Chapter 8

Dairy Products: Cheese and Yogurt

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Chapter Outline

I. INTRODUCTION

The production of cheese began thousands of years ago in the Middle East (Scott *et al.*, 1998). Cheese making was subsequently introduced into Europe during the period of the Roman Empire, where it was produced either in monasteries or on farms. Factory production of cheese began in the middle of the nineteenth century in both Europe and the New World, and beginning in the 1930s, many established varieties were defined by national standards of

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identity, such as the Appellation d'Origine Contrôlées (AOC) system in France, the Denominazione di Origine Controllata (DOC) in Italy, and the international European Community Protected Designation of Origin (PDO) standards. Standards of identity were strengthened internationally by the Stresa Convention in 1951, and later updated by European Community rules (Echols, 2008). The Center for Dairy Research, Wisconsin, online cheese directory lists more than 1400 varieties of cheese, many differing only in shape, size, degree of ripening, type of milk, condiments used, packaging, and/or region of production. Of the main commodity cheeses the most popular globally is Mozzarella, mainly due to its use on pizza.

In addition to cheese, there are a large number of fermented milk products differentiated by manufacturing processes and starter microorganisms. The main cultured product consumed in Australia, Canada, the UK, and the USA is yogurt, while other varieties of cultured milk and cream products are more popular in the Scandinavian and Eastern European countries. Figure 8.1 illustrates the principal types of fermented milk products. This chapter will discuss the manufacturing procedures, the cultures used, and the biochemical changes that take place during the production of yogurt and cheese. Figure 8.2 illustrates the principal ingredients and processes involved in cheese making.

II. MILK COMPOSITION

Cheese and yogurt depend on the growth of bacteria to produce acidity, flavor compounds, and ripening enzymes. Milk is a good source of nutrients, including carbon, nitrogen, and macrominerals; many micronutrients such as vitamins and microminerals are also available (Jenness, 1988). However, milk is unique with respect to its sugar; lactose is naturally present only in milk. Most microorganisms lack the enzyme lactase, which is required to break down lactose into its component sugars, glucose, and galactose. Lactic acid bacteria (LAB), which possess lactase, readily break down lactose and use glucose as an energy source. Furthermore, some LAB are able to convert galactose to glucose. LAB, therefore, have a competitive advantage in milk.

Milk composition data for the most common dairy species are given in Table 8.1. Cheese- and yogurt-making principles are similar for milk of all species, with some modifications required to account for the higher milk solids in species such as buffalo and sheep and for differences in the properties of caseins and enzymes, among other factors. For example, goat's milk has smaller fat globules, which allows higher fat recovery (McSweeney *et al.*, 1993b), and relative to cow's milk, the milk of most goat breeds is low in α_{s1}-casein (Moatsou *et al.*, 2004). The practical effect is that goat's milk is more suitable than cow's milk for some varieties such as Feta, but less suitable for other varieties such as Cheddar, in which flavor development is strongly dependent on the breakdown of α_{s1}-casein by rennet.

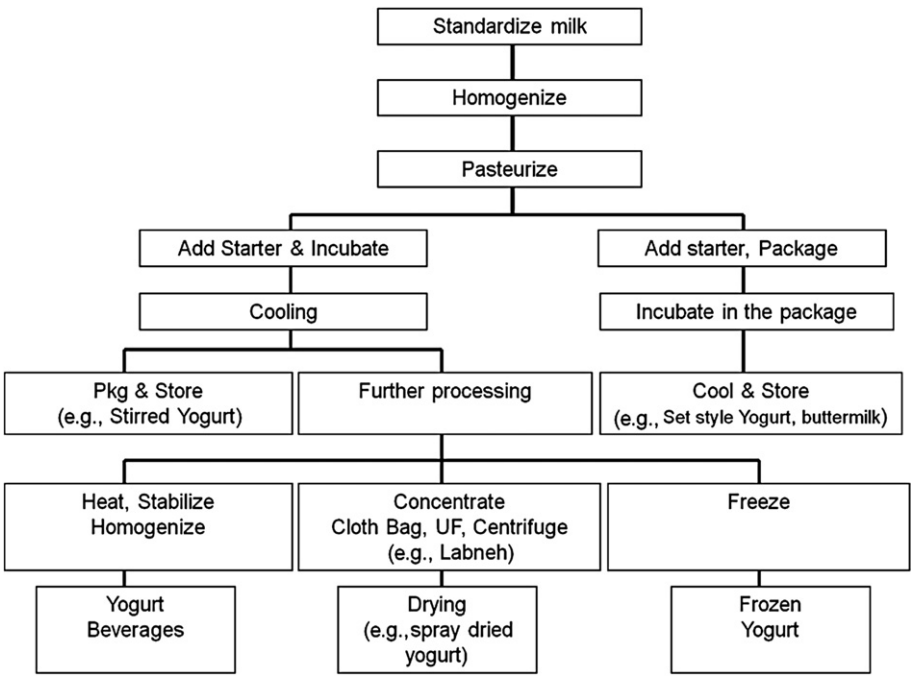

FIGURE 8.1 Principal processes used to differentiate milk into fermented dairy products. *(Adapted from Robinson* et al., *2002.)*

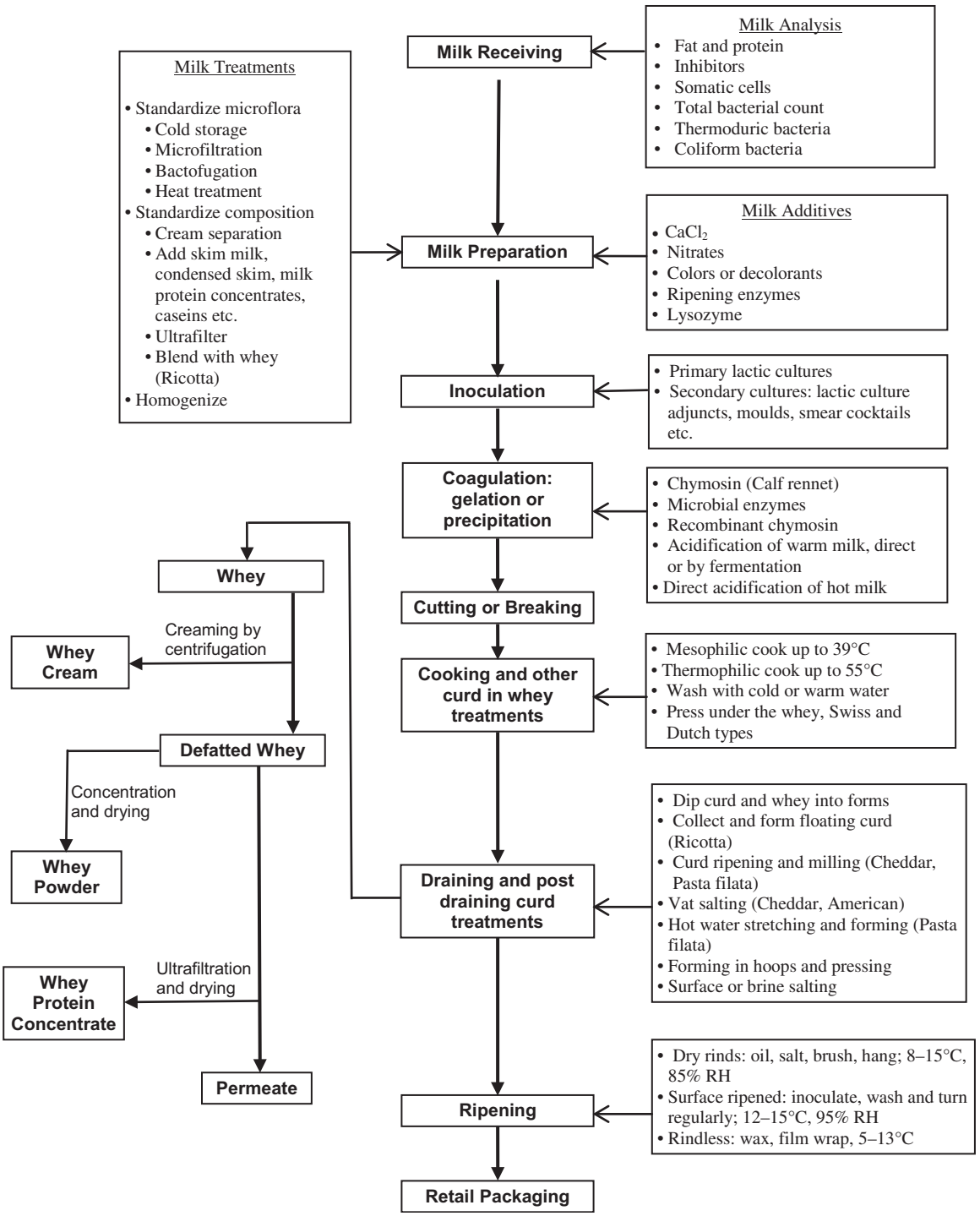

FIGURE 8.2 Flowchart of cheese-making processes.

Buffalo's milk has a higher buffering capacity than cow's milk, which means that the acidification process in the former has to be suitably modified (Ahmad *et al.*, 2008). Owing to differences in the composition of cow's and buffalo's milk, the changes to casein micelle and curd rheology during acidification were found to be qualitatively similar but quantitatively different (Ahmad *et al.*, 2008; Hussain *et al.*, 2011).

There are also important within-species differences. Throughout modern history, farmers have selectively bred dairy cattle to increase milk production or fat content or both. More recently, genetic selection has focused on other

TABLE 8.1 Typical Gross Composition (kg/100 kg) of Cow, Sheep, Water Buffalo, and Goat Milk

	Cow	Dairy Sheep	Water Buffalo	Goat
Fat	3.9	7.2	7.4	4.5
Total protein	3.2	4.6	3.8	3.2
Casein	2.6	3.9	3.2	2.6
Whey	0.6	0.7	0.6	0.6
Lactose	4.6	4.8	4.8	4.3
Ash	0.7	0.9	0.8	0.8
Total solids	12.4	17.5	16.83	12.8

Adapted from Wong *et al.* (1988).

milk properties, such as increasing the proportion of milk protein to fat (PF ratio). With respect to other solids, mineral content (mainly calcium, magnesium, and phosphorus) generally varies in proportion to protein content, and lactose content is relatively stable. Since lactose is largely a wasted component, increasing protein and fat by feed or genetic selection has economic advantages in terms of feed conversion, milk transportation costs, and waste handling.

Season is yet another factor that affects milk composition (Hill, 2011). In general, in the northern hemisphere, fat content reaches a minimum in August and a maximum in October. The protein content changes roughly in parallel with fat content, but because seasonal variations in protein are smaller, the result is a higher PF ratio during summer and a lower ratio in the winter. Casein number (casein as a percentage of total protein) is relatively constant over the seasons; that is, casein varies seasonally but mostly in proportion to the total protein. However, there is a significant positive correlation between total protein and casein number. The average value for casein number in Ontario, where Holstein Friesian is the dominant breed, was reported to be 77.0% ($N = 1067$, SD 1.07%, range 70.31−81.06%). Based on these data, casein can be predicted from total crude protein ($N \times 6.38$) as follows (Hill, unpublished):

$$\text{Casein} = (0.833 \times \text{total protein}) - 0.208$$

Milk fat is unique with respect to its diversity of fatty acids. As indicated in Table 8.2, some noteworthy lipid components include substantial amounts of short-chain fatty acids and rumenic acid (conjugated linoleic acid; C18:2, c9, t11). In its natural state, milk fat is found as globules surrounded by surface-active phospholipid membranes; the latter allow fat particles to remain dispersed in the aqueous milk medium. During the formation of milk gels, as described in Section IV, the fat globules occlude within the protein gel structure and mainly function as shortening agents that soften cheese texture. Short-chain fatty acids tend to be located in the *sn*1 or *sn*3 position (Villeneuve *et al.*, 1996) and for that reason are more subject to lipolysis with associated rancid flavor. (See also the review by Collins *et al.*, 2003a.)

