the presence of aflatoxins in fermented soy products (Watson et al., 1999; Machida et al., 2008). These observations led investigators in to explore, in detail, why *A. sojae* and *A. oryzae* do not synthesize aflatoxins.

The biochemical pathway for aflatoxin biosynthesis consists of at least 23 enzymatic reactions and more than 15 intermediates (Yu et al., 2004a and 2004b). In 2004, the complete gene cluster encoding for aflatoxin biosynthesis in *A. flavus* and *A. parasiticus* was identified and sequenced (Yu et al., 2004a). There are at least 25 aflatoxin biosynthesis genes located within this 70 kb cluster (Amare and Keller, 2014; Cleveland et al., 2009; Figure 14.6.1).

Why are koji fungi non-toxigenic? The absence of enzymatic activities associated with the aflatoxin pathway in *A. sojae* and *A. oryzae* initially led researchers to conclude that the relevant genes were also absent in these species. However, once genomes were sequenced, it was evident that aflatoxin genes were present in koji and soy sauce strains. Several genes (*omtA*, *nor1,* and *ver1*) had high sequence similarity to those from aflatoxin-producing strains of *A. parasiticus* (Watson et al., 1999). However, none of these genes were transcribed, even when cells were grown in alfatoxin-conducive media. Two other genes, *avfA* and *omtB*, were also reported to be present in *A. sojae* and were 99% identical to those found in *A. parasiticus* (Yu et al., 2000). Furthermore, complementation assays showed that the *A. sojae avfA* gene was fully functional. Thus, if aflatoxin genes are present in *A. sojae* and *A. oryzae*, and they appear to be functional, why don't these fungi produce aflatoxin in foods?

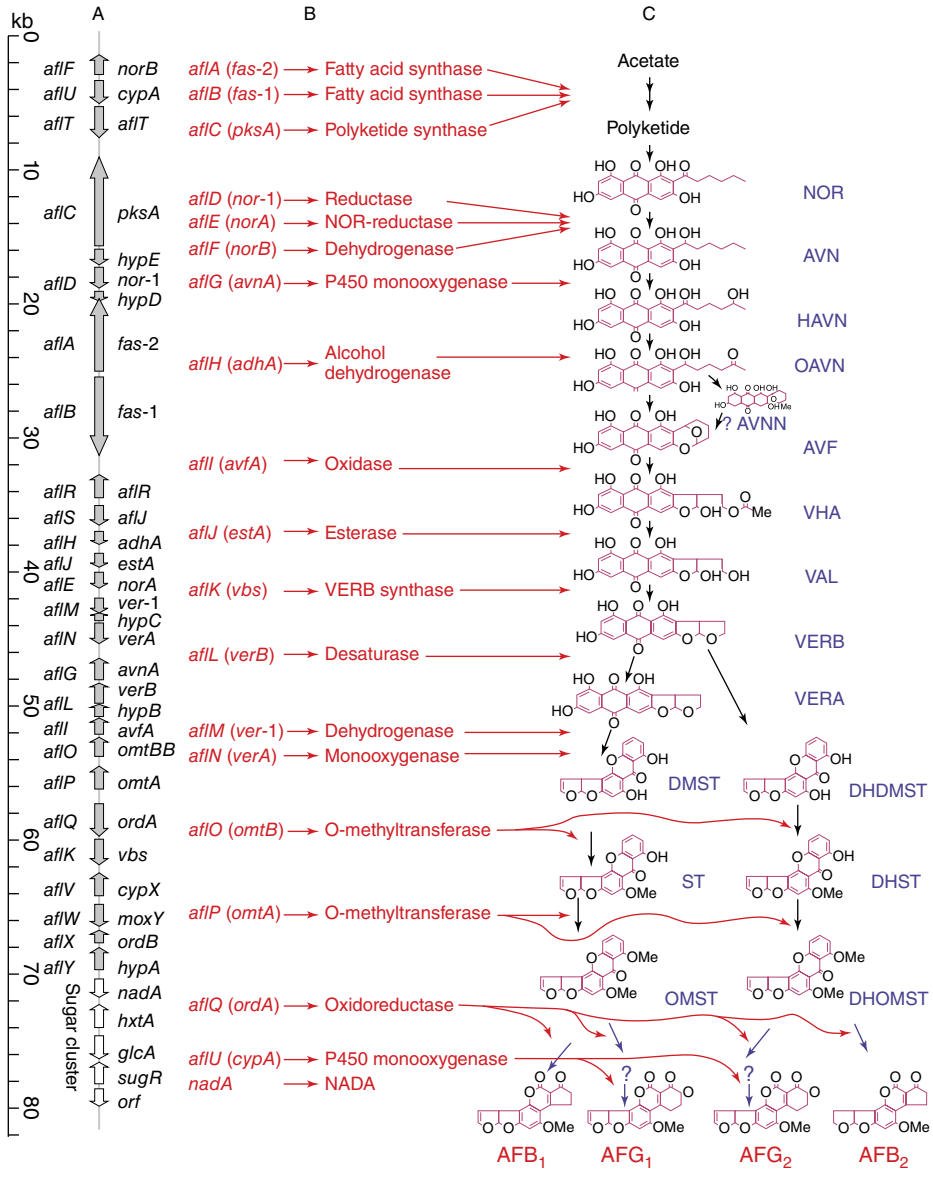


Figure 14.6.1 The *Aspergillus* aflatoxin biosynthetic pathway. The 82 kb aflatoxin gene cluster from *A. parasiticus* and *A. flavus* is shown in panel A (new names on the left and old names on the right), with the direction of gene transcription shown along the vertical line. The encoded aflatoxin biosynthetic enzymes and putative pathway are shown in panels B and C, respectively. From Cleveland et al., 2009, with permission. Refer to the original text for abbreviations.

To answer this question, researchers first had to determine how aflatoxin gene expression was regulated. They discovered that the *aflR* gene product, AflR, acts as a positive regulator by binding to the promoter region of the *afl* gene cluster, thereby

activating transcription of the structural genes. Mutant strains of *A. flavus*, defective in *aflR*, did not express alfatoxin genes, indicating that *aflR* was required for aflatoxin biosynthesis. Although researchers learned that *A. sojae* and *A. oryzae* also express *aflR,* they produce a mutated or non-functional version. Thus, it does not activate *afl* gene expression (Kiyota et al., 2011). Ultimately, despite the very similar genetic organization (Gibbons et al., 2012), koji strains of *Aspergillus* and strains of aflatoxigenic *Aspergillus* differ dramatically in their ability to express toxins. This explains why koji and soy sauce, miso, and other products made using a koji starter are aflatoxin-free.

References

Amaike, S., K.J. Affeldt, and N.P. Keller. 2013. Genetics, biosynthesis and regulation of aflatoxins and other *Aspergillus flavus* secondary metabolites. In: Kempken, F., ed. Agricultural Applications. Springer, Berlin, Heidelberg, pp. 59–74.

Amare M.G. and N.P. Keller. 2014. Molecular mechanisms of *Aspergillus flavus* secondary metabolism and development. Fungal Genet. Biol. 66:11–18.

Cleveland, T.E., J. Yu, N. Fedorova, D. Bhatnagar, G.A. Payne, W.C. Nierman, and J.W. Bennett. 2009. Potential of *Aspergillus flavus* genomics for applications in biotechnology. Trends Biotechnol. 27:151–157.

Gibbons, J.G., L. Salichos, J.C. Slot, D.C. Rinker, K.L. McGary, J.G. King, M.A. Klich, D.L. Tabb, H. McDonald, and A. Rokas. 2012. The evolutionary imprint of domestication on genome variation and function of the filamentous fungus *Aspergillus oryzae*. Curr. Biol. 22:1403–1409.

Kiyota, T., R. Hamada, K. Sakamoto, K. Iwashita, O. Yamada, and S. Mikami. 2011. Aflatoxin non-productivity of *Aspergillus oryzae* caused by loss of function in the *aflJ* gene product. J. Biosci. Bioengin. 111:512–517.

Machida, M., O. Yamada, and K. Gomi. 2008. Genomics of *Aspergillus oryzae*: learning from the history of koji mold and exploration of its future. DNA Res. 15:173–183.

Sheikh-Ali, S.I., A. Ahmad, S.H. Mohd-Setapar, Z.A. Zakaria, N. Abdul-Talib, A.K. Khamis, and M.E. Hoque. 2014. The potential hazards of *Aspergillus* sp. in foods and feeds, and the role of biological treatment: a review. J. Microbiol. 52:807–818.

Watson, A.J., L.J. Fuller, D.J. Jeenes, and D.A. Archer. 1999. Homologs of aflatoxin biosynthesis genes and sequences of *aflR* in *Aspergillus oryzae* and *Aspergillus sojae*. Appl. Environ. Microbiol. 65:307–310.

Xie, L., M. Chen, and Y. Ying. 2016. Development of methods for determination of aflatoxins. Crit. Rev. Food Sci. Nutr. 56:642–2664.

Yu, J., D. Bhatnagar, and T.E. Cleveland. 2004a. Completed sequence of aflatoxin pathway gene cluster in *Aspergillus parasiticus*. FEBS Lett. 564:126–130.

Yu, J., P.-K. Chang, K. Ehrlich, J.W. Cary, D. Bhatnagar, T.E. Cleveland, G.A. Payne, J.E. Linz, C.P. Woloshuk, and J.W. Bennett. 2004b. Clustered pathway genes in aflatoxin biosynthesis. Appl. Environ. Microbiol. 70:1253–1262.

Yu, J., C.P. Woloshuk, D. Bhatnagar, and T.E. Cleveland. 2000. Cloning and characterization of *avfA* and *omtB* genes involved in aflatoxin biosynthesis in three *Aspergillus* species. Gene 248:157–167.

BIBLIOGRAPHY

Babu, P.D., R. Bhakyaraj, and R. Vidhyalakshmi. 2009. A low cost nutritious food "Tempeh" – a review. World J. Dairy Food Sci. 4:22–27.

Baens-Arcega, L., A.G. Ardisher, and C.G. Beddows. 1996. Indigenous amino acid/peptide sauces and pastes with meatlike flavors. In: K.H. Steinkkraus, ed. Handbook of Indigenous Fermented Foods, 2nd edn. Marcel Dekker, New York, pp. 625–633.

Chuon, M.R., M. Shiomoto, T. Koyanagi, T. Sasaki, T. Michihata, S. Chan, S. Mao, and T. Enomoto. 2014. Microbial and chemical properties of Cambodian traditional fermented fish products. J. Sci. Food Agric. 94:1124–1131.

Ebine, H. 1986. Miso. In: N.R. Reddy, M.P. Pierson, and K. Salunkhe, ed. Legume-based fermented foods. CRC Press, Boca Raton, FL, pp. 47–68.

Feng, Y., G. Su, H. Zhao, Y. Cai, C. Cui, D. Sun-Waterhouse, and M. Zhao. 2015. Characterisation of aroma profiles of commercial soy sauce by odour activity value and omission test. Food Chem. 167:220–228.

Hajeb, P. and S. Jinap. 2015. Umami taste components and their sources in Asian foods. Crit. Rev. Food Sci. Nutr. 55:778–791.

Handoyo, T. and N. Morita. 2006. Structural and functional properties of fermented soybean (Tempeh) by using *Rhizopus oligosporus*. Int. J. Food Prop. 9:347–355.

Holzapfel W.H. and B.J. Wood. 2014. Lactic Acid Bacteria: Biodiversity and Taxonomy. Wiley-Blackwell. New York, NY.

Jennessen J., J. Schnürer, J. Olsson, R.A. Samson, and J. Dijiksterhuis. 2008. Morphological characteristics of sporangiospores of the tempe fungus *Rhizopus oligosporus* differentiate it from other taxa of the *R. microsporus* group. Mycol. Res. 112:547–563.

Kaneko, S., K. Kumazawa, and O. Nishimura. 2012. Comparison of key aroma compounds in five different types of Japanese soy sauces by aroma extract dilution analysis (AEDA). J Agric. Food Chem. 60.3831–3836.

Kuda, T. 2017. Fermentation of fish-based products: A special focus on traditional Japanese products. In: Starter Cultures in Food Production. B. Speranza, A. Bevilacqua, M. R. Corbo and M. Sinigaglia, eds. John Wiley, Chichester, pp. 355–369.

Lee, S.H., J.Y. Jung, and C.O. Jeon. 2015. Bacterial community dynamics and metabolite changes in myeolchi-aekjeot, a Korean traditional fermented fish sauce, during fermentation. Int. J. Food Microbiol. 203:15–22.

Lopetcharat, K., Y.J. Choi, J.W. Park, and M.D. Daeschel. 2001. Fish sauce products and manufacturing – a review. Food Rev. Int. 17:65–88.

Nout, M.J. and J.L. Kiers. 2005. Tempe fermentation, innovation and functionality: update into the third millennium. J. Appl. Microbiol. 98:789–805.

Ohyama, T. 2013. Traditional and Modern Japanese Soy Foods: Manufacturing, Nutrition and Cuisine of a Variety of Soy Foods for Health and Joy of Taste. Nova Science Publishers, Hauppauge, New York.

Rhee, S.J., J.E. Lee, and C.H. Lee. Importance of lactic acid bacteria in Asian fermented foods. Microb. Cell Fact. 2011:10.

Shurtleff W., and A. Aoyagi. 1979. The Book of Tempeh. Harper and Row, New York.

Steinhaus, P., and P. Schieberle. 2007. Characterization of the key aroma compounds in soy sauce using approaches of molecular sensory science. J Agric. Food Chem. 55:6262–6269.

Steinkraus, K.H. Industrialization of indigenous fermented foods. Marcel Dekker, New York, New York.

Sulaiman, J., H.M. Gan, W.-F. Yin, and K.-G. Chan. 2014. Microbial succession and the functional potential during the fermentation of Chinese soy sauce brine. Front. Microbiol. 5:556.

Tanasupawat, S. and W. Visessanguan. 2014. Fish Fermentation. In: Seafood Processing: Technology, Quality and Safety, 1st edn. I.S. Boziaris, ed. John Wiley, Oxford.

Yoneya, T. 2003. Fermented soy products; tempe, nattos, miso, and soy sauce. In: Handbook of Vegetable Preservation and Processing. Y. Hui , S. Ghazala , D. Graham, K. Murrell, and W. Nip, eds. Marcel Dekker, New York, pp. 251–272.

Zhu, Y. and J. Tramper. 2013. Koji – Where East meets West in fermentation. Biotechnol. Adv. 31:1448–1457.

15 Cocoa, coffee, and cereal fermentations

> The secret to winning the Nobel Prize is now definitely demonstrated: the Barbajada, an Italian mixture of coffee, milk and chocolate.
>
> Francesco Brigo and Raffaele Nardone, from Evidence-Based Medicine, 2014, based on a statistical analysis between coffee and chocolate consumption and Nobel Prizes won.