The general aspects of milk proteins, which are briefly described below, have been reviewed by Fox (2003a). Apart from enzymes and minor proteins, the principal groups of proteins in milk are the caseins and whey proteins, making up roughly 80% and 20% of total lactoproteins, respectively. In addition to dispersed fat globules (1−10 μm diameter), milk's colloidal system contains casein−calcium phosphate particles called casein micelles (about 80−500 nm diameter) dispersed in a solution (serum) of lactose, whey proteins, and minerals. Bovine casein micelles are made up of four different casein fractions, the α_{s1}-, α_{s2}-, β-, and κ-caseins, in an approximate ratio of 4:1:3.5:1.5, respectively. The size of casein micelles, content of caseins, and relative proportions of individual caseins differ between species. The principal whey/serum proteins in bovine milk are β-lactoglobulin (≈ 50%), α-lactalbumin (≈ 20%), immunoglobulins (≈ 3%; up to 10% in colostrum), bovine serum albumin (0.3−1%), and some lactoferrin (< 0.1%). β-Lactoglobulin is the most abundant whey protein in the milk of most mammals; exceptions are humans, rats, mice, guinea-pigs, and camels, in which the protein is absent. α-Lactalbumin is present in the milk of all mammals. Each molecule of β-lactoglobulin contains two intramolecular disulfide bonds and one cysteine residue. The sulfhydryl group is especially important in heated milk chemistry; following thermal denaturation, it participates

TABLE 8.2 Fatty Acid Composition of Milks from Cow, Goat, and Sheep

		Composition (% w/w)					
		Cow[a]		Goat[b]		Sheep[c]	
Fatty Acid	Common Name	Typical	Range	Typical	Range	Typical	Range
C4:0	Butyric	3.9	3.1–4.4	2.2	2.0–2.4	3.5	3.1–3.9
C6:0	Caproic	2.5	1.8–2.7	2.4	2.0–2.7	2.9	2.7–3.4
C8:0	Caprylic	1.5	1.0–1.7	2.7	2.3–3.0	2.6	2.1–3.3
C10:0	Capric	3.2	2.2–3.8	10.0	8.9–11.0	7.8	5.5–9.7
C12:0	Lauric	3.6	2.6–4.2	5.0	3.9–6.2	4.4	3.5–4.9
C14:0	Myristic	11.1	9.1–11.9	9.8	7.7–11.2	10.4	9.9–10.7
C14:1	Myristoleic	0.8	0.5–1.1	0.18	0.17–0.2	0.3	0.2–0.5
C15:0		1.2	0.9–1.4	0.7	0.5–0.9	1.0	0.9–1.1
C16:0	Palmitic	27.9	23.6–31.4	28.2	23.2–34.8	26.0	22.5–28.2
C16:1	Palmitoleic	1.5	1.4–2.0	1.6	1.0–2.7	1.0	0.7–1.3
C18:0	Stearic	12.2	10.4–14.6	8.9	5.8–13.2	9.6	8.5–11.0
C18:1 *cis*	Oleic	17.2	14.9–22.0	–	–	–	–
C18:1 *trans*		3.9	–	–	–	–	–
C18:1 total		21.1	–	19.3	15.4–27.7	21.1	17.8–23.0
C18:2	Linoleic	1.4	1.2–1.7	3.2	2.5–4.3	3.2	2.9–3.6
C18:2 conj	Conjugated Linoleic	1.1	0.8–1.5	0.7	0.3–1.2	0.7	0.6–1.0
C18:3	α-Linolenic	1.0	0.9–1.2	0.4	0.2–0.9	0.8	0.5–1.0
	Minor acids	6.0	4.8–7.5	3.2	2.2–4.6	3.6	3.1–4.3

[a] *Creamer and MacGibbon (1996); MacGibbon (1996)*
[b] *Alonso* et al. *(1999)*
[c] *Goudjil* et al. *(2004)*.

in thiol/disulfide exchange reactions with the intermolecular disulfide of κ-casein. As discussed in Section IV, this reaction severely impairs rennet coagulation and cheese-making properties of heated milk, but is essential for yogurt making. Some properties of caseins and whey proteins are listed in Tables 8.3 and 8.4, respectively, and will be discussed in the relevant sections; the structural features of the casein micelle and the mechanisms of its destabilization in cheese and yogurt making are briefly described in Section IV.

III. MILK QUALITY

Milk quality has a great influence on the quality of the resulting cheese and yogurt products. Here, some biochemical attributes of milk quality that are associated with the quality and safety of cheese and yogurt are described.

A. Types of Microorganisms and their Activity in Milk

Over a period of 1–4 days, warm raw milk (25–40°C) is first fermented by LAB to pH 4.8–4.2 depending on the acid tolerance of the LAB present and the rate of growth of other species (Figure 8.3). As the LAB approach their stationary growth phase, acid-tolerant yeasts and molds begin to utilize the lactic acid that is produced and also metabolize proteins, causing the pH to rise. The released peptides and amino acids further encourage the growth of LAB until they use up most of the lactose. When the lactose is gone, the pH begins to rise, which causes proteolytic

TABLE 8.3 Principal Caseins and Some Properties of Importance to Cheese Making

Name	Symbol	% of Casein	Properties
Alpha-s1 casein	α_{s1}	40	Binds Ca^{2+} strongly
			Readily hydrolyzed by chymosin during cheese ripening
			Less susceptible to natural milk protease, plasmin
Alpha-s2 casein	α_{s2}	10	Binds Ca^{2+} strongly
			Limited susceptibility to chymosin action
			Eight peptide bonds are susceptible to plasmin
Beta-casein	β	35	Binds Ca^{2+} weakly
			Partially soluble in cold milk
			Broken down by plasmin but not chymosin
			Peptide β-CN (f193−209) and related fragments are very bitter
Kappa-casein	κ	15	Does not bind calcium
			Stabilizes casein micelles; its hydrolysis by chymosin initiates milk coagulation
			Covalently binds to whey proteins when milk is heated
			Para-κ-casein is not broken down by chymosin or plasmin

bacteria to proliferate, and along with yeasts and molds they elevate the pH to neutral or slightly alkaline values. Physical changes at these respective stages include coagulation (pH 5.0−4.6) and increased translucence due to proteolytic breakdown and resulting dissociation of casein micelles. In the manufacture of dairy products, these natural biochemical/physical processes are directed to specified ends. The following subsections describe several types of bacteria that are typically present in raw milk, categorized according to how they change the properties of milk. Often these changes are negative (spoilage), but many of these bacteria, along with contaminants transferred to the milk and/or cheese during manufacture, are also important adjuncts to primary and secondary cultures, particularly in aged cheese varieties.

TABLE 8.4 The Principal Whey Proteins: Some Properties of Importance to Cheese Making

Name	% of Whey Protein	Properties
β-Lactoglobulin	40	Its heat-induced (> 70°C) binding with κ-casein interferes with rennet coagulation
		Principal component of Ricotta cheese
α-Lactalbumin	15	Principal protein of breast milk
		Used in infant formulae
Immunoglobulins	6	Present in high proportions in colostrum
Other heat-sensitive whey proteins	4.0	Mainly includes bovine serum albumin
Heat-stable whey proteins	14	Cannot be recovered by heat−acid precipitation as in Ricotta cheese manufacture
Non-protein nitrogen	21	Consists of amino acids, ammonia, urea, and small peptides

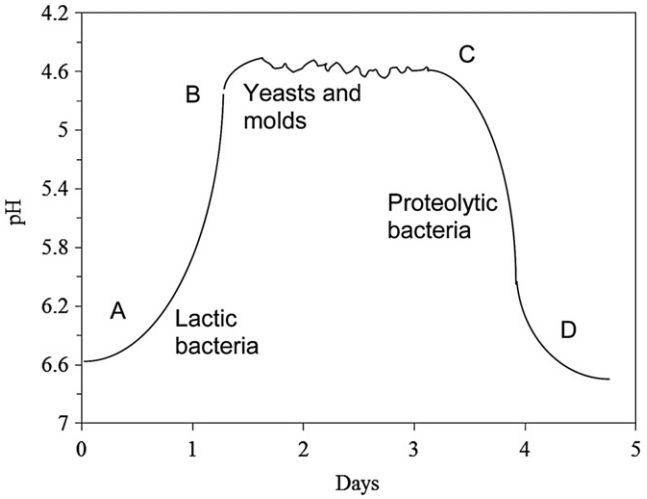

FIGURE 8.3 Natural fermentation of raw milk. A−B: At the natural pH of milk (6.6−6.8) and at temperatures > 20°C, lactic acid bacteria (LAB) rapidly ferment lactose to lactic acid. The lactic acid that is produced lowers pH and inhibits most bacteria, eventually even LAB. B−C: Acid-tolerant yeasts and molds begin to grow and utilize lactic acid, which permits further growth of LAB. This synergistic relation continues until all of the lactose is exhausted. C−D: Eventually, yeasts and molds are joined by proteolytic bacteria. Together they consume lactic acid and/or neutralize it with protein breakdown products. Note that all ripened cheese follows this pattern to a greater or lesser extent depending on the minimum pH reached during manufacture and the highest pH during ripening. The 'textbook' example of this pattern is Camembert cheese, which has a minimum pH of < 4.7 about 24 hours after inoculation and a ripened pH of about 7.5.

1. Lactic Acid Bacteria

LAB are: (1) non-motile, Gram positive, and catalase negative, so simple tests for catalase activity can be used to identify spoilage bacteria in lactic cultures, microaerophilic, or facultative anaerobes, which means that they create and thrive in anaerobic conditions in fermented dairy products; (2) mesophilic or thermophilic, which means that cold storage depletes their numbers and encourages the growth of psychrotrophic spoilage bacteria; and (3) with respect to morphology, cocci (spherical cells) 1 μm in diameter, or rod-shaped cells 1 μm wide and 2−3 μm long. As noted above, LAB have competitive advantages in milk because they are lactase positive, readily able to convert lactose into glucose and galactose. However, LAB prefer temperatures greater than 30°C, so depending on their initial counts psychrotrophic bacteria, including some coliforms and pseudomonads, may outgrow LAB at room temperatures. Lactic cultures are discussed further in Section V.

2. Psychrotrophic Bacteria

Psychrotrophic bacteria grow at less than 7°C. Common species in cold stored milk, which is the storage norm in most jurisdictions, are *Micrococcus*, *Bacillus*, *Staphylococcus*, *Pseudomonas*, *Flavobacterium*, and coliforms. *Pseudomonas* spp. are the most common and typically have the greatest impact on quality. *Pseudomonas fluorescens* is able to grow slowly at near 2°C and, along with other psychrotrophic species of this genus, it produces heat-stable lipases and proteases that remain active even after the bacterial cells have been killed by pasteurization (Lelievre *et al.*, 1978; Law *et al.*, 1979; Hicks *et al.*, 1982; Ellis and Marth, 1984; Cromie, 1992). Elaboration of heat-stable enzymes is maximal in the stationary phase, which most often occurs during the growth of *Pseudomonas* spp. in dirty equipment.

3. Gas Formers

Gas-producing microorganisms such as yeasts, propionibacteria (Todesco *et al.*, 2000), and heterofermentative LAB (Laleye *et al.*, 1987) cause undesirable openness in cheese. The floating curd defect in cottage cheese is also due to gassy LAB. The early gas defect and barney flavor in cheese can be caused by coliform bacteria such as *Enterobacter aerogenes* (Abo-Elnaga, 1971; Nieuwoudt and Bester, 1975; Bester, 1976; Melilli *et al.*, 2004), and late gas defect in Dutch and Swiss type cheese is usually due to *Clostridium tyrobutyricum* (Nieuwoudt and Bester, 1975).