For nearly all of the fermented foods and beverages described in this text, fermentation results in products having dramatically different organoleptic properties than the raw materials from which they were made. Examples should by now be evident: milk into cheese, meat into sausage, and cereal grains into bread, beer, whiskey, and wine. Although enzymatic, as well as chemical and physical reactions are often necessary to produce many of these foods and beverages, microorganisms serve a prominent and obvious role. Above all, they produce the acids, ethanol, and other metabolic end products for which these products are known.

For chocolate, coffee, and other products described in this chapter, fermentation is no less important, but perhaps it is less obvious. Many students are surprised to learn that the rich confection they so often crave or the stimulating beverage they drink every morning is a fermentation-derived product. So important is the fermentation step in the manufacture of chocolate and coffee that without fermentation, these products would lack the unique flavors and aromas that make them so appealing. Fermentation also serves another important, but perhaps under-appreciated role. As will be described in more detail later, freshly-harvested cacao beans are surrounded by a pulpy material that renders the beans unusable. Microorganisms degrade the pulp and therefore act as biological process aids during cocoa processing.

GENERAL HISTORY

Although cocoa and coffee are now produced in similar areas, their histories are very different. The cacao tree originated in the Western hemisphere. Cacao was cultivated, processed, and consumed by indigenous populations, long before it was "discovered" by European explorers in the sixteenth century. It was only after the so-called Columbian Exchange that

Microbiology and Technology of Fermented Foods, Second Edition. Robert W. Hutkins.

cacao farming spread to other parts of the world. Indeed, despite its American origins, more cacao is now produced in Africa and Asia than anywhere else.

Coffee cultivation, in contrast, began in Africa, where it too was discovered by Europeans. Eventually, coffee also traversed the Atlantic, but in the opposite direction as cacao. While several African regions continue to be major producers, Brazil, Colombia and other South and Central American countries now account for most of the world production. Interestingly, Southeast Asia has become a major producer of both cacao and coffee.

Even as cacao and coffee farming spread around the world, the global popularity and economic importance of cocoa and coffee have led to considerable scientific research. As noted below, community sequencing has revealed important new insights into the microbial ecology of these products. In addition, the genomes for both the cocoa bean and coffee bean have been sequenced, and relevant traits identified. Finally, while the popularity of coffee and cocoa is certainly due to their many organoleptic, cultural, and social features, research has recently focused on nutritional benefits, as described below.

CURRENT ECONOMICS

The importance of cacao and coffee to the producer countries cannot be overstated. Most are considered developing countries whose economies are often dependent on cacao and coffee production. Much is produced on small farms, supporting millions of people. Ultimately, the processed cacao and coffee beans are shipped to western countries (the US and Europe are the largest importers) where they are converted into chocolate and coffee products. However, the origin of the beans has become of considerable importance. In part, this is because where the cacao or coffee was grown affects the functional qualities of the product, as noted below. Another reason, however, has less to do with these technical aspects of quality. Rather, chocolate and coffee are among only a few products marketed on the basis of the welfare of the farmers and workers who produced the raw materials. Thus, there is now an entire market devoted to so-called fair-trade or ethically-labeled chocolate and coffee that is made from crops grown and produced by small farmers who receive fair prices for their products (Box 15.1).

Box 15.1 Fair trade and other ethical labels

Coffee has been globally exported, imported, and traded for hundreds of years. Although coffee beans have long been valued based on quality, they were also considered as a commodity product. Thus, except for the occasional advertising slogan, the actual origin of the beans had not, until recently, been of much concern. Now, if one visits the local coffee shop, the location where the beans had been grown may well be indicated (Figure 15.1.1).

In addition to identifying the country of origin, coffee is also frequently labeled with a so-called ethical label. The most common is the "fair trade" designation. Indeed, fair trade coffee is among the hottest segments of the coffee business. Remarkably, imported fair trade coffee into the US has increased by an annual average of nearly 50% between 2004 and 2014 (Fair Trade USA, 2014). Overall, however, it is still a small market. Only about 5 out of every 100 cups of coffee brewed in the US is

Figure 15.1.1 Location matters: coffee menu at a popular coffee house.

considered fair trade. Nonetheless, these products are now considered mainstream and are widely available in coffee houses and major retail stores (Hainmueller et al., 2015).

As to what the fair trade label actually means, the answer is not so easy to decipher. Indeed, there are several agencies (including Fair Trade USA) that certify coffee farms as "fair trade". Each apply somewhat different criteria but in general, fair trade implies that farm workers are treated fairly and receive a fair or even premium price for their products.

Fair trade farms for coffee, as well as cocoa, tea, cotton, and other agricultural products exist throughout the developing world. Often, these products are produced via organic or sustainable agriculture. For some products, like bananas, rain forest sustainability and worker conditions are major factors.

The fair trade label is just one of dozens of so-called ethical labels attached to foods. In addition to worker- and environment-friendly labels, there are also labels for humane treatment of animals used for milk, meat, and eggs. Common examples include free-range eggs, "dolphin-safe" tuna, and grass-fed beef. In a way, even "local"

and "non-GMO" labels can be considered as ethical labels. So called "ethical consumerism" has become a significant market (Carrington et al., 2012; Lotz et al., 2013).

Fair trade and other eco-labeled foods may cost a bit more than conventional products, yet many consumers are more than willing to pay. In one recent study, consumers were given two cups of coffee and asked which one they preferred (Sorkvist et al., 2013). Although the two coffees were identical, one was eco-labeled. Not surprisingly perhaps, a significant majority preferred the eco-labeled coffee. In fact, a few people chose and were willing to pay more for the eco-friendly coffee, even though they admitted they liked the flavor of the regular coffee more.

A similar study with chocolate reported the same result - consumers thought that chocolate tasted better when it was labeled as Fair Trade (Schuldt et al., 2012). In fact, the fair trade-labeled chocolate was also viewed as lower calorie and healthier.

Psychologists call this phenomenon the "halo effect" (Schuldt et al., 2012). If a food is labeled as organic or healthy or produced ethically or a portion of the profits are given to charity, consumers tend to perceive that product more favorably (Bauer et al., 2013). This is especially true for people who recycle, drive hybrids, or are predisposed to "progressive socioeconomic values".

And you thought ordering your morning coffee was already too complicated!

References

Bauer, H.H., D. Heinrich, and D.B. Schäfer. 2013. The effects of organic labels on global, local, and private brands: more hype than substance? J. Bus. Res. 66:1035–1043.

Carrington M.J., B.A. Neville, G. J. Whitwell. 2014. Lost in translation: Exploring the ethical consumer intention–behavior gap. J. Bus. Res. 67:2759–2767.

Hainmueller, J., M.J. Hiscox, and S. Sequeira. 2015. Consumer demand for fair trade: evidence from a multistore field experiment. Rev. Econ. Stat. 97:242–256.

Lotz, S., F. Christandl, and D. Fetchenhauer. 2013. What is fair is good: evidence of consumers' taste for fairness. Food Qual. Prefer. 30:139–144.

Schuldt, J.P., D. Muller, and N. Schwarz. 2012. The "fair trade" effect: health halos from social ethics claims. Soc. Psychol. Person. Sci. 3:581–589.

Sörqvist, P., D. Hedblom, M. Holmgren, A. Haga, L. Langeborg, A. Nöstl, and J. Kågström. 2013. Who needs cream and sugar when there is eco-labeling? Taste and willingness to pay for "eco-friendly" coffee. PLoS One 2013, 8 (12):e80719.

Finally, it is necessary to emphasize perhaps one of the most important challenges faced by cocoa and coffee producers – namely the effect of climate change on the production of these crops. Nearly all of the major growers and corporations involved in coffee and cocoa production are now developing adaptation and mitigation strategies to address this challenge.

COCOA

Cocoa origins

Cocoa was first consumed as a food around 3500 years ago. Although the "when" can only be estimated, there is less uncertainly about the region "where" cocoa was first consumed. The original home of the cacao tree, *Theobroma*, from which cocoa is derived, is now thought

to be near the Amazon region of South America. [Note to readers, although cacao and cocoa are technically different materials, they are often used interchangeably.] Cultivation of this tree eventually spread north through Central American to the Yucatan peninsula of Mexico. However, cacao as a food most likely originated in Mesoamerica, a region that include Central America and central Mexico. The cacao tree is a tropical perennial plant that grows best within narrow geographical latitude, about 20 degrees north or south of the equator. The trees require consistent rainfall (150 to 250 cm per year), high humidity, and reasonable shade – all typical of the rain forests within these regions. Thus, Mesoamerica was particularly well-suited for growing cacao. Despite these limitations, however, cacao was also adaptable to other regions around the world, as noted below.

Historians regard the culture that lived in Mesoamerica 3000 years ago as one of the earliest (and most advanced) human civilizations. Perhaps a good example of the wisdom of the indigenous population was their appreciation for the cacao. Plantations devoted to the cacao tree were established around 100 CE by the Mayan Indians. The cacao was not consumed as the modern-day chocolate, but rather as a beverage or tonic. Cacao cultivation and cocoa processing subsequently spread throughout the region, into Mexico and South America. By the time, Cortez arrived in Mexico City in 1519, the Aztecs had developed sophisticated technologies for cacao bean processing, including the all-important fermentation, roasting, and grinding steps. Although initially unimpressed by the bitter beverage, the explorers returned to Spain with the beans. The addition of sugar compensated for the bitterness, and made the drink more palatable. Eventually, by the 18th century, solid cocoa products, the forerunners of chocolate, were developed, and quickly became popular (and expensive). The new-found appreciation for cacao was noted by the genus name assigned by the taxonomist, Carl Linnaeus in 1753 – *Theobroma cacao*, "food of the gods".

Production and consumption

As noted above, cocoa is now cultivated throughout the world. Despite its origins in the Western hemisphere, however, most of the cocoa is now grown and produced within specific regions in Africa and Asia (see below). There are two main types of cacao, from which all other cacao varieties are derived. Both types are taxonomically classified as *Theobroma cacao*, but they are distinguished based on various botanical traits, such as seed appearance and plant productivity.

Forastero trees are responsible for most of the world supply of cacao. Native to the Amazon region, these trees have been planted around the world and have adapted well to the local conditions. Thus, Forastero cacao and hybrids derived from this variety are now grown in Africa, Malaysia, as well as Brazil and other South American countries. They are very productive (meaning they have good yields) and are moderately disease- and pest-resistant. Forastero beans are sometimes referred to as "bulk" cocoa. However, this rather ordinary designation should not be interpreted as a quality attribute, per se, since excellent chocolate is made from these beans. Rather the "bulk" designation relates to the popularity of this bean as a trade commodity in the global market.

The other main type of cacao is Criollo. These trees are less productive and less resistant to diseases and pests. Regardless of these challenges, however, they are still cultivated (sometimes exclusively) throughout Central America, where they originated, as well as in several South American countries. That these beans are so often preferred is due to their excellent flavor. Indeed, Criollo cacao beans are classified as "fine" or "flavor" cacao, in contrast to the Forastero bulk cacao beans.

There is a third popular type of cacao, a hybrid called the Trinitario. These trees originated in the Caribbean country of Trinidad. They are rather in-between Forastero and Criollo with respect to the productivity and disease-resistance. In addition to Trinitario, there are many other hybrid types grown around the world.

The composition, flavor, color, and aroma characteristics of the beans have a profound influence on the quality of the chocolate. Quality is itself a complex property for all fermented foods, but perhaps more so for chocolate given its often passionate popularity among nearly every region and every demographic. Chocolate manufacturers have long known that chocolate quality (reviewed later in this chapter) is dependent on traits expressed by the cacao bean. Thus, two cacao genome sequencing projects were initiated in the 2000s, and the finished genome sequences were published in 2011.

As described above, cacao production has spread around the world, and West Africa has emerged as the largest region for cacao production even though cacao was not introduced until the nineteenth century. More than 70% of the entire world supply of cocoa (about 4 million tonnes) is produced in just four West Africa countries, Ivory Coast, Ghana, Nigeria, and Cameroon (Figure 15.1). The Ivory Coast alone accounts for more than one-third of all cocoa produced world-wide. Cacao was brought to Southeast Asia in the 16th century. Within this region, Indonesia, Papua New Guinea, and Malaysia are now the main producers. Overall, this region is the second largest producer of cacao, accounting for nearly 15% of the world supply.

As noted above, cocoa from the producer countries is mostly exported to Europe, North America, Australia, and New Zealand. On a consumption basis, Switzerland, Germany, and Australia are the largest consumers, with Ireland and the United Kingdom not far behind (Figure 15.2).

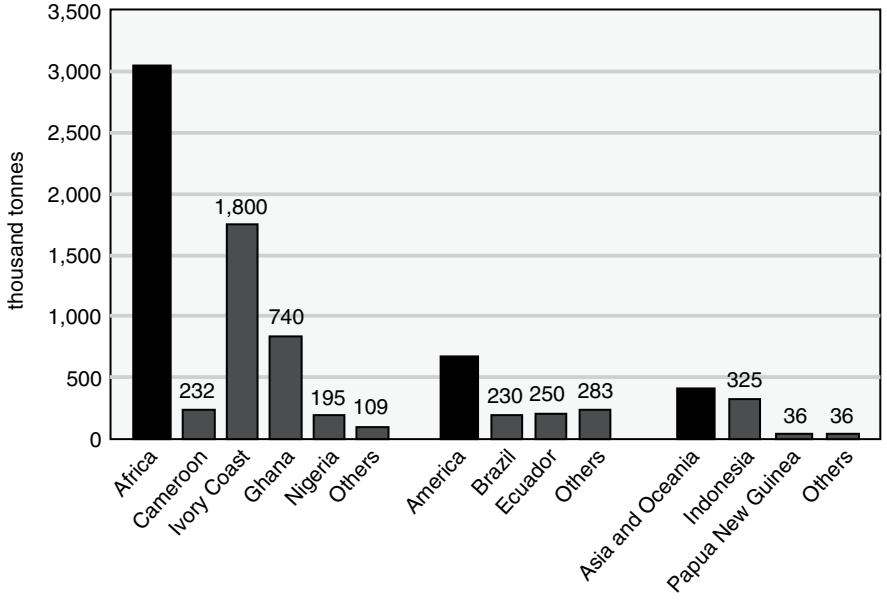


Figure 15.1 Major global producers of cocoa (2014–15). Source: International Cocoa Organization (www.icco.org).

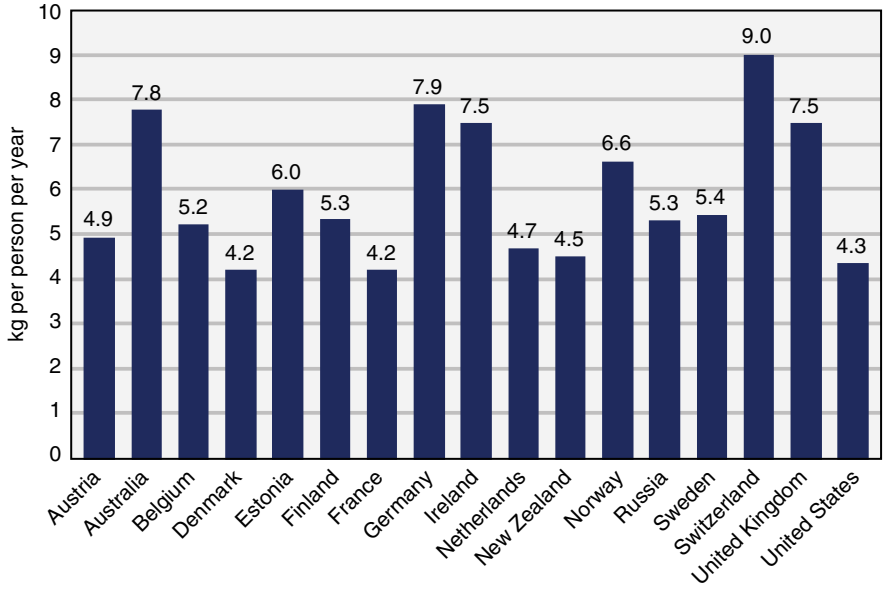


Figure 15.2 Global cocoa consumption (per capita in 2014). Data from the CBI Trade Statistics and Euromonitor.

Post-harvest processing

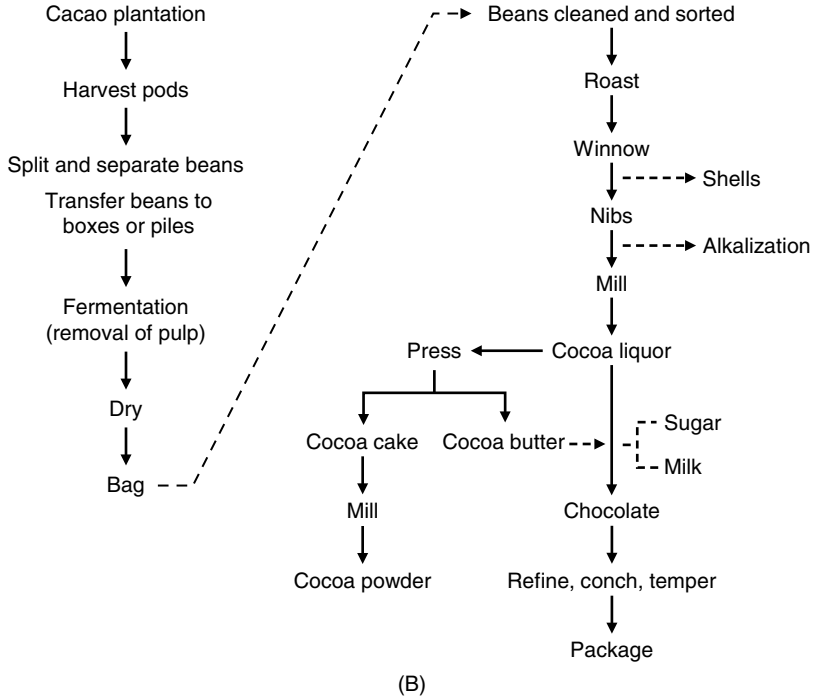
The flavor, aroma, and color of freshly harvested cacao beans are so far removed from cocoa or chocolate it's even hard to imagine one is made from the other. Un-fermented, un-dried, un-roasted, and un-processed cacao is fairly described as unpleasant, with a bitter and astringent flavor. The cacao bean itself is a misnomer, as it is not really a bean, but rather is a seed from the fruit-bearing cacao tree. The so-called beans are contained within thick, fibrous pods that develop as "flowers" from the trunk and branches of the tree. An average pod is about 20 cm long by 10 cm wide, but they vary in size and some can be nearly twice as large. Each pod can contain as many as 40 to 60 beans. Each bean contains an embryo consisting of two cotyledons (later these will be the "nibs") surrounded by a seed coat. However, the pod interior not only contains the beans, but also a soft, thick, pulpy "mucilaginous" material that envelop all the beans (Figure 15.3A). This pulp is comprised of simple sugars, pectin, and citric acid.

The early cacao processing steps occur on the farm and are not highly technical. Processing begins as soon as the pods are harvested and split (Figure 15.3B). The beans are then collected and either placed into boxes or trays or heaped into piles. Boxes and piles contain about 1000 kg of beans and are typically maintained at ambient conditions. It is at this point when the all-important fermentation step occurs. Following fermentation, the beans are dried, bagged, and shipped. The conversion of these beans into cocoa and chocolate is described below.

Fermentation principles

It has long been known that the cacao fermentation is essential to produce cocoa. Micro-organisms perform several critical functions. First, they degrade the pulpy material that surrounds the beans. Second, they produce fermentation end-products, including ethanol and lactic and acetic acids. Third, these fermentation acids, along with heat generated by the

(A)



(B)

Figure 15.3 Cocoa bean processing, from pod surrounded by mucilage (A) through fermentation and processing (B). Sources: Bhattacharjee and Kumar, 2007 and the International Cocoa Organization (www.icco.org).

fermentation, result in the death of the bean. Up to this point, the bean was alive and respiring and the inner tissues were intact. Once the bean is no longer viable, acids diffuse into the tissues, enzymes are released, and bean constituents are degraded. Ultimately, the products of fermentation and enzyme hydrolysis will serve as precursors or reactants for flavor and color formation during subsequent processing steps. Collectively, the fermentation is complete within about five days.

Cacao microbial ecology

Prior to harvesting, the inside of the pods are sterile. Thus, the cacao fermentation depends entirely the indigenous microorganisms ordinarily present in the cacao handling

and processing environments. The exterior of the pods, the knives and utensils used to cut or open the pods and remove the beans, the boxes, the hands of the workers, the air – all contribute microorganisms into the beans. Included are yeasts, lactic acid bacteria, Enterobacteriaceae, acetic acid bacteria, bacilli, and fungi. The inoculum, therefore, is never the same from batch-to-batch. Nonetheless, the cacao fermentation is still rather predictable and consistent.

Like other natural fermentations, the cacao fermentation can be characterized as a succession, with one group of organisms producing end-products that create an environment conducive for other subsequent groups. The conditions and composition of the starting material also influence the succession. Initially, the main substrate source is the sugar-rich pulp (containing glucose, fructose, and sucrose). This high sugar-containing medium, combined with a low pH (usually < 4.0 due to citric acid) and low oxygen atmosphere, provides a highly suitable, if not selective environment for wild yeasts. Yeasts serve an essential role, not only by their growth and metabolism, but also by establishing a suitable environment for the acetic acid bacteria that follow.

Yeasts

Among the yeasts that initiate the fermentation are species of *Pichia*, *Candida*, *Hanseniaspora* (anamorph, *Kloeckera*), *Debaryomyces*, and *Saccharomyces*. They can be detected almost immediately after the beans are removed from the pods. Within about 24 hours, the total yeast population increases about 3 to 4 logs (from about $10^{4–6}$ to $10^{7–9}$), peaking at around 36 hours. The main functions of the yeasts during this first stage are to degrade the pulp, ferment sugars, produce ethanol, and form volatile flavor and aroma compounds. Expression of pectinolytic enzymes by yeast enhances digestion of the pulp and bean tissues, exposing the interior to oxygen and providing access of other organisms into the mass interior. Consistent with the succession theme, the fermentation is initiated by fast-growing, but ethanol-sensitive yeasts, such as *Kloeckera* (the same genus that initiates the wine fermentation!). These yeasts are inhibited by the ethanol and give way to more ethanol-tolerant *Saccharomyces* and *Candida*. After about 48 hours the yeast population begins to decline, reaching low or undetectable levels within 4 or 5 days.

Lactic acid bacteria

In addition to yeasts, lactic acid bacteria are also present as part of the autochthonous microbiota. They begin growing almost immediately after the yeast have peaked. The cacao environment, however, is even more selective at this stage, given the low pH (3.5–4.0) and the presence of ethanol (0.7–0.9%). The temperature also continues to rise above 35°C. Thus, only acid-tolerant, ethanol-tolerant mesophilic species will be able to grow. Among the lactic acid bacteria isolated during the lactic acid phase are both homofermentative and heterofermentative species, including *Lactobacillus plantarum*, *Lactobacillus fermentum, Lactobacillus plantarum,* and *Leuconostoc mesenteroides.*

Even after the yeast phase, there is still plenty of fermentable sugar available for the lactic acid bacteria. Metabolism results in formation of lactic acid, acetic acid, CO_2, and ethanol. This lowers the pH even further, making the environment inhospitable to all but the most acid tolerant organisms. Thus, their tenure is short, with most lactic acid bacteria peaking at around 48 hours, and then decreasing at rates similar to the yeasts.

Acetic acid bacteria

Among the bacteria that can tolerate low pH and grow in this restrictive environment are acetic acid-producing bacteria. This acidic environment, also high in ethanol and lactic acid, is perfectly fine for these bacteria. In particular, *Acetobacter pasteurianus*, one of the organisms used in vinegar fermentations is also one of the key organisms responsible for this phase of the cacao fermentation. Along with other *Acetobacter aceti* and other *Acetobacter* species, they perform the same metabolic function as for vinegar – oxidizing ethanol to acetic acids. However, acetic acid bacteria can also consume lactate, producing acetoin and other small molecules.

Acetic acid bacteria are critical to successful cacao fermentations for several reasons. First, the acetic acid diffuses into the bean, bringing about its demise (helped by the heat generated by fermentation). The acetic acid also enhances acid hydrolysis of cacao proteins. The products of protein hydrolysis, mainly amino acids, serve as essential precursors for flavor and color development. Specifically, they react with sugars during the roasting steps to form Maillard browning reaction products. Ultimately, the accumulated acetic acid is inhibitory to the acetic acid bacteria themselves, as well as any yeasts and lactic acid bacteria that might still be present. Finally, the temperature may increase above 50°C by the end of the acetic acid phase, which also inhibits acetic acid and other bacteria. Thus, at the end of the fermentation phase, the cacao beans are rather well preserved even before they are dried and roasted (the next two steps).

Other microorganisms

Recall that the entire fermentation is performed in open areas, with significant exposure to airborne organisms. Thus, there is considerable potential for fungi and *Bacillus* spores to be present in the beans. Bacilli, in particular, appear to be consistently present in fermenting cacao beans, although no functional role has been demonstrated. These spore-forming bacteria, as well as fungi, may ultimately be deleterious to cacao flavor and quality.

Collectively the organisms involved in the cacao fermentation – yeasts, lactic acid bacteria, and acetic acid bacteria function as a consortium. If any one group is absent, the fermentation may be impaired and the finished product may lack typical cocoa flavor. Many factors may influence the rate and uniformity of the fermentation, starting with the cacao (e.g., type, farm, location, health, and stage of ripening). The initial microbial population and proportion of different groups can vary considerably. How the beans are handled also affects fermentation. In some regions, for example, the beans are stirred and aerated, whereas other farms leave the beans undisturbed.

The microbiota is especially relevant as a quality attribute, in part, because microorganisms are responsible for generating the substrates that serve as precursors for flavor-generating reactions (see below). Thus, the cacao microbiome (i.e., the entire genetic content) is so important that it has been the subject of considerable research (Box 15.2). In particular, by identifying the organisms and their functional role it may be possible to control the fermentation and improve cocoa quality.

The sometimes inconsistent performance of wild or natural fermentations has led to interest in developing and using starter cultures for controlled cacao fermentations. As with other fermentations that require succession events, this is not an easy proposition. Given the substantial market for chocolate, especially high quality products that can be consistently produced to meet exact specifications, such efforts may be worth the investment.

Box 15.2 Cocoa microbiota, the essential ingredient to quality chocolate

Despite the essential role of fermentation in the processing of cocoa beans, research on the microbiota involved in this fermentation has been limited. Moreover, the functional role of specific members of this microbiota had been, for many years, under-appreciated. This situation, however, is no longer the case – the cocoa fermentation is now the subject of considerable fundamental as well as applied interest. In large part this is because the fermentation is now viewed as one of the more important factors affecting chocolate quality (Bortolini et al., 2016). Thus, determining "who's there" and "what are they doing" are among the questions currently being addressed.

Cocoa fermentations rely on a natural succession of several different groups of microorganisms. Ordinarily, this succession and the metabolic changes that occur as a result of microbial growth are repeatable and consistent (Figure 15.2.1; De Vuyst and Weckx, 2016). However, the cocoa fermentation is not so simple to study. The fermentation involves aerobes and anaerobes, bacteria and fungi, with different members dominating at different times. Thus, like other wild fermentations, cocoa can be a difficult raw material to interrogate. Thanks to the advent of culture-independent methods, and next generation sequencing, in particular, several research groups have conducted detailed analyses of the cocoa fermentation.

In one recent study, investigators examined four different cocoa samples from three different countries using high-throughput sequencing, i.e., 16S rRNA amplicons (Bortolini et al., 2016). Despite the geographical distinctions as well as different sampling times, similar lactic acid and acetic acid bacteria were identified. Several species were highly abundant, including *Lactobacillus fermentum* and *Lactobacillus plantarum* and *Acetobacter syzgii* and *Acetobacter senegalensis.* Enterococci were also present. However, only rather modest differences in overall microbial diversity were observed.

Another recent study combined cultural methods for isolation and molecular typing for identification (Visintin et al., 2016). Microbiotas that developed during both heap and box methods of fermentation were examined. As with the 16S based methods, the primary bacteria identified by cultural methods were strains of *L. fermentum*, *L. plantarum*, Acetobacter pasteurianus and *A. syzygii*. Differences between the two methods of fermentation were also observed, mainly on total counts of lactic acid bacteria and yeast, and less so based on diversity.

There is one obvious advantage of culture methods – namely, that organisms having a suitable cocoa fermentation phenotype can be isolated and propagated. Such strains could be potentially be used as possible starter cultures (De Vuyst and Weckx, 2016). Recently, these researchers sequenced the genome of two lactobacilli isolated from a cocoa bean fermentation (Illeghems et al., 2015a). Genome analyses revealed that both strains possessed unique and relevant carbohydrate and amino acid utilization genes, as well as other genetic features that may confer environmental advantages during the cocoa bean fermentation. Ultimately, these authors suggest, it should be entirely possible to develop starter cultures that ferment cocoa beans in a consistent and uniform manner, minimizing variations in fermentation rate and off-flavor development (De Vuyst and Weckx, 2016).