Yeasts occur spontaneously in milk and are common contaminants during the cheese-making process. Yeasts commonly associated with dairy products include *Kluyveromyces lactis*, *Saccharomyces cerevisiae*, *Pichia anomala*,

and *Debaryomyces hansenii* (Klein *et al.*, 2002). Some of their undesirable effects are the formation of yeast slits or holes, development of fruity flavor in many cheese varieties, and browning on and under the rind in white and blue mold-ripened cheeses (Nichol *et al.*, 1996). On the positive side, yeasts contribute peptidases (Klein *et al.*, 2002), which assist in interior ripening. In bloomy cheeses, yeasts importantly reduce acidity, encouraging the growth of the white mold *Penicillium candidum* or *P. camemberti*. In smear-ripened (washed rind) cheeses, yeasts not only reduce the acidity, encouraging growth of *Brevibacterium linens*, but also contribute peptidases to the ripening process (Bockelmann and Hoppe, 2001). They also influence the hue and intensity of color development by *B. linens*. Furthermore, yeasts are the dominant flora in some smear cheeses such as Portuguese sera cheese (Macedo *et al.*, 1993).

Coliform bacteria are usually present in raw milk and have been traditionally used as indicators of sanitation or good manufacturing practice (GMP). Alternative GMP markers are total counts for the family Enterobacteriaceae, which includes enteric pathogens commonly associated with milk such as pathogenic serotypes of *Escherichia coli* 0157:H7 and various *Salmonella* spp. With the notable exception of *E. coli* 0157:H7, which can survive in acidic milk products, most bacteria in this group do not tolerate low pH and compete poorly with LAB, so their numbers decrease in the presence of a growing lactic culture (Bester, 1976). Poor sanitation combined with poor acid development, however, can result in excessive early gas (spongy cheese) with associated barney flavors. Early gas can also be caused by *Propionibacterium* spp. (Todesco *et al.*, 2000) and heterofermentative LAB.

Clostridium tyrobutyricum is a thermoduric (survives pasteurization) spore-forming organism of legendary fame among cheese makers. It causes gas formation (carbon dioxide) during the later stages of ripening of Swiss- and Dutch-type cheeses, and sometimes in others such as hard Italian varieties. The resulting craters and cracks in the cheese are termed 'late gas defect'. European cheese makers frequently check raw milk for thermoduric and/or spore-forming bacteria. Five hundred spores per liter of milk are sufficient to cause late gas defect. Control strategies include: (1) added egg lysozyme, which requires an egg allergy declaration in many jurisdictions (see summary in Fox, 1993); (2) culture adjuncts that at least partly inhibit *Clostridium* spp.; these are commercially available and research in this area is continuing (Christiansen *et al.*, 2005; Martínez-Cuesta *et al.*, 2010); (3) spore removal by bactofugation or microfiltration; (4) addition of nitrate salts, the traditional remedy, which is falling out of favor owing to subsequent formation of nitrosamines when cheese is heated and a general consumer distaste for additives; and (5) for hard Italian cheeses, preripening at cool temperatures (Spolaor and Marangon, 1997).

4. Pathogenic Bacteria

This summary of pathogenic bacteria associated with fermented dairy products is adapted from Hill and Warriner (2011a, b). *Listeria monocytogenes*, *Salmonella* species, and enteropathogenic *E. coli* represent the greatest concern for cheese makers (Johnson *et al.*, 1990a–c). With respect to yogurt and most fermented dairy beverages, such as buttermilk, the risk associated with pathogenic bacteria is greatly reduced by high heat treatment, which is normally well in excess of required pasteurization levels, and closed processing systems that largely mitigate the risk associated with postpasteurization contamination. Sources of contamination in raw milk are shown in Figure 8.4.

Listeria monocytogenes probably represents the highest risk associated with cheese, because both the severity and probability of listeriosis are high relative to other cheese-borne diseases. On the severity side of the equation, the death rate associated with listeriosis is about 30%, and for those who survive, there may be serious complications such as spontaneous abortion. On the probability side of the equation, the rate of incidence of *L. monocytogenes* in raw milk is relatively high; its thermal tolerance is close to pasteurization (72°C, 16 seconds); it survives, if not thrives, under the conditions of cheese making; and it is able to grow at refrigeration temperatures (i.e. cheese ripening and storage temperatures). Similarly, *Salmonella* species and *E. coli* O157:H7 are relatively high risk in cheese because they are frequently present in raw milk, grow at, or at least tolerate, the cold temperatures and acidity of cheese and other fermented milk products, and may persist for several months during ripening.

Staphylococcus aureus is a frequent biological contaminant in cheese, originating from raw milk or from personnel, but is listed as low risk because both growth and toxin production are readily suppressed by competing lactic cultures and cheese acidity (Johnson *et al.*, 1990a, b). Similarly, *Campylobacter jejuni* is widespread in the environment and occurs in raw milk, but does not normally survive the cheese-making conditions. So, it is considered low risk for cheese making provided that either heat treatment or fermentation (reduced pH) is included in the manufacturing process. *Yersinia enterocolitica* apparently tolerates cheese-making conditions; however, recent studies suggest that virulent strains, especially in North America, do not occur frequently in milk (Kushal and Anand, 2001, 2006).

Bacillus cereus is a nuisance because it forms difficult-to-remove bacterial films on milk processing equipment, survives pasteurization, and grows well at refrigeration temperatures. However, it is considered low risk because

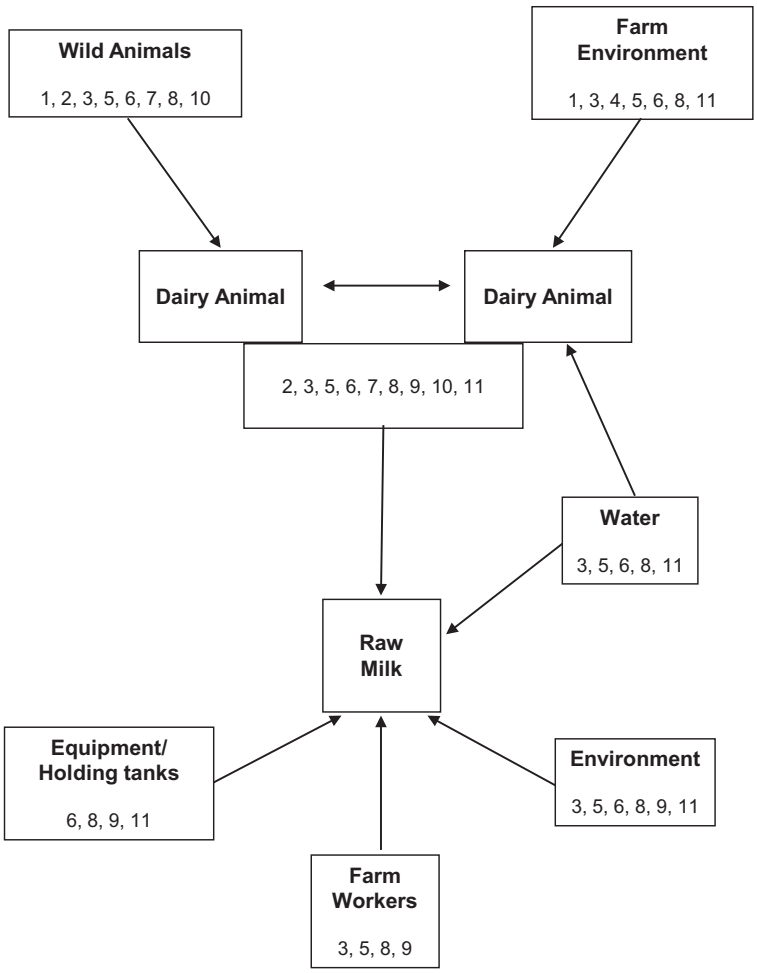

FIGURE 8.4 Sources of contamination in raw milk. 1: *Bacillus* spp.; 2: *Brucella*; 3: *Campylobacter jejuni*; 4: *Clostridium botulinum*; 5: *Escherichia coli* O157:H7; 6: *Listeria monocytogenes*; 7: *Mycobacterium tuberculosis*; 8: *Salmonella*; 9: *Staphylococcus aureus*; 10: *Coxiella burnetii* (Q fever); 11: *Yersinia enterocolitica*. *(Courtesy of K. Warriner, University of Guelph, Canada.)*

production of its diarrheal toxin in milk is associated with obvious spoilage and cell counts typically greater than 10 million/ml (Griffiths, 1990; Sutherland and Limond, 1993; Sutherland, 1993). It is also interesting that pasteurization of milk encourages germination of *B. cereus* spores, so its numbers may be higher in pasteurized milk than in raw milk (Stewart, 1975; Ahmed *et al.*, 1983).

In summary, it is critical to approach all raw milk processing with the assumption that pathogenic bacteria are present and must be eliminated or reduced to safe levels. Considering the average incidence rates in various reports, it seems likely that greater than 10% of cow's milk is contaminated with one or more pathogenic bacteria before it leaves the farm. A South Dakota study found that 26% of bulk tank samples were contaminated with one or more of *E. coli* O157:H7, *L. monocytogenes*, *Salmonella* spp., or *C. jejuni* (Jayarao and Henning, 2001). In Tennessee, 25% of bulk tanks were contaminated with one or more of *L. monocytogenes*, *C. jejuni*, *Y. enterocolitica*, or *Salmonella* spp. (Rohrbach *et al.*, 1992). The pooling process during milk transport and storage ensures that the proportion of contaminated milk reaching the cheese vat is usually much higher, and new rapid and more accurate molecular detection and quantification techniques will certainly increase the estimates of incidence rates. Furthermore, some of the traditional pathogens that provided the initial motivation for milk pasteurization, such as *Corynebacterium diphtheriae* and *Coxiella burnetii*, still occur in raw milk [EFSA Panel on Animal Health and Welfare (AHAW), 2010].

B. Antibiotics

Lactic cultures are very sensitive to the antibiotics used on dairy farms for therapeutic and prophylactic treatment of various diseases, especially mastitis. Common antibiotics used on dairy farms include β-lactams (penicillin), sulfonamides, streptomycin, tetracycline, and amphenicols. In most jurisdictions, increasing penalties have greatly

reduced antibiotic residues in milk. Nevertheless, antibiotic testing of cheese milk before unloading raw milk for bulk milk trucks is still commonly done. In many jurisdictions one of several available growth inhibition assays is still used as the official test, usually based on the inhibition of *Bacillus stearothermophilus*. These tests have the advantage of a broad detection range but are non-specific, slow, and not very sensitive. Commonly used rapid and more sensitive test kits include: (1) the Penzyme Test Kit for β-lactam antibiotics, based on the binding of penicillin by DD-carboxypeptidase; (2) Charm II tests, which test a wide range of antibiotics based on *B. stearothermophilus*, which contains natural receptor sites for antibiotics on or within its cells; the radioactive labeled (^{14}C or ^{3}H) antibiotics are displaced by antibiotics present in milk; and (3) immunoassays, enzyme-linked assays, or enzyme-linked immunosorbent assays. High-performance liquid chromatography is typically used as the reference method for other assays. Several recent reviews on antibiotic testing are available on sulfonamides (Wang *et al.*, 2006; Zhang and Wang, 2009), β-lactams (Cui *et al.*, 2007; Samanidou *et al.*, 2007), tetracycline (Pastor-Navarro *et al.*, 2009), chloramphenicols (Santos and Ramos, 2006), and also an overview of veterinary residues in milk (Nag, 2010).

C. Mastitic Milk

Mastitis is an infection of the cow's udder that has negative impacts on milk quality. Pooling milk dilutes the effect of single infected cows and herds, but in most jurisdictions the cumulative effect of mastitis, especially subclinical mastitis, is significant. Olson, as cited in Eck and Gillis (2000), estimates a 1% loss in cheese yield if 10% of the milk is from cows with subclinical mastitis. Furthermore, as noted below, the quality effects of mastitic milk are probably of more economic importance than the yield effects. Causative organisms include human pathogens such as *E. coli* and *S. aureus*. Non-bacterial infections such as by *Prototheca* also cause high somatic cell counts (SCCs) and are associated with high bacterial counts; see University of California extension report (Kirk and Mellenberger, 2011).

Mastitis is indicated by an increased number of somatic cells in milk. SCC greater than 100,000/ml in cows and about one million in goats (Rupp *et al.*, 2011) indicates clinical or subclinical mastitis. The association of SCC with milk quality in goat's milk is not clear. In cow's milk, SCC of less than 500,000 cells/ml, perhaps as low as 100,000, is associated with reduced cheese yield and reduced quality of fluid milk and other dairy products (Barbano *et al.*, 1991; Klei *et al.*, 1998; Ma *et al.*, 2000; Santos *et al.*, 2003). When counts exceed 1,000,000 cells/ml, altered milk composition and reduced cheese yields are obvious. Principal changes include increased activity of plasmin (an alkaline protease indigenous to milk) and plasminogen, accelerated protein breakdown, elevated milk pH, increased mineral content, reduced casein content, and higher levels of immunoglobulins.

IV. CHEMISTRY OF MILK COAGULATION

The essential step in the making of cheese and yogurt is to induce a clotting reaction in milk, primarily involving the casein proteins. In most cheeses, coagulation of milk is brought about by an enzymatic method, i.e. by the addition of rennet. For thousands of years rennets were extracted from the inner stomach lining of young calves. Chymosin, the aspartic proteinase contained in crude rennet extracts, is now largely produced by fermentation methods using genetically modified (GM) microbes. Yogurt and some cheese varieties are solely or predominantly acid coagulated products in which gelation of caseins, sometimes together with denatured whey proteins, is induced by slow acidification of milk by lactic cultures. Enzymatic and acid coagulation of milk are discussed in separate sections below.

A. Enzymatic Coagulation of Milk

Casein proteins naturally exist in milk as highly hydrated supramolecular colloidal aggregates, the casein micelles, with an average diameter of about 200 nm. The structure and function of casein micelles have been extensively studied and reviewed (Dalgleish, 1998, 2007, 2011; Horne, 1998, 2002, 2006; Walstra, 1999; Holt *et al.*, 2003; de Kruif and Holt, 2003; Dalgleish *et al.*, 2004; Farrell *et al.*, 2006; Fox and Brodkorb, 2008). The casein micelle is made of several thousand individual casein molecules organized around nanodomains of inorganic calcium phosphate. Two-thirds of the calcium in milk exists as these nanoclusters of casein−calcium phosphate. Whereas the calcium-sensitive α_s- and β-caseins are assembled within the micelle's interior, the calcium-insensitive κ-casein largely resides on the micelle's surface. Micelle integrity is due to casein−calcium interactions, hydrogen bonding, and electrostatic and hydrophobic interactions. The κ-casein protein is the key to a stable casein colloidal system in milk; its negatively charged and flexible C-terminus (106−169) protrudes from the micelle's surface, forming

a highly hydrated 'hairy' layer that provides electrostatic and (especially) steric stability to the micelle (Holt and Dalgleish, 1986; Horne, 1986, 2002; Walstra, 1990; de Kruif, 1992). At low temperatures, the hydrophilic N-terminal of β-casein may also contribute to micelle stability against aggregation (Roefs *et al.*, 1990).

When rennet is added to milk, after a certain lag period, the milk rather abruptly begins to coagulate and becomes a three-dimensional gel. The primary phase is entirely proteolytic, in which chymosin hydrolyzes the C-terminal part of κ-casein. The hydrophilic peptide, referred to as caseinomacropeptide (CMP), diffuses away from the micelle (para-casein) into the serum phase of milk. Loss of the hairy layer causes micelles to become unstable and this initiates the secondary clotting phase, i.e. the aggregation of para-casein micelles into a three-dimensional gel. The chemistry of enzymatic coagulation of milk ends here, i.e. with the formation of the casein curd. However, in a subsequent phase, sometimes called the tertiary phase, the newly formed gel continues to become firmer and eventually begins to release whey (syneresis) (see reviews by Dalgleish, 1992, 1993; Hyslop, 2003; Horne and Banks, 2004).

The primary phase in the coagulation of milk by rennet can be approximated to a first order rate reaction (Dalgleish, 1992). The rate increases with enzyme concentration and temperature (so long as the enzyme is stable). The early part of the reaction, i.e. until about three-quarters of κ-casein is proteolyzed, was found to vary linearly with rennet concentration (van Hooydonk and Walstra, 1987; Fox *et al.*, 2000).

In the secondary phase, the destabilized micelles aggregate due to a loss of steric hindrance and reduced electrostatic repulsion ($\approx$ 50% decrease in zeta potential). The onset of clotting requires that at least 85−90% of κ-casein is proteolyzed (Dalgleish, 1979; Chaplin and Green, 1980). Using diffusing-wave spectroscopy, Sandra *et al.* (2007) monitored rennet gelation in undiluted milk at its natural pH. They suggested that partially renneted micelles continue to diffuse freely until there is a significant breakdown of κ-casein 'hairs' and only when $>$ 70% of the hairs have been cleaved do the micelles become restricted in motion (owing to decreased electrostatic repulsion), but they do not aggregate yet. The real onset of aggregation requires more extensive proteolysis ($\approx$ 90% CMP release) and a space-filling gel is formed only when about 95% of κ-casein has been cleaved. Several models have been proposed to explain the mechanism of micelle aggregation (Horne and Banks, 2004), but there is no clear consensus about the nature of attractive forces that drive the aggregation reaction; calcium bridges, hydrophobic interactions, and van der Waals' forces are likely to be involved. Aggregation of destabilized micelles is greatly inhibited in the absence of free Ca^{2+} and at temperatures below 15°C, but rennet can remain active (although greatly reduced) even at temperatures as low as 0°C. This temperature dependence of the clotting reaction suggests the role of hydrophobic interactions in para-casein aggregation.

The clotting of milk by rennet and the strength of the resulting gel are influenced by several factors. Optimum temperature and pH for rennet action and micelle aggregation are about 30°C and 6.5, respectively. Free Ca^{2+} is essential for the milk to clot and produce a firm gel; ionic calcium (as $CaCl_2$), up to 1 mM, is routinely added to cheese milk. Concentrations of ions such as Na^+ (as NaCl), content of casein, presence of homogenized fat, and denatured whey proteins are also important in the clotting process and influence the final texture of rennet gels. These factors have been discussed previously by several authors (Dalgleish, 1993; Green and Grandison, 1993; Hyslop, 2003; Horne and Banks, 2004; Janhøj and Qvist, 2010).

Heat treatment of milk is a routine practice in the dairy industry and is an essential step in the manufacture of yogurt and some soft cheeses. The classic casein/whey protein interaction that occurs when milk is heated above 75°C is the covalent binding (through thiol/disulfide exchange reactions) of denatured whey proteins with κ-casein on the micelle surfaces and the formation of soluble whey protein/κ-casein complexes in the serum (O'Connell and Fox, 2003). The heat-induced changes in casein micelle structure unfavorably alter its rennet functionality. Cheese produced from heated milk has poor texture and unacceptable organoleptic properties. The rennet curds are soggy and ragged in appearance, with poor stretching and melting properties (Singh and Waungana, 2001). Heat-treated milk is therefore seldom used in cheese making. It is now well established that the proteolytic action of rennet (rate of CMP release) is similar in unheated and heated milk (Vasbinder *et al.*, 2003; Anema *et al.*, 2007; Sandra and Dalgleish, 2007; Kethireddipalli *et al.*, 2010) and is therefore not the reason for heat-impaired rennet clotting. However, heating milk severely impairs the aggregation of renneted micelles and this is routinely attributed to the binding of denatured whey proteins to the casein micelles and to the heat-induced losses of ionic calcium. Kethireddipalli *et al.* (2010, 2011) demonstrated that the poor clotting ability of heated milk can be attributed not only to the heat-induced association of denatured whey proteins with micelle surfaces, but also to a further binding in the course of renneting, of the soluble fraction of whey protein/κ-casein complexes to the micelles. It was suggested also that the heat-induced binding of denatured whey proteins impairs clotting to a greater extent than similar levels of rennet-induced micellar binding of serum protein complexes, possibly because of the distinct ways of protein binding,

i.e. through covalent linkages in the former and by a largely hydrophobic interaction in the latter. In addition to heat-induced serum protein complexes, certain unknown serum components were implicated in clotting inhibition. Further, it was shown by these authors that decreased levels of ionic calcium in heated milk has no significant effect on clotting inhibition; heated milk samples, with or without restored ionic equilibrium, had similarly long clotting times and produced weak gels with comparable low elastic moduli. These new findings on heat-induced milk protein interactions may help researchers to develop novel cheese varieties from heat-impaired milk.

B. Acid Coagulation of Milk

Yogurt, fresh acid cheeses, and acidified milk drinks are popular fermented foods produced throughout the world. Commercial production of yogurt involves gradual acidification at 40−45°C of previously heat-treated milk (85°C, 10 minutes) by lactic cultures. Fermentation of lactose to lactic acid by the thermophilic starter bacteria, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, gradually causes the pH of the milk to drop from about 6.7 to below 5.0. Titration of negative charges on the surfaces of casein micelles collapses the κ-casein hairy layer, destabilizes the micelles, and induces clotting as the isoelectric point is approached (pH ≈ 4.6). As the pH is reduced, the colloidal calcium phosphate (CCP) is dissolved and this alters/weakens the micelle's internal structure. The chemistry of progressive acidification of milk can be observed in three distinct pH regions from pH 6.7 to 4.6 (Lucey, 2004a): (1) as pH is lowered from 6.7 to 6.0, there is a decrease in the net negative charge on the micelle surface and reduced electrostatic repulsions; CCP and micelle integrity are preserved; (2) a decrease in pH from 6.0 to 5.0 further neutralizes the micelle's surface and with the shrinkage/collapse of κ-casein hairs, micelles are sterically and electrostatically destabilized; CCP is completely dissolved when a pH around 5.0 is reached and this increases the internal flexibility of micelles (Donato *et al.*, 2007); and (3) at pH less than 5.0, as the destabilized micelles come closer, hydrophobic interactions come into play (Horne, 1998, 2001); gelation occurs at pH 4.9 in unheated milk, but at a higher pH (≈ 5.2) in heat-treated milk. Hydrophobic interactions are important in the initiation of clotting, but subsequent cooling/refrigeration causes the gels to swell, increasing the particle contact area, and thereby gel firmness/strength (storage modulus). Because hydrophobic interactions are weakened at low temperatures, this suggests that gel integrity may be due to other forces such as electrostatic and van der Waals' interactions. For reviews on acid gelation of milk, the reader may refer to Dalgleish *et al.* (2005), de Kruif *et al.* (1995), Lee and Lucey (2010), Lucey (2002, 2004a, b), Lucey *et al.* (1997), and van Vliet *et al.* (2004).

Heating milk (and subsequent cooling) prior to acidification is the key to producing yogurts with desired texture and consistency. Unheated milk forms very weak acid gels, probably because the interface between aggregating micelles is still dominated by κ-casein hairs, which although collapsed, could prevent attractive interactions owing to their hydrophilic tendency (Li and Dalgleish, 2006). In heated milk, the attachment of heat-denatured whey proteins to micelle surfaces and the presence of serum-dispersed whey protein/κ-casein particles facilitate extensive cross-linking of gels, greatly increase gel strength and stiffness, and reduce syneresis (Lucey *et al.*, 1998; Donato *et al.*, 2007). Heat-treated acid milk gels also set at a higher pH, around 5.2 compared to unheated milk gels (setting pH ≈ 4.9), mostly because of the higher isoelectric pH (≈ 5.3) of β-lactoglobulin than that of caseins (Lucey *et al.*, 1997; Guyomarc'h *et al.*, 2003).