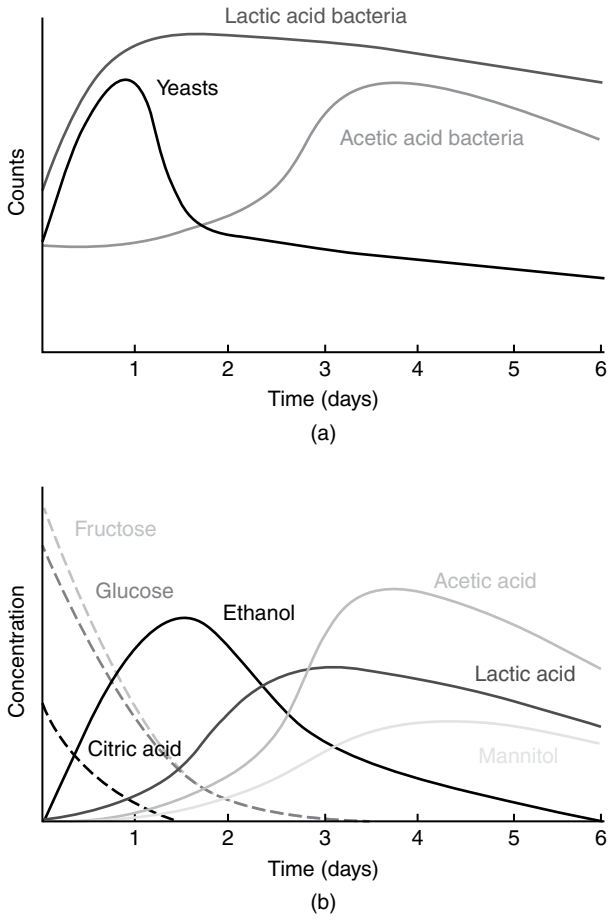


Figure 15.2.1 Idealized growth (a) and metabolic changes (b) that occur during spontaneous cocoa bean fermentations. From De Vuyst and Weckx, 2016, with permission.

The importance of acetic acid bacteria and yeasts cannot be understated, as their role in forming flavor precursors and other end-products is well established (Adler et al., 2014; Maura et al., 2016; Meersma et al., 2016; Visintin et al., 2016). Both culture-based and culture-independent studies have shown the populations of these organisms are very diverse. Among the yeasts, species of *Hanseniaspora*, *Candida, Saccharomyces*, *Schizosaccharomyces*, and *Pichia* are particularly common, not only during the cocoa fermentations, but also in the environment and on equipment (Maura et al., 2016).

Finally, in contrast to 16S sequencing that identifies abundances of taxa, metagenome approaches not only can describe taxonomic composition, but they can also reveal potential metabolic pathways that function during cocoa fermentations (Illeghems et al., 2015b). In this study, *L. plantarum* dominated the cocoa bean fermentation, and was also responsible for important metabolic functions, including heterofermentation of sugars. In addition, the metagenome also included metabolic genes contributed by

enterobacteria and acetic acid bacteria. Given that the cocoa ecosystem is a competitive environment, it was not surprising that genes encoding for bacteriocin production and stress responses were also present.

Finally, another recent study demonstrated that *L. plantarum* and *Leuconostoc mesenteroides* were the dominant lactic acid bacteria in fermented cocoa beans from Ivory Coast (Ouattara et al., 2017). The researchers also observed that several isolated strains metabolized citrate and expressed the enzyme, citrate lyase. Thus, they suggested that successful cocoa fermentations relies, in part, on citric acid utilization.

References

Adler, P., L.J. Frey, A. Berger, C.J. Bolten, C.E. Hansen, and C. Wittmann. 2014. The key to acetate: metabolic fluxes of acetic acid bacteria under cocoa pulp fermentation-simulating conditions. Appl. Environ. Microbiol. 80:4702–4716.

Bortolini C., V. Patrone, E. Puglisi, and L. Morelli. 2016. Detailed analyses of the bacterial populations in processed cocoa beans of different geographic origin, subject to varied fermentation conditions. Int. J. Food Microbiol. 236:98–106.

De Vuyst, L., and S. Weckx. 2016. The cocoa bean fermentation process: from ecosystem analysis to starter culture development. J. Appl. Microbiol. 121:5–17.

Illeghems, K., L. De Vuyst, and S. Weckx. 2015a. Comparative genome analysis of the candidate functional starter culture strains *Lactobacillus fermentum* 222 and *Lactobacillus plantarum* 80 for controlled cocoa bean fermentation processes. BMC Genomics. 16:766.

Illeghems, K., S. Weckx, and L. De Vuyst. 2015b. Applying meta-pathway analyses through metagenomics to identify the functional properties of the major bacterial communities of a single spontaneous cocoa bean fermentation process sample. Food Microbiol. 50:54–63.

Maura, F.Y., T. Balzarini, P.C. Borges, P. Evrard, L. De Vuystc, H.-M. Daniel. 2016. The environmental and intrinsic yeast diversity of Cuban cocoa bean heap fermentations. Int. J. Food Microbiol. 233:34–43.

Meersman, E., J. Steensels, N. Struyf, T. Paulus, V. Saels, M. Mathawan, L. Allegaert, G. Vranckenc, and K.J. Verstrepen. 2016. Tuning chocolate flavor through development of thermotolerant *Saccharomyces cerevisiae* starter cultures with increased acetate ester production. Appl. Environ. Microbiol. 82:732–746.

Ouattara, H.D., H.G. Ouattara, M. Droux, S. Reverchon, W. Nasser, and S.L. Niamke. 2017. Lactic acid bacteria involved in cocoa beans fermentation from Ivory Coast: Species diversity and citrate lyase production. Int. J. Food Microbiol. 256:11–19.

Visintin, S., V. Alessandria, A. Valente, P. Dolci, and L. Cocolin. 2016. Molecular identification and physiological characterization of yeasts, lactic acid bacteria and acetic acid bacteria isolated from heap and box cocoa bean fermentations in West Africa. Int. J. Food Microbiol. 216:69–78.

Drying

The purpose of the drying step is to remove sufficient moisture such that the water activity (a_w) is too low to support growth of microorganisms. At the end of fermentation, the beans contain about 55–60% moisture, with a water activity of about 0.99). The goal is reduce the moisture to 7–8% (with water activity < 0.80).

The most common way to dry the fermented beans relies on sun and air. The beans are simply spread out over a flat surface to a depth of about 6–10 cm. The beans are routinely stirred with rakes to facilitate drying. Depending on the ambient temperature, length of day, and sunlight, the beans will be dry within about one week. To prevent rain from re-wetting

the beans, portable canopies or covering can be wheeled into place. Convection drying has been introduced in some cacao farms, providing weather-independent technology, but this is not common. Slower drying rates, as achieved by sun drying, are generally considered to produce beans with the highest quality.

At the end of the drying step, the beans will contain about 6–8% moisture, 45–55% fat, 12–14% starch, 10–12% protein, 2–4% polyphenolics, 2–4% free amino acids, about 1–2% organic acids (citric, acetic, and lactic), and less than 1% sugars (glucose and fructose). After the beans are dried, they are bagged and prepared for shipping to distributors. The processor receives these beans and begins the process of converting them to a variety of cocoa and chocolate products.

Roasting

Roasting the beans is one of the most important steps in chocolate processing since this is when much of the cocoa flavor and color are formed. It is worth re-stating a previous comment from above, namely, that fermentation is mandatory to produce the substrates for the reactions that occur during roasting. Roasting is usually preceded by several other important steps, including bean evaluation, cleaning, sieving, breaking, sorting, and winnowing (the process of separating beans from husks). However, it is also possible to pre-roast the whole beans before the winnowing step to enhance husk separation. At the end of these processes, the broken beans (called nibs) are separated from the shells, husks, dirt, and other debris. In addition, some processors apply steam to inactivate microorganisms. Another option is to treat the nibs with an alkaline solution (usually carbonate). This process, called the Dutch process, increases the pH to near neutral. Despite enhancing the dark color of the nibs, the process actually produces cocoa with a less intense, but smoother flavor.

There are many variations to the roasting step. Depending on the manufacturer, nibs can be roasted for short time at high temperatures (170°C) or for longer times at lower temperatures (90°C). Nibs can also be ground into a liquor (see below) and then roasted. In either case, whether roasting the nibs or the liquor, the same critical reactions occur. First, Maillard reaction products are formed from amino acids and reducing sugars. These products enhance both color and flavor development characteristic of chocolate. Roasting also decreases moisture from about 8% to nearly 2% and causes volatile compounds to vaporize. Among the volatiles lost during roasting is acetic acid (which has a vinegar aroma), as well as several other organic constituents that contribute to the bitterness of chocolate.

Chocolate processing

Following the roasting step, the nibs are ground in various types of milling devices to form a product known as cocoa liquor (or chocolate liquor). As noted above, this liquor can also be formed prior to roasting. The cocoa liquor has the same composition as the nibs, but becomes fluid as the cocoa fat melts as a result of the heat generated during grinding. Cocoa liquor can be further heated to 100°C to enhance microbial stability and cooled and packaged.

Although cocoa liquor looks like chocolate, it is not quite there. Indeed, it is way too bitter to consume. Instead, the liquor is usually pressed to separate the cocoa fat (cocoa butter) from the non-fat cocoa solids (cocoa powder). As noted above, the nibs and cocoa liquor made from nibs contain about 50% fat. Pressing under hydraulic pressure releases about 80–90% of this fat into the cocoa butter; the remainder stays within the cocoa cake.

The latter is then milled into a powder resulting in cocoa power or simply cocoa. It is generally not used directly for chocolate, but rather is used in the confectionary and processed foods industry.

To make chocolate, portions of cocoa liquor and cocoa butter are mixed together, along with one or more additional ingredients. Among the added ingredients are sugar, milk, and emulsifiers. The chocolate is then passed through rollers to reduce the particle size of the chocolate and enhance the smoothness of the final product.

This refining step is followed by another heating process, ranging from 50 to 80°C and from several hours to a day or more. This process is called "conching", and it serves several important functions. During conching, the fat from the cocoa butter (and milk if present) becomes more evenly distributed, improving the flow and rheological properties. In addition, undesirable volatile compounds, especially volatile acids are removed, improving flavor. Conching also removes water (by evaporation) and enhances emulsification when lecithin or other emulsifiers are added.

The final step in chocolate manufacture is called tempering. The purpose of tempering is to adjust the form and structure of fat crystals such that the chocolate has the desired appearance, texture, and smoothness. In the absence of a tempering step, a serious defect called "bloom" may occur. This is primarily a visual defect characterized by the appearance of "blotches" caused by fat crystals. Ordinarily, cocoa fat consists in one of several polymorphic forms, with each having a particular crystalline structure. Depending on the specific fatty acid composition, cocoa fat solidifies in one form or another. Thus, while cocoa fat in the so-called "V" form gives the best chocolate appearance and "snap", others result in chocolate that are either too hard, are gritty, or that are prone to bloom formation. Tempering involves moderate heating and cooling cycles so that the appropriate type and number of crystals are formed and undesirable crystals are absent or undetectable.

Chocolate quality

Many factors contribute to the quality of chocolate, starting with the cacao bean. The type of bean, where the beans were grown and under what climatic conditions, and how and when they were harvested – all have a major influence on chocolate quality even before fermentation or processing has occurred. It's fair to say that cacao beans are to chocolate as grapes are to wine. Thus, the terroir concept has now been applied to cacao (Box 15.3). Finally, as noted above, the two main types of beans (Forastero and Criollo) are categorized based on their quality and flavor attributes.

Post-harvest processing certainly has a major influence on chocolate quality. All of the steps described above (i.e., roasting, grinding, conching), as well as the quality of added ingredients, affect the final product. Ultimately, chocolate quality is assessed based on several sensory properties, including flavor and aroma. However, hardness, melting properties, "snap" and other physical properties are also important. The absence of flavor and appearance defects (especially bloom) is expected for quality chocolate. Recently, sophisticated chemical methods have been used to measure quality in a more objective manner (Box 15.4).

Nutritional quality

One of the reasons for the popularity of chocolate is due to the association of moderate chocolate consumption with positive changes in health. Dark chocolate, in particular, has high concentrations of flavanols and other polyphenolic compounds. These have anti-oxidant

Box 15.3 A sense of place – the terroir of chocolate and coffee

Coffee and chocolate beans have been grown around the world, and then globally exported, imported, and traded for hundreds of years. However, for most consumers, the actual origin of the beans has not, until recently, been of much concern. Now, if one visits a coffee shop or purchases coffee at a grocery store, the location where the beans had been grown is often advertised or is a prominent part of the label. The "place" from which these products originated, the so-called terroir, has become as much a part of the coffee and cacao trade as it has for wine.

Of course, as described in Chapter 11 for wine, terroir means much more than simply the country the raw materials were grown. Obviously, substantial differences exists in the type and quality of wine made from different regions in France, Italy, Spain, or other wine-production countries. Likewise, for coffee and cacao beans grown in Colombia, Brazil, or Ivory Coast, the specific region, and even the location of specific farms can also affect product quality. Altitude, spoil type, sunlight, rain – all those factors that affect wine grapes, would also affect coffee and cacao.

In addition, terroir for coffee and cacao would also account for the climatic conditions that prevailed during a particular growing season. Terroir would include ripeness of the beans at harvest, the sorting of ripen from immature beans, and of course the cultivar. Terroir would essentially include all of the particular traits conferred by the beans into the final products.

In the larger sense, however, terroir conveys more than origin or climate or cultivar. As Nesto, 2010 stated "location is the starting point for terroir". Human interaction, Nesto contends, also influence the final product. Thus, according to this view, even roasting, grinding, conching in the case of chocolate, and other processing steps would also contribute to terroir.

Reference

Nesto, B. 2010. Discovering terroir in the world of chocolate. Gastronomica: the journal of food and culture. 10 (1):131–135.

Box 15.4 Better cocoa through chemistry

The quality of nearly all fermented foods depends on the quality of the raw materials. High quality raw cocoa beans are certainly necessary to produce excellent chocolate. However, it's not the raw cocoa beans, per se, but rather the fermented and dried beans that are ultimately obtained and used by chocolate manufacturers. Thus, assessing flavor, aroma, and other quality attributes of the dry, fermented beans is of utmost importance. This task has been mainly left to expert tasters, whose evaluations are subjective and often inconsistent (Teye et al., 2015). There are, unfortunately, few fast and accurate objective methods for predicting chocolate quality based on the phenotypic properties of the dry fermented beans.

Recent, several rapid methods have been proposed for assessing cocoa properties and quality (Krähmer et al., 2015; Teye et al., 2015). These methods rely on near infrared spectroscopy (NIRS). Although based on rather sophisticated analytical chemistry principles, NIR methods are fast, require minimal sample preparation, and they can be included as part of routine quality control procedures. While NIR can quantify a range of molecules, there are several fermentation end products that are particularly relevant to cocoa. For example, one NIRS method (Krähmer et al., 2015) measured lactic and acetic acids, as well as moisture, carbohydrates, free amino acids, phenolics, and pH. Not only do these values reflect the extent of fermentation, but they also be used as organoleptic markers for cocoa flavor and quality.