In some fresh acid cheeses such as cottage cheese, quark, and fromage frais, a small amount of rennet may also be used as coagulant. Studies on milk gelation by simultaneous acidification and renneting have received greater attention during recent years (Lucey *et al.*, 2000; Gastaldi *et al.*, 2003; Li and Dalgleish, 2006; Cooper *et al.*, 2010; Tranchant *et al.*, 2001). This method produces gels that are firmer and also set earlier (at a higher pH) than pure acid gels. The enzymatic removal of less than 25% of the micellar hairy layer has a synergistic effect on micelle destabilization by acid and enzyme. As the pH drops, the charge at the micelle's surface decreases and the CMP layer collapses, and so the extent of κ-casein breakdown needed to cause aggregation becomes smaller, in contrast to the requirement of over 90% proteolysis in pure rennet gels. Or, the κ-casein that needs to be cleaved to produce a reactive 'hot spot' will be smaller (Li and Dalgleish, 2006). With weakened steric hindrances due to the collapse of κ-casein hairs, even a small gap in the surface layer will allow interaction between the inner surfaces of micelles. The casein aggregates that are formed are larger and have increased connectivity (Cooper *et al.*, 2010) and so the combination gels are stronger. Depending on the concentration of rennet used (10−25% proteolysis), combined gels set at pH values between 5.0 and 5.3, in contrast to the lower gelation pH (4.8−4.9) of acid gels (Li and Dalgleish, 2006; Cooper *et al.*, 2010). Addition of small amounts of rennet during acidification of preheated milk (≥ 85°C for 5 minutes) also has the effect of improving gel firmness and increasing the pH of the onset of gelation. The inter-micelle bonds in these gels are not entirely made up of cross-linked denatured whey proteins (as in acidified heated

milk), but the inner surfaces of the partially renneted micelles may also be involved (Li and Dalgleish, 2006). This principle is exploited in the commercial manufacture of thermo quark (Schulz-Collins and Senge, 2004).

Heat−acid coagulation, a third type of coagulation, is primarily acid induced, but no fermentation is involved and the acid is added to hot milk at temperatures in the range of 75−100°C. Some of the thermally labile whey proteins are coagulated along with the casein and recovered in the cheese. Although total protein recovery increases by only a small amount (about 2−5%, compared to rennet coagulation), yield increases are largely due to the higher water-holding capacity of the denatured whey proteins. Increasing milk protein content also increases the proportion (percent recovery) of protein in the cheese (Hill *et al.*, 1982). Acid coagulation at high temperatures requires less acidification, so the final cheese pH is in the range of 5.2−6.0. Due to the interaction of denatured whey proteins with caseins, the cheese is rendered unmeltable, but has excellent frying/cooking properties; examples of such cheeses are Ricotta and Indian Paneer.

V. CULTURES FOR CHEESE AND YOGURT

A. Functions of Cheese Cultures

Culture refers to prepared inocula of bacteria, yeasts, and/or molds that are directly added to milk and/or incorporated into the fermented dairy product at a later stage during its manufacture. Specific LAB are selected as primary acid formers and are referred to as primary cultures; other cultures including yeasts, molds, certain bacteria, and other LAB may be added as secondary or adjunct cultures for specific purposes (see Cogan *et al.*, 2007, for a review). In broad terms, cultures have five purposes in fermented milk products: (1) to develop acidity; (2) to develop typical flavors such as the diacetyl and acetaldehyde flavor of fermented milks and certain cheeses; (3) to promote ripening of cheese (Section VII); (4) to mitigate specific quality or safety concerns such as the late gas defect in cheese caused by *Clostridium tyrobutyricum* or a food hazard such as listeriosis; and (5) for health-promoting properties such as from probiotics.

McKay and Baldwin (1975) were the first to demonstrate that many properties that are of technological importance to cheese making are encoded on the plasmids of lactococci. As listed by Callanan and Ross (2004) in their review of starter culture genetics, plasmid-encoded properties include the ability to contribute enzymes necessary for casein hydrolysis and for the transport and metabolism of lactose and citrate, the ability to produce bacteriocins, and the ability to combat phages. The rapidly growing area of LAB genetics is beyond the scope of this chapter; a search of *Food Science and Technology Abstracts* for LAB genetics or genomics returned more than 1500 papers during 2006−2010. For example, a review by Broadbent and Steele (2007a) illustrates the importance of genomics research in understanding and directing flavor development in cheese.

B. Classification of Lactic Acid Cultures

Table 8.5 lists the Latin names of some common lactic cultures. Both homofermentative and heterofermentative cultures produce lactic acid as the principal metabolite. However, heterofermentative cultures also produce substantial amounts of carbon dioxide (CO_2) and certain flavor compounds such as diacetyl (the principal flavor note in sour cream and buttermilk). Gas formation by heterofermentative cultures creates the desired open texture in some cheese types such as Gouda and Havarti.

The other major divider is mesophilic versus thermophilic cultures. The optimum growth range for mesophilic cultures is 30−35°C; acid production is slow or absent at temperatures less than 20°C and growth is inhibited at temperatures greater than 39°C. In general, cheeses that do not employ high temperatures (> 40°C) at the cooking stage utilize mesophilic cultures. These varieties include Cheddar, soft ripened cheese, most fresh cheese, and most washed cheeses.

Thermophilic cultures prefer temperatures in the approximate range of 40−45°C, but can survive temperatures of 55°C or higher, as required in cooked cheese varieties like Swiss and Italian types. These cheeses are generally cooked at temperatures exceeding 45°C before the separation of curd and whey. Cell counts decrease rapidly at temperatures less than 20°C, so bulk thermophilic cultures cannot be chilled to extend their shelf-life. The two most common thermophilic mixed cultures are *Streptococcus thermophilus* with *Lactobacillus delbrueckii* subsp. *bulgaricus*, and *S. thermophilus* with *Lb. helveticus* (Table 8.5).

Thermophilic starters grow together in an associative or symbiotic relationship, in which the growth rate and acid production of the mixed cultures are faster than for either culture on its own (Carrasco *et al.*, 2005). The proteolytic

TABLE 8.5 Properties and Functions of Some Lactic Cultures

Culture	Properties and Functions
Mesophilic Cultures	
Lactococcus lactis ssp. *cremoris*; *Lactococcus lactis* ssp. *lactis*	As a blend, these two form the most common mesophilic and homofermentative culture used in many low-temperature cheese varieties including fresh cheese, Cheddars, American Colby types, Dutch varieties, soft-ripened varieties, and others
Leuconostoc mesenteroides ssp. *cremoris*; *Lactococcus lactis*	Heterofermentative cultures. Often mixed with citrate fermenting *L. lactis* ssp. *cremoris*/*lactis* for cultured butter and buttermilk, and for cheese with small holes such as Havarti and Gouda
Thermophilic Cultures	
Streptococcus thermophilus; *Lactobacillus delbrueckii* ssp. *bulgaricus* or *Lactobacillus helveticus* or *Lactobacillus delbrueckii* ssp. *lactis*	Commonly used coccus/rod blend for high-temperature cheese varieties. *Lb. helveticus* is galactose positive and helps to reduce browning in pizza cheese; also, being proteolytic, it can accelerate ripening in Cheddar cheese. *Lb. delbrueckii* ssp. *lactis* is an alternative to *L. helveticus* and *L. bulgaricus* where less acid is preferred, as in 'stabilized' Brie cheese, and some mild and probiotic yogurts

activity of rods produces amino acids and peptides that stimulate the growth of cocci, and cocci in turn produce formic acid, which is required by rods (Galesloot *et al.*, 1968; Veringa *et al.*, 1968; Higashio *et al.*, 1977; Kikuchi *et al.*, 1984; Suzuki *et al.*, 1986). Cocci also have higher temperature and lower acid tolerances, so the rod/cocci balance is also influenced by temperature and pH. For example, in cooked cheese, cocci dominate the initial growth when the temperature and pH are high after curd separation; then, as the temperature and pH decrease, the rods flourish. Also of technological significance are the differences in galactose fermentation: *Lb. helveticus* readily metabolizes galactose, whereas *Lb. delbrueckii* subsp. *bulgaricus* does not (Turner and Martley, 1983; Matzdorf *et al.*, 1994; Mukherjee and Hutkins, 1994; Hassan, 2000; Baskaran and Sivakumar, 2003). This is important because faster metabolism of galactose reduces Maillard browning, so pizza color, for example, can be modified by culture selection.

C. Secondary and Adjunct Cultures

There are two general approaches to directed specified technological outcomes using cultures. The first is to select or engineer primary cultures to be used not only for acidification, but also for additional specified functionalities. For example, a heterofermentative mesophilic culture can function as a primary acid former, as well as produce an open cheese structure and characteristic flavors. The second approach is to select and/or engineer other bioagents with specific functionality. Some of these functional agents are described below.

Large hole (eye) formation in Swiss cheese and its cousins is caused by *Propionibacterium freudenreichii* subsp. *shermanii*. The growth and gas production of this bacterium are induced by warming young cheese to 20−25°C for 1−2 weeks. Cheese permeability and elasticity are important and are determined by a range of factors including cheese composition and the extent of proteolysis (Grappin *et al.*, 1993; Ekinci and Gurel, 2008). Eye formation occurs when the rate of gas production exceeds its rate of permeation through cheese. If the cheese is not sufficiently elastic, cracks rather than eyes will form.

Bloomy (white) rinds are formed mainly by *Penicillium camemberti* Thom, which is an amalgamation of two closely related phenotypic forms, namely, *P. album* (grey−green color) and *P. caseiocolum* or *P. candidum* (white) (see review by Chamba and Irlinger, 2004). According to these authors, other white molds found on cheese include *P. thonii*, *P. nalglovensis*, and *P. verrucosum*. The characteristic mushroom flavor of bloomy varieties is due to production of 1-octen-3-ol (Spinnler and Gripon, 2004).

With the exception of so-called stabilized Brie, soft ripened cheeses such as Camembert, Brie, blue, and Feta are initially acidic, reaching a minimum pH of less than 4.8 (Table 8.6). Stabilized Brie typically reaches a minimum pH of 5.2. The lag phase of white mold is reduced by 'contaminating' or adding yeasts that consume lactic acid and

TABLE 8.6 Some Properties of Cheese Categorized According to Type of Coagulation and Procedures used for pH and Moisture Control[a] (Hill, 2007)

Category	Examples	Coagulation[b]	MNFS[c]	pH[d]	Calcium[e]	Ripening
1. Acid coagulated	Fresh: cottage, quark, cream. ripened: Valençay, Harzer	Predominantly acid coagulation at pH 4.6–4.9; rennet: 0–1200 IMCU/1000 liters of milk	72–80%; a_w 0.980–0.997; controlled by cooking and washing treatments	4.3–4.9; inhibition of culture by low pH, high temperature cooking, rapid cooling and/or washing	50–350	Normally consumed fresh; or mold and/or smear ripened
2. Heat–acid coagulated	Cooking cheeses: Paneer and Channa, Ricotta, Requeson; cream: Mascarpone	Whey proteins coprecipitate with caseins and inhibit melting	75–84%; a_w 0.975–0.997; increases with whey protein content, decreases with cooking after acidification	5.0–5.8; amount of acidulant added; 3–6% lactose in cheese due to absence of fermentation		Consumed fresh, unless hot packed, pickled, or packed in sugar syrup
3. Fresh: rennet coagulated	Hispanic white frying cheeses; Italian fresh cheese	Rennet+; little or no culture; cutting pH 6.4–6.6; milk may be salted before renneting	60–80%; controlled by cooking, stirring out, milling before draining, vat salting; syneresis often occurs in the package	5.8–6.6; little or no culture; high pH prevents melting		Consumed fresh; high pH limits shelf-life; Hispanic varieties may develop yeasty flavor
4. Soft ripened	Feta, Camembert, blue	Rennet+++; culture+++; ripening time+++; cutting pH 6.5–6.4	60–70%; a_w 0.940–0.980; syneresis induced by acid development and by salting	4.5–4.8; acid inhibition of culture, salting and cooling	400–600	2–8 weeks
5. Mesophilic washed	Gouda, Edam, Colby, Havarti, Montasio, and many others	Rennet++; culture−++; ripening time++; cutting pH 6.6–6.5	55–65%; a_w 0.950–0.970; controlled by cooking, temperature of wash water, rate of acid development, curd handling, salting treatments	4.8–5.2; washing step to remove lactose	500–700	2 weeks–12+ months
6. Mesophilic unwashed	Cheddar, Provolone	Rennet++; culture++; ripening time++; cutting pH 6.6–6.5	52–60%; a_w 0.940–0.960; controlled by cooking, curd ripening, rate of acid development, and salting	5.0–5.3; rate of acid development and moisture determines residual lactose; draining pH is critical	500–700	1–24+ months
7. Thermophilic	Swiss and hard Italian types	Rennet+; culture+; ripening+ or none; cutting pH > 6.6	39–52%; a_w 0.900–0.960; controlled by high-temperature cooking (52–55°C)	5.1–5.3; acidity and moisture determine residual lactose; draining pH is critical	600–800	1–24+ months

a_w : water activity.
[a] *Representative data from various sources are given to define broad ranges and trends only.*
[b] *+ symbols indicate amounts of rennet and culture and ripening time relative to other categories.*
[c] *Moisture in non-fat substance.*
[d] *Minimum pH reached during manufacture or in the first days of ripening 5 mM/kg non-fat solids.*
[e] *Calcium content in mM per Kg of nonfat cheese solids.*

reduce acidity. Proteolysis by white molds further reduces acidity, so the pH on the surface and interior ripened portions of bloomy cheese increases rapidly to 6.8−8.0. Combined with low calcium content and high moisture (Table 8.6), the increase in pH softens the casein matrix, creating the characteristic creamy texture of bloomy cheeses (Vassal *et al.*, 1986; Spinnler and Gripon, 2004). Increased pH also explains the relatively high incidence of food-borne illnesses associated with bloomy rinds, especially by *E. coli* O157:H7 and *Listeria monocytogenes*; these organisms survive the low initial pH and then multiply when the pH increases during ripening (Griffiths, 1989; El-Gazzar and Marth, 1991a; Ramsaran *et al.*, 1998).

Blue cheese is produced through interior veining by blue/green molds, mainly *Penicillium roqueforti*, and is usually of rennet-coagulated type. However, there are also some acid-coagulated varieties, mostly the Chevre type, which are surface ripened with blue mold. Blue molds produce a similar effect as described above for white molds (but to a lesser extent), i.e. they increase the pH and soften the texture. For surface-ripened Chevre-type cheese, proteolysis and increased surface pH tend to create a gelatinous layer under the rind.

Commercially available strains of *P. camemberti* and *P. roqueforti* vary in their lipolytic and proteolytic properties. β-Oxidation of free fatty acids during lipolysis produces methyl ketones and their secondary alcohols, some of which are important flavor notes (Collins *et al.*, 2004). Proteolysis includes all principal caseins. Some *P. roqueforti* strains are more lipolytic and produce the sharp rancid flavor notes typical of Danish blues; other proteolytic strains that are less lipolytic produce more mellow notes, such as in Stilton.

Smear/washed/mixed rinds are formed by complex mixtures of yeasts, various coryneform bacteria such as *Brevibacterium linens*, various species of *Micrococci* and *Staphylococci*, sometimes molds, and others (see review by Chamba and Irlinger, 2004). Similar to the bloomy varieties, the surface pH of smear/mixed rind varieties increases during ripening, but generally not to the same extent. There is evidence that *Listeria monocytogenes* is able to grow on some smear-ripened cheeses (El-Gazzar and Marth, 1991b; Farrag and Marth, 1992; Rudolf and Scherer, 2001). In a challenge study using 24 market cheeses (Genigeorgis *et al.*, 1991), *L. monocytogenes* was found to survive but did not grow on cheese surfaces with pH less than 5.5.

Ripening adjuncts include bacterial or yeast cultures added in addition to the regular lactic acid cultures, attenuated cultures that are not intended to grow but only to contribute their enzymes, and bacterial culture adjuncts, such as lactobacilli and pediococci, that are intended to grow during cheese ripening and also contribute enzymes. For example, in cooked cheese varieties, *Lb. bulgaricus* is the primary acid former, as well as a proteolytic ripening agent, but in mesophilic aged cheeses such as Cheddar, *Lb. bulgaricus* and sometimes other lactobacilli such as *Lb. casei* are mainly used as flavor adjuncts that contribute aminopeptidases (Simova and Beshkova, 2007; Slattery *et al.*, 2010). The search for ripening adjuncts is currently an active area of research, often employing non-starter bacteria and yeasts from traditional cheeses (see Section VII and reviews: Laleye *et al.*, 1990; Klein *et al.*, 2002; Broadbent and Steele, 2007b; Nieto-Arribas *et al.*, 2009; Law, 2010a; Milesi *et al.*, 2010; Morales *et al.*, 2010).

LAB bacteriocins are grouped as lantibiotics, small heat-stable non-lantibiotics, and large heat-stable bacteriocins (see summary by Parente and Cogan, 2004). Many lactococci produce bacteriocins which, when combined with other control factors, can help to control the growth of non-starter (Rea *et al.*, 2003) and pathogenic bacteria such as *L. monocytogenes* (Dal Bello *et al.*, 2010). In recent years this has been a very active area of investigation, with much effort focused on identifying new sources of bacteriocins associated with LAB and their potential application in various food products such as ready-to-eat meats. Examples include: (1) identification of bacteriocin-producing species of *Lactobacillus*, *Pediococcus*, and *Enterococcus* from various sources (Todorov, 2008, 2010; Tome *et al.*, 2009; Todorov *et al.*, 2010, 2011a−c); (2) use of nisin-producing lactococci strains to improve the safety of low-salt Domiati (Ayad, 2009); (3) description of the lantibiotic mutacin 1140 produced by *Streptococcus mutans* (Smith, 2002); (4) description of pentocin 31-1, an anti-listeria bacteriocin produced by *Lactobacillus pentosus* (Zhang *et al.*, 2009); (5) description of bacteriocins produced by *Lb. acidophilus* and their pH dependence (Soomro and Masud, 2008); (6) production of enterocin by *Enterococcus faecium* in Cheddar cheese (Rea *et al.*, 2003); (7) description of macedocin produced by *Streptococcus gallolyticus* subsp. *macedonicus* (Leroy and De Vuyst, 2010); (8) a 2010 PhD dissertation (Martin-Visscher, 2010) reporting that each of the four LAB bacteriocins (nisin, gallidermin, CclA, and enterocin 710C) killed one or more of *E. coli* DH5α, *Pseudomonas aeruginosa* ATCC 14207, and *Salmonella typhimurium* ATCC 23564; and (9) inhibition of late gas defect (*Clostridium beijerinckii* INIA 63) in Manchego cheese by nisin- and lacticin 481-producing *Lactococcus lactis* subsp. *lactis* INIA 415 (Garde *et al.*, 2011). Cultures imparting health functionalities will be discussed in Section VIII, B.

Exopolysaccharides produced by some non-starter and starter bacteria may be important health-promoting and texture-building agents in fermented milk products and particularly in improving the texture of low-fat cheese (see

reviews by Vlahopoulou *et al.*, 2001; Zisu and Shah, 2005; Hassan, 2008; Robitaille *et al.*, 2009; Welman, 2009; Costa *et al.*, 2010; Badel *et al.*, 2011).

D. Culture Management

Provided that milk is not chilled and that the cheese maker has lots of patience, it is possible to make fermented dairy products without additional cultures, but the normal practice is to add domesticated cultures for the manufacture of cheese from both pasteurized and raw milk. In terms of increasing direction or definition, cultures are selected and maintained in three general ways: natural starters, mixed strain starters, and defined strain starters.

1. Natural Cultures

The practice of using natural cultures based on traditional culture handling techniques has been preserved, particularly among artisan cheese makers, and in some cases by national and/or international cheese identity standards such as the AOC. For both primary LAB cultures and secondary cultures, the simplest type of natural handling technique is back-slopping, in which a small portion of a fermented product is used to inoculate a new batch. For example, finished yogurt can be used as the source of LAB culture for a new batch of yogurt, and bloomy cheese surfaces can be inoculated by placing the young cheese in the curing area with older cheese. Similarly, smear cultures for washed rind cheeses can be transferred via wooden curing shelves or hoops, or by washing young cheese with brine that was previously used to wash older cheese. Another example of a type of back-slopping is the Sicilian Ragusano cheese, in which the PDO requires milk fermentation in a wooden vat called a tina. Lortal *et al.* (2009) found that most of the interior surface of the vat is covered in a biofilm, a matrix of exopolysaccharides, which is the source of the primary starter.

Natural primary starters are prepared from both milk and whey. The various techniques were reviewed by Limsowtin *et al.* (1996) and summarized by Parente and Cogan (2004). For both milk and whey natural starters selective pressure is applied by heat treatment, incubation temperature, and incubation time. For example, Parente and Cogan (2004) reported that a traditional milk culture composed mainly of *S. thermophilus*, but also containing a number of other LAB, can be prepared by thermization (62−65°C) followed by incubation at 37−45°C. Similarly, natural whey-based culture for Parmigiano Reggiano and Grana Padano, composed mainly of aciduric *Lb. helveticus* with lower counts of other thermophilic LAB (*S. thermophilus*), is prepared by incubating fresh whey at 45°C until the pH reaches a low of 3.3. Another example is the AOC-prescribed whey starter (called Recuite) for Comté cheese. According to Dr. Sébastien Roustel (personal communication), dried calf intestines are heated in fresh whey at 54−55°C for 34 minutes and fermented at 42°C overnight. Vinegar may be added to encourage aciduric LAB. The LAB counts in whey are usually 10^{6-7}/ml after cheese manufacture and 10^{9-10}/ml in the Recuite after fermentation.

Natural cultures have the disadvantage of inconsistent performance and the advantage of inherent phage resistance, although phages may still cause a slow vat by attaching to dominant strains. Natural starters, including spontaneous starters (starters that grow spontaneously in uncooled raw milk), are also used as co-starters or adjunct starters. One example is AOC Gruyere, which must be made from a blend of evening and morning milk not cooled to less than 15°C. The spontaneous culture is then supplemented with mixed cultures. Another example is the common practice of subpasteurization heat treatment of Cheddar cheese milk to reduce pathogens and select for thermoduric pediococci and lactobacilli which contribute aminopeptidases and other enzymes.

Natural cultures are also rich sources of LAB genetics. Rapid identification techniques such as the randomly amplified polymorphic DNA (RAPD) technique make it possible to screen large numbers of samples. A 2011 survey of fermented yak's, goat's, sheep's, and cow's milk products in Mongolia, on the basis of 16s RNA analysis, identified a total of 664 strains and 10 species of LAB, of which *S. thermophilus* and *Lb. helveticus* were the most abundant species (Yu *et al.*, 2011). Similarly, Morales *et al.* (2010) identified and characterized the lipolytic and proteolytic properties of 10 halotolerant/halophilic LAB in Mexican Cotija and doble crema cheeses. See also characterization of LAB in artisanal goat cheese (Colombo *et al.*, 2010) and an artisanal Corsican cheese (Casalta *et al.*, 2009).