In another study, Fourier transform (FT)-NIRS was similarly used to assess cocoa quality parameters (Teye et al., 2015). Chemometrics was also incorporated into this study. In chemometrics, chemical data is combined with computational and statistical data to identify patterns among different sample sets and to track chemical changes that occurs over time. Thus, in this study, the researchers were able to distinguish between unfermented, partially fermented, and fermented cocoa beans based on the FT-NIR spectra.

In addition to NIR approaches for identifying cocoa attributes and predicting cocoa quality, proteomics has also been used. Because products of protein hydrolysis are very important in cocoa flavor development, these methods are generally based on characterizing peptides, amino acids, and other nitrogenous compounds. In one very recent study, extracts of cocoa beans from various regions were analyzed during the fermentation period (Hue et al., 2016). The results showed that proteins were clearly degraded during fermentation, yielding peptides and free amino acids. However, total nitrogen also decreased during fermentation, presumably due to migration of some constituents from the bean to the shell.

A MALDI-TOF approach was similarly used to characterize fermentation-derived cocoa peptides that presumably serve as flavor precursors (Voight et al., 2016). Amino acid sequences of more than 50 peptides were obtained. Ultimately, by reacting these peptides with suitable sugars and amino acids, these researchers suggested that it may be possible to identify which of these peptides contribute to chocolate flavor and aroma formation.

References

Hue, C., Z. Gunata, A. Breysse, F. Davrieux, R. Boulanger, and F.X. Sauvage. 2016. Impact of fermentation on nitrogenous compounds of cocoa beans (*Theobroma cacao* L.) from various origins. Food Chem. 192:958–964.

Krähmer, A., A. Engel, D. Kadow, N. Ali, P. Umaharan, L.W. Kroh, and H. Schulz. 2015. Fast and neat – Determination of biochemical quality parameters in cocoa using near infrared spectroscopy. Food Chem. 181:152–159.

Teye, E, X. Huang, L.K. Sam-Amoah, J. Takrama, D. Boison, F. Botchway, and F. Kumi. 2015. Estimating cocoa bean parameters by FT-NIRS and chemometrics analysis. Food Chem. 176:403–410.

Voigt, J., K. Janek, K. Textoris-Taube, A. Niewienda, and J. Wöstemeyer. 2016. Partial purification and characterisation of the peptide precursors of the cocoa-specific aroma components. Food Chem. 192:706–713.

activity and are considered to be heart-healthy. Chocolate also contains stimulatory compounds, including caffeine and theobromine that may reduce blood pressure. Recent evidence from both epidemiological and clinical studies has shown that dark chocolate (as little as 30 g per day) may not only reduce risks of myocardial infarctions, heart failure, and cardiovascular mortality, but can also improve cognition, relieve stress, and improve mood. As if consumers needed an excuse to consume chocolate!

COFFEE

Coffee history

As noted above, the history of coffee shares several details with that of cacao. Both were indigenous to specific regions (but on separate continents) of the world and were well established crops before their "discovery" by Europeans. Eventually, they both became prized commodities in Europe, and their cultivation spread across the world. Interestingly, they are now both grown and produced in similar regions.

The coffee plant (it's actually a shrub) was first grown in what is now Ethiopia (in the Eastern part of Central Africa). A beverage form of coffee was consumed by local populations, and by the 1400s, consumption had spread to other regions, including Turkey, Persia, and Egypt. Eventually, in the 1500s, coffee reached the European market, presumably by travelers who had sampled coffee during visits to Northern Africa. Coffee shops began to emerge in the seventeenth century, and soon coffee had become one of the most popular beverages in Europe, perhaps second only to alcoholic products.

Until the mid-1600s, coffee was unknown outside of Africa, the Middle East, and Europe. In the mid-1600s, European colonists and traders soon began to grow coffee in other regions, in particular the New World. Coffee was first grown in Haiti, Jamaica, Cuba, and other Caribbean islands. Farms or plantations then formed in South American in the early 1700s, and by the end of the century, Brazil had become a major producer. Later, Colombia and several Central America countries began to produce coffee. Coffee farming in Asia and the Far East began in the eighteenth century. At the same time, less coffee was produced in Africa and by the 1900s coffee exports from Africa were virtually non-existent. This situation has changed dramatically in the past 50 years.

Until near the end of the nineteenth century, all coffee was purchased from wholesale or retail suppliers in the form of unroasted or so-called "green" coffee beans. The roasting and grinding steps were performed in the home. However, by the early 1900s, a coffee industry had developed, and new products, including ground coffee in cans and instant coffee were introduced.

The very first coffee beverages were made by adding boiled water to crushed or ground beans, a practice still popular in much of the world. Percolated and drip-style coffee began in the early 1800s, although they were not widely used. Indeed, it was not until the 20th century that these production methods were adopted in American homes. Espresso machines were introduced at the end of the nineteenth century and these coffees became popular almost immediately in Europe and eventually around the world. They obviously remain so today.

Production and consumption

Regardless of region or country, most of the world supply of coffee is still produced on small farms. These small farms also perform the early processing steps (drying and fermentation), then bag and ship the coffee to manufacturers and processors. Surprisingly, as noted above,

the coffee processing industry did not begin until the end of the nineteenth century. Before then, consumers purchased green coffee beans and performed the roasting step in the home. Now the roasting step has become an important step as it allows manufacturers to produce a range of different types of products. Roasting also allows manufacturers to distinguish their products from their competitors.

The main coffee producing countries are in South America (Figure 15.4). However, Africa is still a major producer as are Indonesia and India. Like cacao, coffee grows within a narrow latitude, about 2 degrees on either side of the equator. Altitude is also important for coffee – some coffee-growing regions in Colombia are over 1800 meters (6000 feet) above sea level. Although coffee can be grown at even higher altitudes, yields are lower.

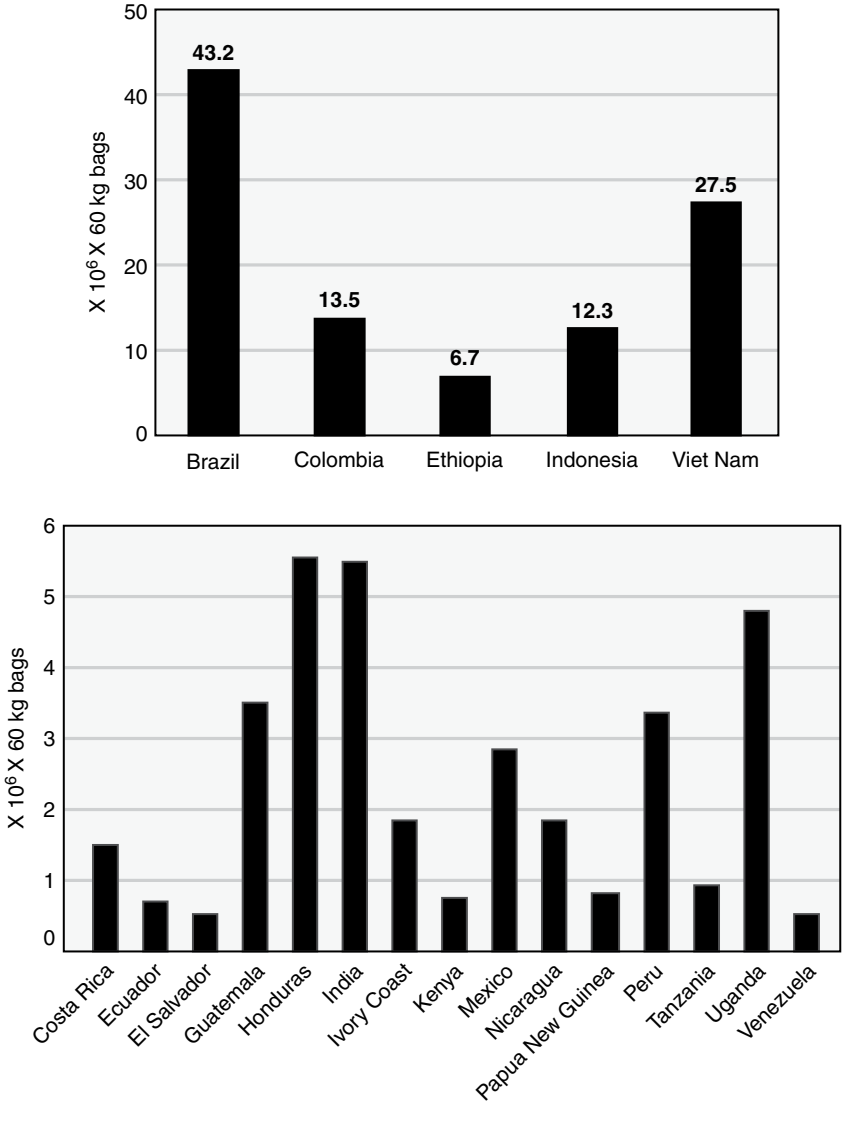


Figure 15.4 Global coffee production (2015). Shown are the top five producers (upper panel) and the next 15 largest producers (lower panel). Sources: International Coffee Organization.

It is important to note that there are two main species of coffee, and different countries generally produce one or the other of these. The most widely-produced and widely-consumed coffee is *Coffea arabica*, known in coffee trade as "Arabica". About 75% of all coffee produced is Arabica. This is the main type of coffee grown in Brazil, Colombia, and other South American countries, particularly in higher elevations. However, many varieties of *C. arabica* exist, each with their own particular qualities.

The other species of coffee is *Coffea canephora*. It is grown primarily in Indonesia, Ivory Coast, and Uganda. Brazil, the leading producer of Arabica, also produces this species as well. This species is known by the descriptive name "Robusta", reflecting its bolder, more acidic, and more pronounced bitter flavor. Robusta grows at lower elevations and is generally more disease-resistant. Robusta also contains about twice as much caffeine as Arabica, which contributes to its bitterness. As an aside, the bitterness associated with coffee is not necessarily a negative organoleptic property, as many consumers like bitterness in coffee and chocolate, as well as other fermented food and beverage products (Box 15.5).

Box 15.5 Some like it bitter

Given the strong and apparently innate human aversion to bitterness, it is surprising that coffee is so popular. Many of the plant materials that early humans must have sampled tens of thousands of years ago contained alkaloids and other bitter-tasting compounds that also happened to be poisonous. Thus, bitterness aversion is an evolved trait, whose function is to warn a hungry human that what you are about to eat is toxic and injurious to health (Behrens and Meyerhof, 2011).

Indeed, of the five tastes detected by humans (the others are sweet, sour, salty, and umami), humans are most sensitive to bitter (Beauchamp, 2016). The taste threshold for bitter is about 1000 times less than for other tastes (Meyerhof, 2005), and there are more receptors for bitter than any other taste (Beckett et al., 2014). Moreover, while there are only a few human genes that encode for sweet and umami taste receptors, there are 25 genes (called TAS2R) encoding for bitterness receptors (Behrens and Meyerhof, 2011).

Why then, if humans are genetically programmed to be on the watch for and avoid bitter-tasting foods, do some individuals actually like bitter flavors? Is bitterness-liking a learned trait or are there genetic reasons why some individuals like bitter-tasting foods? Of course, liking bitter is not unique to coffee. Indeed, many of the fermented foods described in this text have bitter flavors. Examples include aged cheese, where bitterness is derived from bitter-tasting peptides, beer, where hops provide the bitter flavor, and olives that undergo a process step to reduce the naturally-containing bitter glycosides. As noted even earlier in this chapter, bitter flavor is also a prominent attribute of chocolate.

As noted above, there are currently 25 TAS2R bitterness receptor genes in the human genome. The target molecules for many of these receptors are known and include caffeine, peptides, quinine, strychnine, humulone, and saccharin. One way for researchers to assess bitter sensitivity is via the use of two specific bitter compounds, phenylthiocarbamide (PTC) and 6-n-propyl-2-thiouracil (PROP). Thus, among a population of human subjects, there are PTC/PROP tasters and non-tasters. This suggests that a genetic component for bitter coffee-liking might exist.

Indeed, one specific TAS2R receptor, TAS2R43, is particularly sensitive to caffeine. In contrast, the TAS2R38 receptor (which detects PROP), is not associated with coffee liking (Becket et al., 2014). In one recent study, more than 4000 individuals completed preference surveys and were scored as coffee-likers or non-likers (Pirastu et al., 2014). Each subject was then genotyped based on polymorphisms of all 25 TAS2R genes. The results revealed that individuals who expressed the wild type allele for TAS2R43 generally were likers. Individuals with a mutation or SNP associated with this gene (i.e., they carried the H212R allele) were generally non-likers. A subset of subjects was also tested for caffeine perception. Subjects who were non-likers and had low caffeine perception also carried this non-functional allele. No other TAS2R receptor appeared to be linked to coffee-liking, however. Thus, the authors concluded that coffee-liking or non-liking, as well as caffeine perception, was associated with the H212R allele.

If the "original" biological function of TAS2R taste receptors was to warn a human that a food he or she was about to eat was bitter and should be avoided, then what has changed such that bitterness is no longer such a threat? In fact, for some individuals, bitter-liking has become their personal preference, whether it's for coffee, aged cheese, or beer.

There are some obvious behaviors that may account for evolved bitter-liking. Many coffee drinkers, for example, may have started out by adding plenty of cream and sugar, then being gradually weaned. Likewise, early chocolatiers discovered they could attract new customers (especially children) by adding sugar and milk to the chocolate to reduce bitter flavor. The current trend toward dark chocolate with high cocoa content suggests many of those children have grown up and now appreciate chocolate without the milk. Bitter-liking, especially for coffee, has considerable practical implications, since consumer preferences may ultimately influence breeders, processors, and even baristas!

The phenomenon of bitter-liking, regardless of an individual's TAS2R43 genotype, may have occurred as a result of what Pirastu et al., 2014 and others have referred to as "flavor-nutrient learning". According to this somewhat controversial theory, the positive biological, sociological, and psychological effects of coffee are associated with the bitterness of caffeine. Similar associations may exist for other bitter foods, such as beer and cheese. In other words, while some animal species are hard-wired not to like bitter, other species (i.e., humans) can learn to like certain foods, however bitter they may be (Mol, 2012).

References

Beauchamp, G.K. 2016. Why do we like sweet taste: A bitter tale? Physiol. Behav. 164 (B):432–437.

Beckett, E.L., C. Martin, Z. Yates, M. Veysey, K. Duesing, and M. Lucock. 2014. Bitter taste genetics – the relationship to tasting, liking, consumption and health. Food Funct. 5:3040–3054.

Behrens, M. and W. Meyerhof. 2011. Gustatory and extragustatory functions of mammalian taste receptors. Physiol. Behav. 105:4–13.

Folmer, B. 2014. How can science help to create new value in coffee? Food Res. Internal. 63:477–482.