2. Mixed Strain and Defined Strain Starters

Primary starters prepared under controlled conditions are mixed strain starters (MSS) or defined strain starters (DSS) (for reviews, see Limsowtin *et al.*, 1996, 1997; Parente and Cogan, 2004). In brief, MSS cultures are prepared by

culturing from natural sources, but retain undefined strains that are diverse with respect to properties such as sugar metabolism, citrate metabolism, and bacteriocin production. With respect to phage resistance, MSS may contain both phage-resistant and lytic strains, and may harbor their own phages (Lodics and Steenson, 1993). So diversity provides some protection, but as with natural cultures, the principal strains may become susceptible. With respect to performance, strain ratios are constantly evolving, again similar to natural cultures, so properties such as rate of acid development and enzyme activities during ripening may not be consistent.

DSS are selected from mixed cultures by intensive subculturing to obtain more accurately directed and repeatable performance with respect to flavor and texture development. The original DSS starters in New Zealand were based on a single phage-resistant strain with daily rotations to prevent phage build-up. However, phages still occurred and the daily rotations were tedious. So, multiple strains were introduced. Current practices for DSS cultures rely less on rotation and more on improved phage resistance. Reduced rotation also improves consistency of performance.

In summary, it seems that the distinction between natural, MSS, and DSS cultures will become less important with the application of rapid techniques to isolate, accurately identify, and characterize functional properties of large numbers of microbial species and strains, and the opportunity to use that information to create complex but more defined culture blends that mimic natural cultures and more accurately account for other microbial fermentation and ripening agents. In other words, with increasing ability to intelligently target an overall balance of fermentative and ripening activities, culture strategies can become more holistic. A good example of this is the recent development of cultures for smear (washed rind) ripened cheese. It is well known that washed rinds may harbor *L. monocytogenes* (Rudolf and Scherer, 2001; Wagner *et al.*, 2006) and this is exacerbated by the common practice of using different types of back-slopping to transfer smear from old to young cheese. This has resulted in commercial cultures and ongoing research to prepare mixed or defined strain cultures to replace back-slopping (Bockelmann and Hoppe, 2001; Hannon *et al.*, 2004) or to otherwise mitigate the risk. A 2011 report that anti-listerial strains were isolated from the washed rind of raclette cheese (Roth *et al.*, 2011) suggests that it may be possible to improve safety by supplementing natural smear cultures with anti-listeria cultures. This could be quite important considering the large number of cheeses with PDO or otherwise specified natural smears and the growth of the artisan cheese industry in many countries.

3. Culture Handling in the Plant

Cultures are typically prepared for cheese milk and yogurt inoculation as bulk set cultures (one transfer and scale-up from the commercial culture) or direct-to-vat cultures (requiring no scale-up at the cheese plant). High cell counts for bulk set cultures can be obtained by internal pH control using buffers or by external pH control by titrating with sodium or ammonium hydroxide. Inclusion of phosphate salts in the culture media effectively prevents the proliferation of bacteriophages in bulk cultures (Suarez *et al.*, 2007). Industrial experience suggests that bulk mesophilic cultures can be cooled to near 4°C and stored for up to 7 days. Thermophilic cultures should not be cooled to less than 20°C and have a shelf-life of only about 24 hours.

4. Bacteriophages

Commercial experience suggests that bacteriophages continue to pose a significant challenge to cheese makers, especially for mesophilic cultures and for processing in open vats that are exposed to the environment. Common phages associated with dairy cultures are c2, 936, and p335 (Ahn *et al.*, 2009). Both lysogenic and lytic life cycles occur. With a short latent period of 30−50 minutes and a large burst size of 50−175, lytic phages can multiply quickly and destroy a culture within hours. Culture growth stops when phage levels reach 10^3−10^7/ml. Culture failure due to phage can be recognized by an initial normal acid development, followed by a decrease or termination of culture growth at a later stage. This is different from inhibition due to antibiotics, which is characterized by no or slow initial growth; if antibiotic inhibition is not severe, culture growth and acid development by resistant strains or mutants may resume with time.

Means to prevent culture infection with bacteriophage include: (1) culture rotation, which is effective because bacteriophages are highly (although not completely) strain specific; (2) general good hygiene, especially with respect to whey spillage (removing or pasteurizing whey immediately after separation from the curd); (3) routine tests for normal acid development, when the culture currently in use is grown in skimmed milk containing a little whey from the most recent vat; and (4) selection of LAB with inherent phage resistance. To this end, there is a great deal of

ongoing work to characterize LAB phages (see reviews by Limsowtin *et al.*, 1997; Martin *et al.*, 2008; Suarez *et al.*, 2008; Ahn *et al.*, 2009; Powell, 2010; Quiberoni *et al.*, 2010; Zinno *et al.*, 2010).

VI. CHEESE MANUFACTURE

The essential ingredients in cheese making are milk and a protein coagulant such as rennet and/or acid; the acid is normally produced by LAB. Rennet and acid alone, or in combination, cause milk proteins to aggregate and eventually transform fluid milk to a semi-firm gel (Section IV). When this gel is cut into small pieces (curds), the whey (mostly water and lactose) begins to separate from the curds. The curds are then differentiated by various cheese-making procedures to produce all of the many different cheese varieties. Exceptions to gelation are the heat-acid coagulated varieties (Table 8.6, Category 2) in which curd is recovered as falling or floating (precipitated) aggregates.

Properties of some representative cheese varieties are listed in Table 8.6, grouped into families according to basic manufacturing procedures. Detailed descriptions of the cheese families can be found in Hill (2007). Cheese recipes representing each of the families in Table 8.6 can also be found in Hill (2011). Using these categories, most cheese can be classified into technological groups, but the categories themselves cannot be applied rigidly. For example: (1) pasta filata varieties vary widely in composition, manufacturing techniques, and degree of ripening, so they do not fit well into any category; and (2) the manufacture of Cheshire types is similar to Cheddar up to the point of draining, but after that their high acid development is similar to Feta.

With respect to quality, the objectives of cheese making are: (1) to achieve an optimum composition with respect to moisture, acidity (pH), fat, protein, and minerals (especially calcium); (2) to establish the correct ultrastructure of cheese at the microscopic level; and (3) to ripen cheese to the desired flavor and texture. Objectives (1) and (2) are achieved by varying initial make procedures, and by doing so, objective (3) is achieved (Adda *et al.*, 1982; Green and Manning, 1982; Lawrence *et al.*, 1983, 1984). These variations in initial make procedures are mostly different means of controlling the rate and extent of acid development and the rate and extent of moisture release. Figure 8.2 is a flowchart illustrating the principal unit operations that may be applied to cheese making depending on the variety. A detailed discussion of these alternative operations is beyond the scope of this chapter; rather, the following paragraphs describe the principles that can be applied to control the most important cheese-making parameters, namely, pH history, moisture, calcium, texture, and flavor. Much of what is presented in this section is discussed in greater detail in Hill (2007).

A. Moisture Control

Cheese making is the process of removing moisture from a rennet or acid coagulum which is made of fat globules (unless the milk is skimmed) and water droplets trapped in a matrix of casein micelles. Cheese is, therefore, primarily a concentrate of milk protein (casein) and fat. The many cheese-making operations are directed to this process of removing water from the milk gel by inducing syneresis. Syneresis refers to the contraction of the protein network with resulting expulsion of water (whey) from the curd. Contraction is primarily due to increased hydrophobic interactions, which are strengthened by a rise in temperature up to a maximum at about 80°C. This means, for example, that thermophilic (cooked) cheese varieties have short make procedures because the higher cooking temperatures (up to 52°C) dry the curds quickly. Contraction is also encouraged by reduced electrostatic repulsion caused by decreasing the pH towards the isoelectric point of caseins (pH 4.6). The whey contains lactose, whey proteins, lactic acid, and some of the milk minerals. Moisture content, therefore, influences the final cheese pH because it determines the amount of residual/fermentable lactose in cheese; and the pH history, in turn, influences cheese moisture by affecting the rate and extent of syneresis. The final cheese moisture is also strongly influenced by packaging and ripening conditions (Section VII).

B. pH Control

Proper development of acidity, as indicated by the pH history, is the most important process control tool that determines cheese safety and quality. Critical process control points are pH at draining, the pH at salting for varieties that are salted before forming, the pH at demolding or at 24 hours after manufacturing, the minimum pH during 1−14 days after manufacture, and the pH of the ripened cheese. Some typical pH profiles are presented in Table 8.7. With respect to minimum pH, which is usually reached within 7 days of ripening, most rennet-coagulated varieties can be categorized into three groups: (1) fresh varieties with little or no acid development and a minimum pH $> 5.8-6.5$

TABLE 8.7 pH versus Time Profiles for Several Cheese Varieties (Hill, 2007)

	Swiss Type		Gouda		Cheddar MNFS 53%		Cheddar MNFS 57%		Feta		Cottage	
Operation	**Time**	**pH**	**Time**	**pH**	**Time**	**pH**	**Time**	**pH**	**Time**	**pH**	**Time**	**pH**
Add starter	0	6.60	0	6.60	0	6.60	0	6.60	0	6.60	0	6.60
Add rennet	15 min	6.60	35 min		60 min	6.55	30 min	6.55	75 min	6.50	60 min	6.50
Cut	45 min	6.55	70 min	6.45	90 min	6.50	75 min	6.50	115 min		300 min	4.80
Drain or dip into forms	150 min	6.35	100 min		210 min	6.20	195 min	6.3	130 min	NA	360 min	
Milling	NA	NA	NA	NA	360 min	5.40	315 min	5.45	NA	NA	NA	NA
Pressing	165 min	6.35	130 min		420 min	5.35	390 min	5.40	NA	NA	NA	NA
Demolding	16 h	5.30	8 h	5.40	24 h	5.20	10	5.20	24 h		NA	NA
Minimum pH	1 wk	5.20	1 wk	5.20	1 wk	5.10	1 wk	5.10	1 wk		NA	NA
Retail	6 mo	5.6	6 mo	5.6	24 mo	5.50	4 mo	5.3	6 wk	4.4	2−14 d	5.2

MNFS: moisture as a percentage of the non-fat solids in the cheese.

(Table 8.6, Category 3); (2) soft ripened varieties with minimum pH < 5.0 (Table 8.6, Category 4); and (3) varieties with minimum pH > 5.0 (Table 8.6, Categories 5−7).

Increased acidity induces syneresis (due to reduced charge repulsion between casein micelles), causes solubilization of CCP, and disrupts casein micelles (Section IV). This alters curd texture and results in reduced lactose levels in the curd due to fermentation (conversion to lactic acid) and syneresis (lactose removed with the whey). Acid development takes place mainly within the curd because most bacteria, following coagulation, are trapped in the gel matrix. The minimum pH value usually occurs within 3−14 days after manufacture and is dependent on: (1) the initial extent of acid development; (2) the amount of fermentable lactose that remains during early curing; and (3) the activity and concentration of ripening agents that utilize lactic acid and/or cause proteolysis. Also important for pH control are salting, which reduces the rate of acid development, and the ability of the culture to ferment galactose.

C. Calcium Control

More calcium is retained at a higher draining pH. For example, the calcium content of Swiss cheese (draining pH 6.3−6.5) is greater than that of Cheddar (draining pH 6.0−6.2). The important parameter is the ratio of total calcium to casein or calcium to non-fat solids (NFS), which is easier to measure (see Table 8.6). Little calcium is retained in Feta cheese, a fact that needs some explanation. Feta curds are dipped into the forms early, while the pH is still quite high. However, the moisture is also high because no cooking has taken place. The decrease in pH while the cheese is in the forms combined with the high salt levels used in Feta greatly increase syneresis and associated moisture removal. The net result is that a great deal of moisture (whey) is removed at the low pH and high salt concentration and most of the calcium phosphate is also removed with it. This is also true for other soft ripened cheeses such as blue and Camembert. In addition, soft ripened cheese making procedures have a long fermentation time before rennet addition and an extended setting time. The acid development before cutting encourages the release of minerals into the whey. The degree of solubilization of micellar calcium phosphate determines the extent of casein micelle disruption which, in turn, determines the basic cheese structure.