Mol, A. 2012. Layers or versions? Human bodies and the love of bitterness. In: Routledge Handbook of Body Studies. Routledge, Oxford, pp. 119–129.
Pirastu, N., M. Kooyman, M. Traglia, A. Robino, S.M. Willems, G. Pistis, P. d'Adamo, N. Amin, A. d'Eustacchio, L. Navarini, C. Sala, L.C. Karssen, C. van Duijn, D. Toniolo, and P. Gasparini. 2014. Association analysis of bitter receptor genes in five isolated populations identifies a significant correlation between TAS2R43 variants and coffee liking. PLOS One. 9(3): e92065.

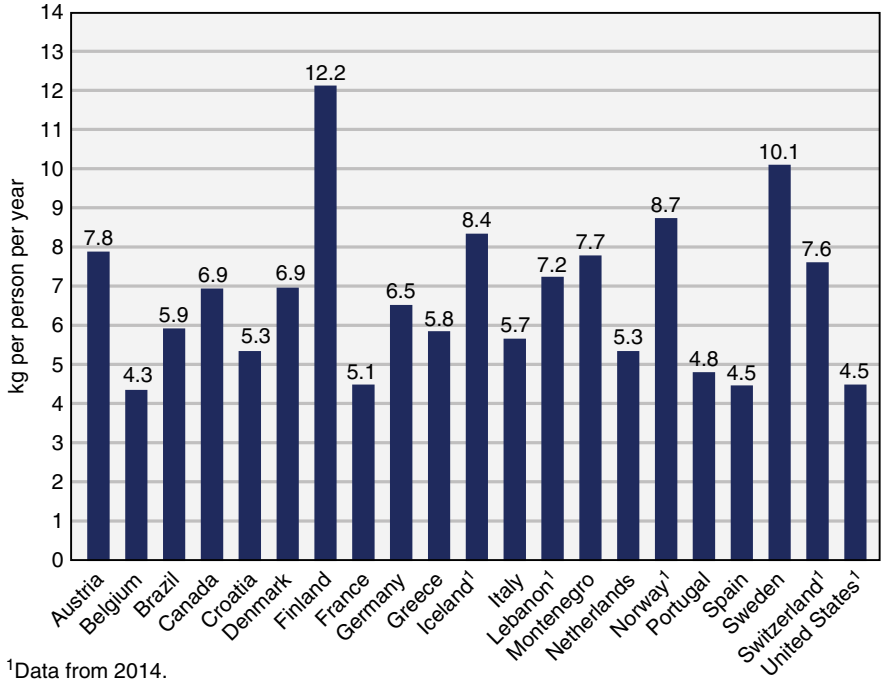


Figure 15.5 Per capita coffee consumption (top 20 countries + US). Data from the International Coffee Association for 2015–16.

Just as for cocoa, the main coffee consuming countries are in North America and Europe (Figure 15.5). On a per capita basis, the Scandinavian countries consume the most coffee (5 of the top 8 consuming countries). Nonetheless, despite the appearance of coffee shops at every turn, coffee consumption has been rather flat since 2000. For example, coffee consumption in the US during the 1940s was more than double that of 2015.

Coffee processing and fermentation

The coffee bean has the appearance of other fruits in that they grow as clusters. Thus, the fruits of coffee trees are called cherries or berries (Figure 15.6). They start off as green and take on a red appearance as they ripen. Each cherry is surrounded by a thick and bitter skin called an epicarp (Figure 15.7). Beneath this skin is a sweet pulpy material called the mesocarp. This material is rich in sugars and serves as the substrate for the subsequent fermentation. The bean seeds (two per fruit) are contained within the mesocarp. They are surrounded by a protective skin called the parenchyma, and a parchment-like material

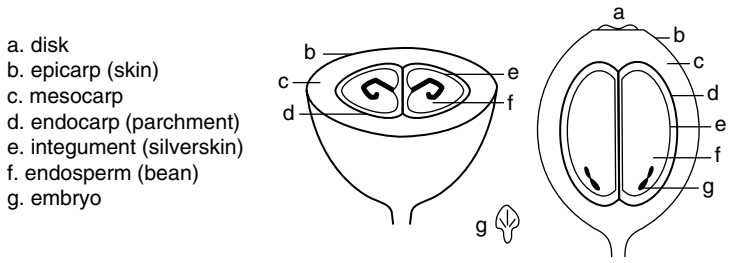


Figure 15.6 Anatomy (transverse and longitudinal sections) of a coffee berry. Adapted from Wintgens, 2012, with permission.

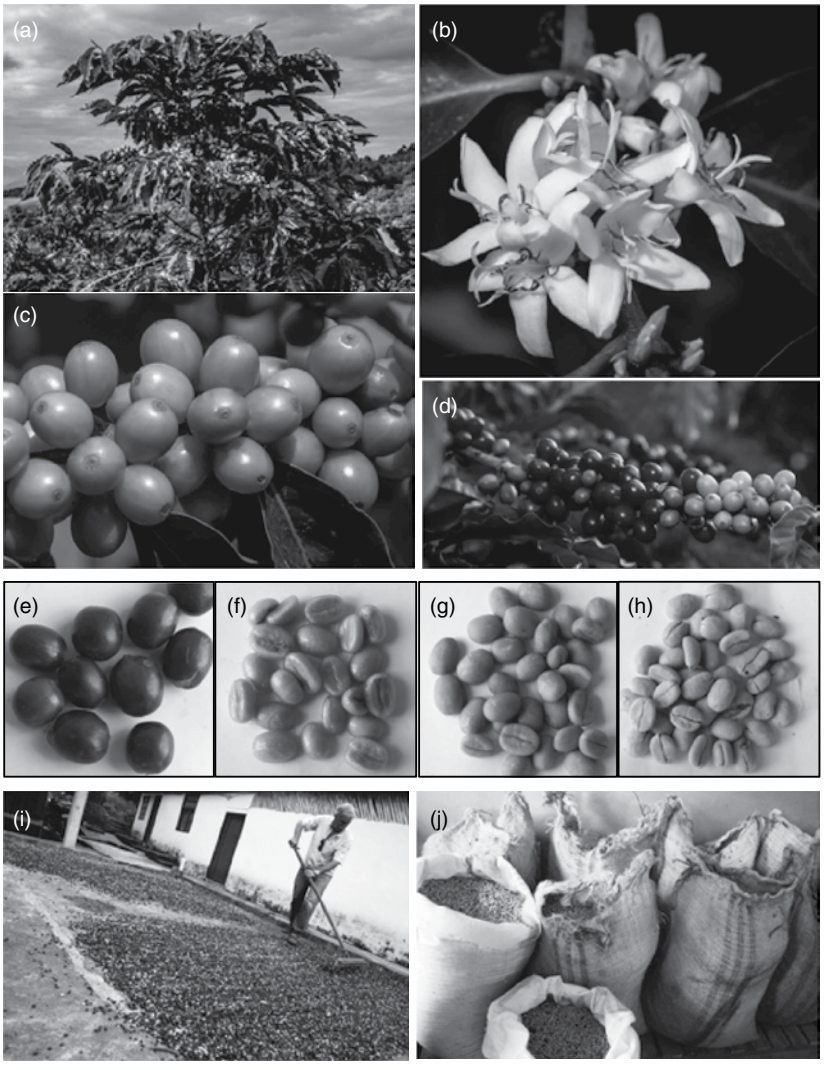


Figure 15.7 Production of coffee beans on typical Colombian farm. Coffee tree (a), coffee flower (b), and coffee beans (c and d). Stages of coffee beans, from cherry (e), with mucilage (f), without mucilage (g), and dried beans (h). Collection of coffee beans after fermentation and drying (i) and in bags (j). Figures courtesy of Alejandra Ramirez-Hernandez and Fernando Ramirez.

called the endocarp. The processing of the fruit begins immediately after the coffee is harvested. However, harvesting itself is important, since the stage of ripening will influence the finished product.

The main goal of processing is to degrade and remove the outer portions of the coffee bean, similar to that of cacao. A secondary goal is to generate flavor compounds, although the importance of this function is arguable. Indeed, some coffee researchers have suggested that perfectly fine coffee can be made sans fermentation. Nonetheless, there are three main ways to achieve these goals, and all involve a fermentation step (Figure 15.8). The dry method relies primarily on a long fermentation period while the beans are exposed to sun-drying. In contrast, the wet method combines mechanical de-pulping and shorter fermentation in water. The semi-dry method includes mechanical de-pulping followed by dry fermentation. Each of these processes is described below.

The dry (or natural) method is more commonly used for Robusta, although depending on the region, Arabica may also be processed by this method. The dry method requires ample sun and little rain. This method is sometimes considered as producing beans having lower quality. However, this view is not universal – the long fermentation period may also add flavor complexity to the beans that is lacking in coffee made by the wet method. Nonetheless, this dry method is easy and inexpensive and is still widely used. The process involves first sorting the beans, based on maturity, and then spreading them on flat surfaces to a depth of about 10 cm. The beans are exposed to the sun in an open air environment for up to 3–4 weeks. The beans are stirred or mixed regularly (i.e., daily). In the evening they are piled or heaped, then re-spread the next day.

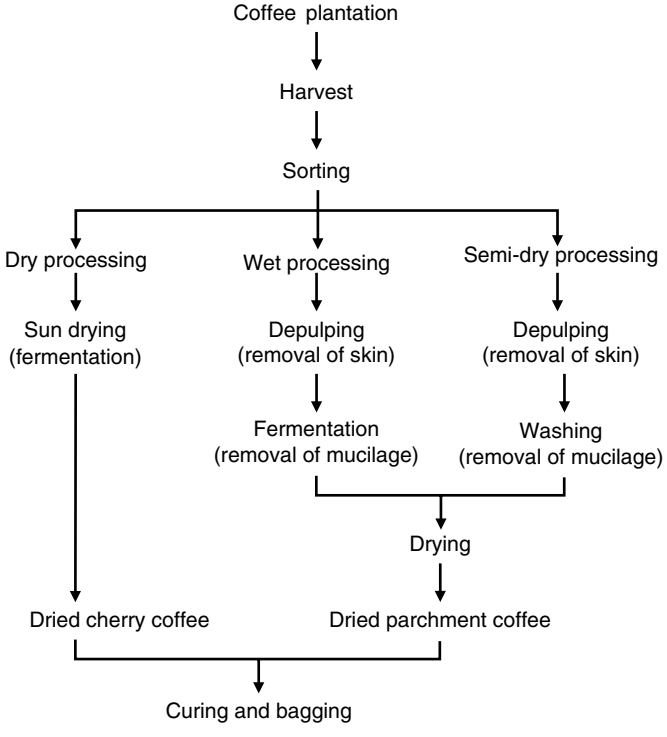


Figure 15.8 Three types of coffee processing. Adapted from Pereira et al., 2017, with permission.

The fermentation begins as soon as the beans are harvested. A complex microbiota, containing organisms from the beans, environment, and workers, contributes to the fermentation (Box 15.6). Gram-positive and Gram-negative bacteria, aerobes and facultative anaerobes, will be present, as will yeasts and other fungi. While lactic acid bacteria may be present, the coffee fermentation relies more on Enterobacteriaceae, acetic acid bacteria, bacilli, and other plant epiphytic bacteria. Among the major groups of yeasts are *Pichia*, *Debaryomyces*, and *Candida*. The fermentation is mainly aerobic.

Box 15.6 The coffee microbiota

Numerous studies have demonstrated the presence of a large and diverse population of microorganisms inhabiting coffee beans. The functional role of this complex microbiota, however, is not fully established. While the degradation and removal of the pectin and other pulpy material is essential, whether or not microorganisms serve any other purpose during coffee processing is debatable.

At the outset, i.e., before any fermentation has occurred, the coffee microbiota is comprised of bacteria and yeasts, representing hundreds of genera (Gilberto et al., 2016; Silva, 2014). Fungal organism such as *Aspergillus* and *Penicillium* are also present (Iamanaka et al., 2014). Some of these organisms are autochthonous to the coffee cherry, surrounding the fruit during its growth on the tree. Others are introduced, post-harvest, via workers, harvesting tools and equipment, and other environmental sources. Regardless of how they got there, however, the microbiota that ultimately is responsible for fermentation depends more on how the beans are processed rather than on the composition of the initial population. Thus, for coffee beans processed by the dry method a particular microbiota will be formed that is different from that which forms in beans processed by the wet method (Evangelista et al., 2015). Nonetheless, for both the dry and wet methods (and demi-dry), there is a succession of organisms that emerge during the fermentation.

As described previously, the main function of coffee bean processing is to remove the outer material from the bean. The bean is surrounded first by a skin called the silver skin), then a parchment layer, then the mucilage, then the pulp, and finally an outer skin. The main difference between the dry method and wet method concerns how and to what degree this material is ultimately removed.

In the dry method, coffee beans are first usually sorted by various methods, and then are spread onto flat surfaces to a depth of 5–10 cm. The beans are exposed to the sun and the ambient temperature and periodically mixed and allowed to ferment for up to 20–25 days (Evangelista et al., 2015; Lee et al., 2015). This promotes mainly aerobic and facultative bacteria, including several genera from the Enterobacteriaceae, as well as other Gram-negative bacteria. Among the Gram-positive bacteria are *Bacillus* and *Paenibacillus*. In addition, *Kloeckera*, *Candida*, *Pichia*, and other yeasts are also present. These organisms express pectinolytic and other pulp-degrading enzymes, such that the pulp and mucilage material is removed. However, the extent to which specific organisms or even whether it's the bacteria or the yeast that contribute to pectin degradation is not clear (Lee et al., 2015).

There is also uncertainly about the role of lactic acid and other fermentative bacteria during dry method processing of coffee. Clearly, lactic acid bacteria are

present in the fruit (Leong et al., 2014), although their function is not well-established. Species of *Leuconostoc* have been isolated from dry method coffee, but they appear to be more involved in pectin degradation rather for their metabolic contributions (Silva, 2015). Indeed, for coffee processed by the dry method, there is rather minimal microbial activity, other than removal of the pulp and mucilage by microbial pectinases. Finally, the parchment, as noted previously, is not removed during the dry method fermentation and must be removed mechanically after drying.

For coffee processed by the wet method, the outer portions of the beans, i.e., the skin and pulp are removed mechanically by special pulping devices. The mucilage, however, remains stuck to the parchment and the bean. In traditional wet method processing, microorganisms degrade the mucilage, serving the same role as for the dry method. Alternatively, it is now possible to rely on mechanical removal of the mucilage. The latter minimizes the need for microorganisms. Nonetheless, there is still a diverse microbiota that develops that includes Gram-negative and Gram-positive bacteria as well as yeasts. In one recent study using both culture-dependent and independent methods, predominant members of the wet method coffee microbiota included the bacteria, *Enterobacter* and *Bacillus*, and the yeasts, *Pichia* and *Hanseniaspora* (Feng et al., 2016). Somewhat similar organisms were also reported by Evangelista et al. (2015) in wet method coffee, but in addition, the heterofermentative lactic acid bacterium, Leuoconostoc, and two *Enterobacteriacease, Erwinia* and *Klebsiella*, were also present.

As with the cocoa microbiota, a better understanding of the role of the different members of the coffee microbiota might ultimately be useful for developing coffee fermentation starter cultures (Evangelista et al., 2015; Lee et al., 2015).

References

Evangelista, S.R., M.G.C.P. Miguel, C.F. Silva, A.C.M. Pinheiro, and R.F. Schwan. 2015. Microbiological diversity associated with the spontaneous wet method of coffee fermentation. Int. J. Food Microbiol. 210:102–112.

Feng, X., H. Dong, P. Yang, R. Yang, J. Lu, J. Lv, and J. Sheng. 2016. Culture-dependent and-independent methods to investigate the predominant microorganisms associated with wet processed coffee. Curr. Microbiol. 73:190–195.

Gilberto, V.M.P., V.T. Soccol, S.K. Brar, E. Neto, and C.R. Soccol. 2016. Microbial ecology and starter culture technology in coffee processing. Crit. Rev. Food Sci. Nutr. 56.

Iamanaka, B.T., A.A. Teixeira, A.R.R. Teixeira, M.V. Copetti, N. Bragagnolo, and M.H. Taniwaki. 2014. The mycobiota of coffee beans and its influence on the coffee beverage. Food Res. Int. 62:353–358.

Lee, L.W., M.W. Cheong, P. Curran, B. Yu, S.Q. Liu. 2015. Coffee fermentation and flavor – an intricate and delicate relationship. Food Chem. 185:182–191.

Leong, K.-H., Y.-S. Chen, S.-F. Pan, J.-J. Chen, H.-C. Wu, Y.-C. Chang, and F. Yanagida. 2014. Diversity of lactic acid bacteria associated with fresh coffee cherries in Taiwan. Curr. Microbiol. 68:440–447.

Silva, C.F. 2014. Microbial activity during coffee fermentation. In: R.F. Schwan and G.H. Fleet, eds. Cocoa and Coffee Fermentations. CRC Press, pp. 397–430.

While these bacteria and yeasts produce organic acids and other end-products that may contribute to flavor, their primary role is to secrete various pectin- and cellulose-degrading enzymes. The coffee bean pulp is degraded by these enzymes – mainly pectin lyase, polygalacturonidase, and cellulases – facilitating release of the bean from the fruit. The pulp also binds moisture, so its degradation and fermentation also enhances the drying rate. Ultimately, at the end of the process, the beans should have a moisture content of about 10%. Low moisture is critical to prevent growth of fungi during storage. Otherwise, coffee beans could support growth of mycotoxin-producing *Penicillium*, including strains that produce ochratoxin.

While the wet method relies primarily on mechanical removal of the pulp material, a fermentation step is still performed. Most Arabica coffee is processed by this method. The beans are usually first submerged for sorting purposes. Then, one of several types of pulping machines – screen, drum, disk, or others, is used to tear off the pulp. After pulp or exocarp removal, a slippery mucilage layer, about 1–2 mm thick, remains. The solids portion of the mucilage is about 80% pectin and related pectinic acids and about 20% simple sugars. Thus, the submerged beans are allowed to ferment in tanks for about 12–36 hours, although sometime less, sometimes more.

The organisms that participate in this fermentation are introduced via the water, tanks, and those that remain attached to the beans. They produce pectin-degrading enzymes, as described above. Ultimately, a drying step (in the sun) is still necessary to reduce the moisture content to about 10–12%. The semi-dry method combines features of both the dry and wet methods. The pulp is mechanically removed as for the wet method, but then the beans are allowed to ferment while in the dry state.

Despite the seeming simplicity of the coffee fermentation, and the many years in which these natural methods have been used, aberrant microbial activities are not uncommon. In other words, a lot can go wrong. How to control coffee fermentations and to make the process more consistent and predictable is a major challenge. Thus, there is now considerable interest in isolating and characterizing organisms from coffee and then re-introducing them as starter cultures. Such efforts could also provide a basis for producing coffee with particular flavor or aroma attributes (Box 15.7).

Box 15.7 Cultures for coffee and cacao

Coffee and chocolate are among only a few fermented food products for which commercial cultures are not available. Nonetheless, there has been considerable interest in developing starter cultures for these products. The rationale is the same as for other fermented food – cultures can provide consistent processing times, reliable fermentation, and perhaps most importantly, consistent quality. Cultures may also provide opportunities to create customized products having particular flavor characteristics.

Strategies for developing starter cultures for coffee rely first on isolating the relevant organisms and then demonstrating that they have the necessary phenotype. Ultimately, however, they must perform, *in situ*, that is they must have the functional performance properties during an actual fermentation. These approaches are now being employed.

Recall that the main outcome of the coffee fermentation is degradation of the pectin-containing pulp and mucilage that surrounds the beans. This requires the action of pectinolytic organisms. In particular, various yeast species have been suggested to contribute this function including *Saccharomyces cerevisiae*, *Candida parapsilosis*, and *Pichia guilliermondii* (Silva et al., 2013). Recently, the potential application of four specific strains of these yeasts was assessed by the dry fermentation method. Each strain was introduced individually into coffee beans (washed or unwashed) and then held at ambient temperature and relative humidity for two weeks (Evangelista et al., 2013). Although all yeasts persisted during the two-week fermentation period, variations in fermentation end-products as well as sensory descriptions were observed. For example, beans fermented by *C. parapsilosis* UFLA YCN448 and *S. cerevisiae* UFLA YCN727 had a pleasant aroma of caramel, herbs, and fruits. Also, all of the inoculated coffees had more flavor than un-inoculated coffee. The same group also tested these strains by the semi-dry method (Evangelista et al., 2014). Although a more diverse microbiota was present, volatile end-products and flavor profiles were similar to those obtained in the dry method study. The authors concluded that selected yeasts could be used as starter cultures to perform the fermentation step, producing a coffee with unique flavor characteristics.

Another group recently used a similar approach to obtain potential starter cultures for wet processing of coffee (de Melo Pereira et al., 2014). Manually de-pulped beans were allowed to ferment for 48 hours and yeasts were isolated and identified. Yeasts similar to those isolated by Silva et al. (2013) were present, including *Pichia*, *Candida*, *Saccharomyces*, and *Hanseniaspora*. The isolates that could grow and had pectinolytic activity in laboratory experimental conditions mimicking the wet fermentation were subsequently used in the wet fermentation trials. The results revealed that specific strains, either as single or co-culture inoculants, led to enhanced production of volatile aroma compounds compared to the un-inoculated control. In addition, the culture-fermented beans produced brewed coffee having higher sensory scores for several aroma traits.

Collectively, these recent studies suggest that coffee starter cultures have several advantages compared to wild or natural fermentations. Not only do they provide consistent and standardized fermentation conditions, but they also produce beans and coffee having unique and desirable flavor.

References

de Melo Pereira, G.V., V.T. Soccol, A. Pandey, A.B.P. Medeiros, J.M.R.A. Lara, A.L. Gollo, and C.R. Soccol. 2014. Isolation, selection and evaluation of yeasts for use in fermentation of coffee beans by the wet process. Int. J. Food Microbiol. 188:60–66.

Evangelista, S.R., M.G.P.C Miguel, C. S. Cordeiro, C.F. Silva, A.C.M. Pinheiro, and R.F. Schwan. 2014. Inoculation of starter cultures in a semi-dry coffee (*Coffea arabica*) fermentation process. Food Microbiol. 44:87–95.

Evangelista, S.R., Silva, C.F., Miguel, M.G.P.C., Cordeiro, C.S., Pinheiro, A.C.M., Duarte, W.F., and Schwan, R.F., 2013. Improvement of coffee beverage quality by using selected yeasts strains during the fermentation in dry process. Food Res. Int. 61:183–195.

Silva, C.F., Vilela, D.M., Souza, C.C., Duarte, W.F., Dias, D.R., and Schwan, R.F., 2013. Evaluation of a potential starter culture for enhance quality of coffee fermentation. World J. Microbiol. Biotechnol. 29:235–247.

Roasting

Similar to cacao, coffee beans leave the production farms in bags as whole dry beans. They are then exported to coffee processing companies where the processing and roasting steps occur. Initially, after they are received, the beans are cleaned and foreign material (stones and other debris) are removed. The beans are then conveyed to roasters.

As noted above, prior to the early 1900s, consumers purchased green coffee beans and performed the roasting step in the home. Commercial roasters, although available during this time, did not become the primary means for roasting coffee until the 1930s. Continuous roasters, introduced in the 1940s enhanced speed, efficiency, and quality.

Although traditional equipment is still used by small coffee processors, coffee roasting technology has evolved dramatically in the past hundred years. Regardless of how coffee is roasted, the principles remain the same. During the early stages, at temperatures between 100 and 150°C, moisture, CO_2, and volatile compounds in the green beans are lost. This drying phase is followed by much higher temperatures, from 200–260°C. High internal pressures (i.e., within the beans) are formed as the temperature increases that contribute to the catalysis of chemical reactions. Thus, it is primarily during this roasting step when color, flavor, and aroma develop. If unroasted coffee were brewed, it would have very mild, perhaps beany flavor and aroma with a pale color.

There are several general types of reactions that occur during roasting, including Maillard and caramelization reactions. Importantly, these reactions depend on substrates present in the green beans. Thus, the composition of the beans has a major influence on the quality of the roasted beans and ultimately, the brewed coffee consumers drink.

The reactants for the Maillard or non-enzymatic browning reaction are reducing sugars and free amino acids. The sugar content of the beans, even after fermentation, is still about 5%. Although fewer amino acids are present (less than 1% in coffee beans), this is still sufficient to promote the formation of Strecker degradation and other Maillard reaction products. Caramelization, in contrast, is a heat-driven process that involves dehydration of sugars followed by polymerization reactions. Thus, although Maillard and caramelization reactions yield different products, both are responsible for the characteristic properties of roasted coffee beans.

These reactions are also similar to those that occur during the kilning or roasting of malt and cacao. Maillard and caramelization reactions convert the green beans into yellow, then tan, and finally into beans with a light or medium brown appearance. Longer roasting times at high temperatures result in a dark brown color characteristic of French or Italian roast style coffees. However, another there is another type of heat-induced reaction, called pyrolysis that occurs at very high temperatures and is more specific to coffee. Pyrolysis refers to thermal degradation of sugars and formation of aldehydes, esters, ketones, and other organic compounds. Many of these are volatile, giving dark roasted coffee a pronounced aroma.

Grinding

While roasted beans are sold at retail or purchased by coffee shops, most consumers purchase ground coffee. Roller-type mills are usually used, although many different versions and configurations exist. Manufacturers base the grind on the amount of coffee that passes through sieves or screens of varying meshes. The grind, from course to fine, depends ultimately on how the coffee is intended to be brewed. Thus, coffee for percolators is usually coarse, drip-style coffee is medium, and espresso uses finer grind coffee.

Brewing considerations

Not only have specialty coffees become popular, but many coffee consumers have also become interested in the manner in which coffee is brewed. For hundreds of years coffee was brewed according to local or traditional practices. Thus, so-called Turkish coffee was made by steeping grounds in boiling water and consuming the coffee without a separation step. In Europe, espresso-style coffee (including cappuccino and other assorted coffee beverages) has long been made by forcing steam under pressure through the grounds. This style eventually made its way to the rest of the world, where it is now de rigueur for many coffee consumers. There are even affordable home versions of the European espresso maker. In the past decade, machines that use individual pods or capsules to make espresso-type coffee have become popular.

Percolated coffee was introduced in the nineteenth century and soon became the most popular style in the US. However, one of the most important innovations was the electric drip-style coffee maker. This filter-based device made it easy to brew a 12-cup pot of coffee in just a few minutes. They are now commonplace in homes, offices, and in the workplace. More recently, several new ways of brewing coffee have been introduced. In the French press method, a plunger-type device is used to force how water though the grounds. A similar device uses air pressure, and yet, a slightly different method relies on vacuum. As noted above, the pod- or capsule devices are now common in homes and workplaces.

Nutritional benefits

Coffee contains several biologically active compounds, including caffeine, polyphenolics, and Maillard reaction products. That these and other compounds present in coffee could have health benefits has therefore attracted considerable research interest. In particular, based on many epidemiological studies, coffee consumption appears to consistently reduce the risk of several diseases (Box 15.8).

Box 15.8 Health properties of coffee

Coffee is one of the most commonly consumed beverages in the world (Cornelis, 2014). The coffee market has penetrated every continent and nearly every adult demographic group, regardless of age, social status, ethnicity, or religion. Collectively, several factors account for its popularity, including pleasant flavor and aroma, association with social activities, and of course, stimulation by caffeine (which is also addictive). More recently, other reasons have emerged (as if they were necessary) to justify the coffee habit. Specifically, coffee consumption has been associated with several health benefits, and mechanistic explanations have been proposed to support many of these benefits.

Much of the support for the beneficial effects of coffee has been based on epidemiological studies. In general, these epidemiology studies involve comparing disease rates among large populations with varying levels of coffee consumption. Depending on the health outcome or disease being studied, population groups (or cohorts) may be healthy adults or may already have an underlying condition. For example, the HAPIEE cohort study included more than 25,000 individuals from several European countries,

some with a history of cardiovascular disease (CVD), others were healthy (Grosso et al., 2016). The goal was to assess the impact of diet and lifestyle on CVD and other chronic diseases. Their results suggested that moderate coffee consumption (3–4 cups per day) decreases the risk of mortality and CVD. The effect was statistically even stronger when smoking status and alcohol intake was included. Other epidemiology studies, summarized in Table 15.8.1, have similarly revealed that coffee consumption is associated with several positive health outcomes, including reduced rates of certain cancers, obesity, and metabolic syndrome. In contrast, an increased risk of prostate and colon cancers is also associated with coffee consumption. In addition, several meta-analyses (where many studies are combined statistically) have concluded that regular coffee consumption is associated with lower risk of total mortality from all causes (Crippa et al., 2014; Je and Giovannucci, 2014; Zhao, et al., 2015).

It is important to note that while caffeine is the major biologically active compound in coffee, there are hundreds of other compounds that may also have biological activity

Table 15.8.1 Coffee and health: results of recent epidemiological studies.

Disease	Result	Reference
Melanoma	Coffee consumption was associated with reduced risk of melanoma, and ≥4 cups per day reduced risk by 20% compared to no coffee consumed.	Loftfield et al., 2014
Lung cancer	After controlling for smoking, no association was observed	Guertin et al., 2015
Obesity, metabolic syndrome and type 2 diabetes	High coffee intake was associated with reduced risks of all three diseases	Nordestgaard et al., 2015
Dementia	No association observed	Mizra et al., 2014
Colon cancer	Coffee consumption was associated with greater risk of colon cancer among men (but not women)	Yamada et al., 2014
Colorectal adenoma	High coffee consumption was associated with a reduced risk of colorectal adenoma	Budhathoki et al., 2015
Endometrial cancer	Coffee intake was inversely associated with endometrial cancer risk	Merritti et al., 2015
Coronary heart disease	Consumption of over 2 cups per day of Italian-style coffee was associated with increased risk	Grioni et al., 2015
Gastric cancer	Increased coffee consumption was associated with reduced of liver cancer	Ainslie-Waldman, 2014
Gall bladder cancer	Coffee consumption (>1 cup) reduced risk of gall bladder cancer in healthy adults	Larsson et al., 2017
Liver cancer	Daily coffee consumption reduced risk of gastric cancer in high-risk populations	Setiawan et al., 2015
Prostate cancer	Coffee consumption was associated with reduced prostate cancer risk	Liu et al., 2015
Bone fractures	Coffee consumption was not associated with increased risk of fractures in men	Hallström et al., 2014

(de Mejia and Ramirez-Mares, 2014; Ludwig et al., 2014). Among the other major constituents of coffee and their suggested bioactive mode of action are chlorogenic acids, trigonelline, melanoidins, kahweol and cafestol. However, because the concentrations of these other bioactive compounds vary depending on cultivar, growing conditions, and type of processing, ascribing health benefits to any one specific compound is not easy.

References

Ainslie-Waldman, C.E., W.-P. Koh, A. Jin, K.G. Yeoh, F. Zhu, R. Wang, J.-M. Yuan, and L.M. Butler. 2014. Coffee intake and gastric cancer risk: the Singapore Chinese Health Study. Cancer Epidemiol. Biomarkers Prev. 23:638–647.

Budhathoki, S., M. Iwasaki, T. Yamaji, S. Sasazuki, S. Tsugane. 2015. Coffee intake and the risk of colorectal adenoma: the colorectal adenoma study in Tokyo. Int. J. Cancer 137:1097-1115.

Crippa, A., A, Discacciati, S.C. Larsson, A. Wolk, and N. Orsini. 2014. Coffee consumption and mortality from all causes, cardiovascular disease, and cancer: a dose-response meta-analysis. Am. J. Epidemiol. 80:763–775.

Cornelis, M.C. 2014. Gene-coffee interactions and health. Curr. Nutr. Rep. 3:178–195.

de Mejia, E.G. and M.V. Ramirez-Mares. 2014. Impact of caffeine and coffee on our health. Trends Endocrinol. Metab. 25:489–492.

Grioni, S., C. Agnoli, S. Sieri, V. Pala, F. Ricceri, G. Masala, C. Saieva, S. Panico et al. 2015. Espresso coffee consumption and risk of coronary heart disease in a large Italian cohort. PLoS ONE 10(5): e0126550.

Grosso, G., U. Stepaniak, A. Micek, D. Stefler, M. Bobak, and A. Pajak. 2016. Coffee consumption and mortality in three Eastern European countries: results from the HAPIEE (Health, Alcohol and Psychosocial factors In Eastern Europe) study. Public Health Nutrition.

Guertin, K.A., N.D. Freedman, E. Loftfield, B.I. Graubard, N.E. Caporaso, and R. Sinha. 2015. Coffee consumption and incidence of lung cancer in the NIH-AARP Diet and Health Study. Int. J. Epidemiol. 45:929–939.

Hallström H., A. Wolk, A. Glynn, K. Michaëlsson, and L. Byberg. 2014. Coffee consumption and risk of fracture in the cohort of Swedish men (COSM). PLoS ONE 9(5): e97770.

Je, Y., and E. Giovannucci. 2014. Coffee consumption and total mortality: a meta-analysis of twenty prospective cohort studies. Br. J. Nutr. 111:1162–1173.

Larsson, S.C., E.L. Giovannucci, and A. Wolk. 2017. Coffee consumption and risk of gallbladder cancer in a prospective study. J. Natl. Cancer Inst. 109.

Liu, H., G.-H. Hu, X.-C. Wang, T.-B. Huang, L. Xu, P. Lai, Z.-F. Guo and Y.-F. Xu. 2015. Coffee consumption and prostate cancer risk: a meta-analysis of cohort studies. Nutr. Cancer 67:392–400.

Loftfield, E., N.D. Freedman, B.I. Graubard, A.R. Hollenbeck, F.M. Shebl, S.T. Mayne, and R. Sinha. 2015. Coffee drinking and cutaneous melanoma risk in the NIH-AARP diet and health study. JNCI J. Natl. Cancer Inst. 107:dju421.

Ludwig, I.A, M.N. Clifford, M.E.J. Lean, H. Ashihara, and A. Crozier. 2014. Coffee: biochemistry and potential impact on health. Food Funct. 5:1695–1717.

Merritt, M.A., I. Tzoulaki, S.S. Tworoger, I. De Vivo, S.E. Hankinson, J. Fernandes et al. 2015. Investigation of dietary factors and endometrial cancer risk using a nutrient-wide association study approach in the EPIC and Nurses' Health Study (NHS) and NHSII. Cancer Epidemiol. Biomarkers Prev. 24:466–471.

Mirza, S.S., H. Tiemeier, R.F. de Bruijn, A. Hofman, O.H. Franco, J. Kiefte-de Jong, P. J. Koudstaal, and M.A. Ikram. 2014. Coffee consumption and incident dementia. Eur. J. Epidemiol. 29:735–741.

Nordestgaard, A.T., M. Thomsen, and B.G. Nordestgaard. 2015. Coffee intake and risk of obesity, metabolic syndrome and type 2 diabetes: a Mendelian randomization study. Int. J. Epidemiol. 551–565.

Saab, S., D. Mallam, G.A. Cox II, and M.J. Tong. 2014. Impact of coffee on liver diseases: a systematic review. Liver Int. 34:495–504.
Setiawan, V.W., L.R. Wilkens, S.C. Lu, B.Y. Hernandez, L. Le Marchand, and B.E. Henderson. 2015. Association of coffee intake with reduced incidence of liver cancer and death from chronic liver disease in the US Multiethnic Cohort. Gastroenterology 148:118–125.
Yamada, .H, M. Kawado, N. Aoyama, S. Hashimoto, K. Suzuki, K. Wakai, S. Suzuki, Y. Watanabe, and A. Tamakoshi. 2014. Coffee consumption and risk of colorectal cancer: The Japan Collaborative Cohort Study. J. Epidemiol. 24:370–378.
Zhao, Y., K. Wu, J. Zheng, R. Zuo, and D. Li. 2015. Association of coffee drinking with all-cause mortality: a systematic review and meta-analysis. Public Health Nutr. 18:1282–1291.

CEREAL FERMENTATIONS

Many if not most of the fermented foods and beverages described in the previous chapters relied on very simple starting materials. Milk into yogurt and grapes into wine are good examples. Home-made fermented cereal gruels and porridges also fit into this category. However, the main distinguishing feature of the latter products is that they have yet to be industrialized and are generally only made in the home or on a very small scale. Nonetheless, for the mainly African and Asian populations who consume fermented cereals, the functional, nutritional, and cultural importance of these products cannot be overstated.

Fermented cereal products have been consumed for thousands of years. They can be made from nearly every cereal grain, including barley, cassava, maize, millet, wheat, sorghum, and rice (Table 15.1). Legumes are also used. In many parts of Africa, they are food staples, fed to infants at weaning and during all stages of life. Despite the simple ingredients (grain and water) and few processing steps, a wide range of products can be produced. In general, their manufacture starts with sorting, steeping, milling, and sieving. The mixture may be boiled or not, followed by a natural fermentation. They can be consumed directly as gruels, cakes, or mixed with other ingredients. After fermentation, they can also be pasteurized by boiling.

Table 15.1 Fermented cereal products from Africa[1].

Product	Country	Substrate	Organisms[2]
Agidi	Nigeria	Maize/sorghum/millet	LAB, yeasts
Burukutu	Ghana	Sorghum	LAB, AAB, yeasts
Busa	Egypt	Rice/millet	LAB, yeasts
Bushera	Uganda	Sorghum/millet	LAB, yeasts
Doro	Zimbabwe	Millet	LAB, yeasts
Enjara	Ethiopa	Maize, sorghum	Yeasts
Fura	Nigeria	Maize, sorghum, wheat	LAB, Yeasts
Injera	Ethiopa	Maize, sorghum, wheat	Yeasts
Kunu-Zaki	Nigeria	Millet	LAB, yeasts
Nasha	Sudan	Sorghum	Yeasts
Ogi	West Africa	Maize, sorghum, millet	LAB, yeasts
Pito	Nigeria, Ghana	Sorghum, millet	LAB, yeasts

[1] Adapted from Galati et al., 2014 and Kumari et al., 2015.
[2] LAB = lactic acid bacteria; AAB = acetic acid bacteria.

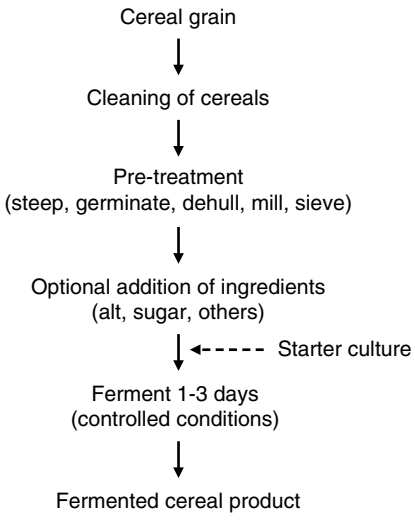


Figure 15.9 Production of fermented cereals, with or without addition of a starter culture. Adapted from Ogunremi et al., 2017, with permission.

The microbiota of fermented cereals consist mainly of lactic acid bacteria, especially species of *Pediococcus*, *Leuconostoc,* and *Lactobacillus*. Among the latter may be amylolytic species capable of hydrolyzing starch. Other bacteria, as well as yeasts are not uncommon. Typical end products include lactic acid, acetic acid, and small amounts of ethanol. The low pH (often less than 4.0) preserves these products and inhibits pathogens and other spoilage organisms.

Fermented cereal products provide calories, vitamins, and fiber. They also contribute live lactic acid bacteria that may have probiotic-like activity. In particular, in regions where serious diarrheal diseases are common, these foods may be protective for infants, children, and other vulnerable population. However, depending on the substrate, these products are usually low in lysine (an essential amino acid), and minerals may be unavailable. Therefore, while these foods have many healthy attributes, their use as a sole source of nutrition, especially for infants, must be carefully assessed.

Despite the long-standing, home-based manner in which fermented cereal products are made, the sensory quality, nutritional properties, and perhaps safety of these products often vary. Therefore, there is now interest in developing starter cultures for fermented cereals, in order to achieve more consistent quality and address consumer expectations. On strategy has been to identify lactic acid bacteria and yeast responsible for the natural fermentation and assess their metabolic and nutritional properties *in vitro*. Ultimately, suitable strains could then be used as "rational" cultures in industrial manufacture of these products (Figure 15.9).

BIBLIOGRAPHY

Achi, O.K., and M. Ukwuru. 2015. Cereal-based fermented foods of Africa as functional foods. Int J. Microbiol. Appl. 2:71–83.

Afoakwa, E.O. 2016. Chocolate Science and Technology. John Wiley, Oxford.

Aprotosoaie, A.C., S.V. Luca, A. Miron. 2016. Flavor chemistry of cocoa and cocoa products – an overview. Compr. Rev. Food Sci. Food Saf. 15:73–91.

Beckett, S.T. 2008. The Science of Chocolate, 2nd edn. RSC Publishing, Cambridge.
Bhattacharjee, R. and P.L. Kumar. 2007. Cacao. In: Genome Mapping and Molecular Breeding in Plants, Vol. 6. Technical Crops, C. Kole, ed. Springer, New York.
Gross, M. 2014 Coffee and chocolate in danger. Curr. Biol. 24:R503–R506.
Franz, C.M.A.P., M. Huch, J.M. Mathara, H. Abriouel, N. Benomar, G. Reid, A. Galvez, and W.H. Holzapfel. 2014. African fermented foods and probiotics. Int. J. Food Microbiol. 190:84–96.
Galati A., F.A. Oguntoyinbo, G. Moschetti, M. Crescimanno, and L. Settani. 2014. The cereal market and the role of fermentation in cereal-based food production in Africa. Food Rev. Int. 30:317–337.
Huch, M. and C.M.A.P Franz. 2015. Coffee: fermentation and microbiota. In: Holzapfel, W., ed. Advances in Fermented Foods and Beverages: Improving Quality, Technologies and Health Benefits. Elsevier, Oxford.
Kumari, S., P. Guleria, and N. Dangi. 2015. Cereal based beverages and fermented foods: a review. Int. J. Enhanced Res. Sci. Technol. Eng. 4:134–145.
Lima, L.J.R., M.H. Almeida, M.J.R. Nout, and M.H. Zwietering. 2011. *Theobroma cacao* L., "the food of the gods": quality determinants of commercial cocoa beans, with particular reference to the impact of the fermentation. Crit. Rev. Food Sci. Nutr. 51:731–761.
Ludwig, I.A., M.N. Clifford, M.E.J. Lean, H. Ashihara, and A. Crozier. 2014. Coffee: biochemistry and potential impact on health. Food Funct. 5:1695–1717.
Mussatto, S.I., E.M.S. Machado, S. Martins, and J.A. Teixeira. 2011. Production, composition and application of coffee and its industrial residues. Food Bioproc. Technol. 4:661–672.
Nielsen, D.S., M. Crafack, L. Jespersen, and M. Jakobsen. 2013. The microbiology of cocoa fermentation. In: R.R. Watson, V.R. Preedy, and S. Zibadi, eds. Chocolate in Health and Nutrition. Springer, New York.
Ogunremi, O.R., K. Banwo, and A.I. Sanni. 2017. Starter-culture to improve the quality of cereal-based fermented foods: trends in selection and application. Curr. Opin. Food Sci. 13:38–43.
Ozturk, G. and G.M. Young. 2017. Food evolution: the impact of society and science on the fermentation of cocoa beans. Comp. Rev. Food Sci. Food Saf. 16:431-455.
Pereira, G.V.M., V.T. Soccol, S. Brar, E. Neto, and C.R. Soccol. 2017. Microbial ecology and starter culture technology in coffee processing. Crit. Rev. Food Sci. Nutr. 57:2775–2788.
Pereira, G.V.M., V.T. Soccol, and C.R. Soccol. 2016. Current state of research on cocoa and coffee fermentations. Curr. Opin. Sci. 7:50–57.
Silva, C.F., L.R. Batista, L.M. Abreu, E.S. Dias, and R.F. Schwan. 2008. Succession of bacterial and fungal communities during natural coffee (*Coffea arabica*) fermentation Food Microbiol. 25:951–957.
Wintgens, J.N. 2012. Coffee: Growing, Processing, Sustainable Production, 2nd edn. Wiley, Oxford.

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