D. Texture (Cheese Body)

When cheese graders refer to cheese texture they often mean the amount and type of openness or holes in the cheese. Here, texture refers to the sensory attributes of firmness, elasticity, brittleness, etc. A typical texture in young cheese

is a strong indication of probable flavor defects later. Therefore, an important objective of cheese making is to develop the proper ultrastructure that will eventually lead to the desired cheese texture (see reviews by Olson *et al.*, 1996; Gunasekaran and Mehmet Ak, 2003; Lucey *et al.*, 2003). The following are the principal determinants of cheese texture.

Percent fat in dry matter (FDM) ranges from 60% for double cream varieties, through 50% for the so-called full-fat varieties, to 30% for part-skim varieties such as pizza cheese. Fat globules physically disrupt and weaken the casein matrix, so higher fat content softens cheese texture. FDM is mostly determined from the relative proportions of fat and protein in milk.

Percent moisture in non-fat substance (MNFS), ranging from about 40% to 80% in rennet-coagulated varieties (Table 8.6), also weakens the casein matrix and softens the cheese.

The pH profile is extremely important to cheese texture. Rennet-coagulated cheese with little acid development (pH $>$ 5.8; Table 8.6, Category 3) is chewy and crumbly, and does not melt or stretch when heated. Hence it can be used in stir fries, for example. Rennet-coagulated cheese with a minimum pH in the range of 5.3−5.0 (Table 8.6, Categories 5 and 6) becomes stretchable or meltable when heated depending on the degree of ripening. As the cheese ages, the protein matrix is broken down so the caseins become more meltable and less stretchable. Finally, rennet-coagulated cheese with minimum pH $<$ 5.0 (Table 8.6, Category 4) is brittle (e.g. Feta and Cheshire). Although low pH solubilizes calcium phosphate and disrupts casein micelles, the casein proteins remain intact and tightly packed owing to reduced charge repulsion. Therefore, while Feta remains brittle, Camembert becomes soft and smooth due to increased alkalinity from the ammonia released during ripening.

Calcium content, and the relative proportions of soluble and insoluble/colloidal calcium, combined with their interactive effects with pH are important determinants of cheese texture (Lee *et al.*, 2005; O'Mahony *et al.*, 2006a, b). The pH history up to the point of draining, along with postdraining syneresis, determine the calcium content of the curd, and the minimum curd pH determines the distribution of soluble and insoluble calcium in cheese. So, together with pH, calcium influences the strength of the casein matrix and, therefore, the texture. In brine-salted cheeses it is important that the Ca^{2+} concentration and the pH of brine are at levels similar to those in cheese. High pH and/or low Ca^{2+} induce more ion exchange (Ca^{2+} into the brine and Na^{+} into the cheese) to produce more sodium caseinate, which holds much more water than calcium caseinate. The result is moist mushy rind (Genigeorgis *et al.*, 1991).

Physical and timing aspects of curd handling are diverse and strongly influence texture and flavor. A few of the many examples that could be cited follow:

- Cheese with eyes (Swiss) requires a smooth fused texture which is obtained by forming and/or pressing the curd under warm whey.
- Vat salting, in which the curd is salted before forming and pressing (Cheddar, Cheshire, and American type varieties,) creates a distinct granular structure that never fuses as smoothly or as completely as brine-salted varieties. Vat salting versus brine or surface salting is probably the most important difference between traditional European varieties and their Americanized imitations such as American Havarti and American Mozzarella.
- One of the more interesting curd handling processes is the stretching operation in the manufacture of pasta filata cheeses and its many ethnic cousins around the world. Traditional pasta filata is mesophilic cheese similar to Cheddar in the early stages of its manufacture. After 'cheddaring', Cheddar is milled, salted, formed, and pressed, whereas the traditional pasta filata types are milled, immersed in hot water, and stretched. The structure and texture of stretched cheese are dependent on all of the factors discussed above and also on other factors: moisture, fat content, plasmin activity, pH history, total and insoluble calcium, temperature and time of heating before stretching, and ripening time (Walsh *et al.*, 1998; Guinee *et al.*, 2001; Feeney *et al.*, 2002; Somers *et al.*, 2002; O'Mahony *et al.*, 2006b; Tunick and Van Hekken, 2006).

E. Flavor Control

In the broadest terms, directed flavor development involves retention and/or addition of ripening agents and controlling their activity over time. See Weimer's recent text (2007) on this topic. Milk heating and clarification treatments determine the number and type of non-starter bacteria present in the milk. A great deal of direction is obtained through selection of cultures, coagulants, and other additives such as lipases. Debittering cultures, for example, reduce bitter protein fragments to shorter, non-bitter peptides and are an important tool to reduce bitterness and extend shelf-life in high-moisture varieties, such as Monterey Jack, and low-fat varieties. See the discussion of ripening agents in Section VII.

All cooking and curd-handling procedures exert specific effects on the various cheese-ripening agents (bacteria and enzymes) and determine the types and numbers/levels of these biological agents that remain in the curd to ripen the cheese. For example, the milk enzyme plasmin prefers neutral to slightly alkaline pH and is inactivated by low pH. Therefore, varieties in which ripening pH is high (e.g. smear-ripened types) have higher retention and greater activity of plasmin. Plasmin activity is also increased by higher cooking temperatures, due to the activation of plasminogen, for example, in traditional Swiss and Italian varieties (Bastian and Brown, 1996; Bastian *et al.*, 1997). On the other hand, calf rennet is more soluble at higher pH, but more active at lower pH. Therefore, rennet retention is higher in varieties that are drained at lower pH and more so in varieties that are cut at lower pH. Rennet activity is also drastically reduced by higher cooking temperatures, as in traditional Swiss and Italian types; therefore, rennet is more active in mesophilic varieties.

Finally, a great deal of flavor and texture development and differentiation among varieties takes place when cheese ripens. See the discussion on cultures (Section V) and ripening (Section VII).

VII. BIOCHEMISTRY OF CHEESE RIPENING

Some varieties of cheese, mostly acid- or acid/heat-coagulated types, are consumed fresh, i.e. soon after the curds are processed. Most other cheeses, which are of rennet type, have to be ripened to the desired texture and flavor over a period ranging from 2 weeks (e.g. Mozzarella) to over 2 years (e.g. extra-mature Cheddar and Parmigiano Reggiano). Cheese is a biologically and biochemically dynamic food system in which the proteins, lactose, and milk fat undergo physicochemical changes that facilitate the conversion of the 'green' curd to mature cheese with its characteristic microstructure, texture, flavor, and desired functionality (Guinee, 2003). The biochemical processes during cheese ripening have been reviewed several times (Fox *et al.*, 1995, 1996, 2000, 2004; Fox and Wallace, 1997; McSweeney and Sousa, 2000; Fox and McSweeney, 2006). The complex process of cheese ripening is discussed under three main headings:

- metabolism of residual lactose and catabolism of lactate and citrate
- lipolysis and catabolism of fatty acids
- proteolysis and amino acid catabolism.

The products of these metabolic pathways undergo further biochemical and chemical modifications. Cheese texture and flavor are mostly the outcome of the above primary reactions, while flavor is also probably due to the modification of primary reaction products (Povolo *et al.*, 1999).

A. Metabolism of Residual Lactose and Catabolism of Lactate and Citrate

Cheese is a fermented dairy product and the metabolism of lactose to lactic acid is essential in the production of all varieties. These reactions were reviewed by Fox *et al.* (1990), McSweeney and Sousa (2000), and McSweeney and Fox (2004). The cheese microbes (starter and/or non-starter) are mainly responsible for the fermentation of lactose (in the vat stage of cheese making) and the breakdown of lactate and citrate. Fresh cheese curd contains only about 1−2% lactose depending on whether the curds are washed (Dutch-type cheeses; ≈ 1% lactic acid) or not (Emmental and Parmigiano Reggiano; ≈ 1.5% acid) and whether the curd is dry-salted (Cheddar type; ≈ 1.5% in young Cheddar). About 98% of lactose is lost in the drained whey (Huffman and Kristoffersen, 1984). Fermentation of the remaining lactose is critical to the manufacture of cheese curd. Unless it is completely metabolized, the residual sugar can lead to undesirable secondary fermentations during ripening (Fox *et al.*, 1990, 2000; McSweeney and Fox, 2004). Lactose trapped in the curd matrix is rapidly and completely fermented by the starter bacteria before the salt/moisture ratio reaches a level that inhibits starter activity (Fox and Kelly, 2003; McSweeney, 2007); that is, when the curd pH is about 6.2−6.4 at molding and prior to salting (McSweeney, 2011); in about 12 hours the pH reaches ≈ 5.0−5.3 (Fox *et al.*, 2000). In the production of Cheddar cheese, the low curd pH of nearly 5.4 and dry-salting before molding retard lactose metabolism (Povolo *et al.*, 1999; McSweeney, 2011), and lactose is slowly, in about a month, fermented by residual starter activity [or by non-starter lactic acid bacteria (NSLAB)], to L(+) lactic acid (McSweeney, 2004).

The catabolic pathway of lactic acid is characteristic of the cheese variety, and the range of reactions that take place has a positive or negative impact on cheese ripening and/or quality. In Swiss-type cheese, the secondary fermentation of lactate by *Propionibacterium freudenreichii* subsp. *shermanii* is of great importance. The organic acids (propionate, acetate) produced contribute to the nutty flavor and the released CO_2 collects at weak points in

the curd and forms the large eyes characteristic of this cheese variety (Martley and Crow, 1996; McSweeney, 2004). Catabolism of lactate is also important in surface mold-ripened (e.g. Camembert and Brie) and smear-ripened (e.g. Tilsit or Limburger) cheeses. The oxidation of lactate to CO_2 and water by *Penicillium camemberti* greatly increases the surface pH of this cheese type, and causes a pH gradient from the surface (pH $\approx$ 7.5) to the core (pH $\approx$ 6.5) and the diffusion of lactate towards the surface (Bonaiti *et al.*, 2004; Spinnler and Gripon, 2004). The characteristic soft texture of this cheese comes from the precipitation of calcium phosphate at the high-pH surface and a concomitant migration of soluble calcium and phosphate to the surface (Vassal *et al.*, 1986; Walstra *et al.*, 2006). In surface smear-ripened cheeses, yeast activity deacidifies the surface and this favors the growth of coryneform bacteria. In Cheddar and Dutch-type cheeses, excessive formation of D/L-lactate (racemic mixture of L(+) and D(−) lactate) by adventitious NSLAB activity produces undesirable white specks of Ca-D-lactate crystals on the surface of mature cheese (Chou *et al.*, 2003; McSweeney and Fox, 2004; Swearingen *et al.*, 2004). Another undesirable, but minor, reaction in cheese (when the packaging material becomes more permeable to oxygen) is the oxidation of lactic acid by some members of NSLAB to acetic or formic acids and CO_2 (McSweeney and Fox, 2004). Late gas blowing and off-flavors are sometimes caused in hard and semi-hard cheese varieties by the anaerobic catabolism of lactate to butyrate, CO_2, and hydrogen by *Clostridium tyrobutyricum*. This defect can be avoided by minimizing the spore count in milk through good hygiene and by the removal of spores by bactofugation or microfiltration; increased levels of NaCl in cheese and lower ripening temperatures can also alleviate the problem (McSweeney, 2007).

Most ($\approx$ 94%) of the citrate in milk is soluble and is lost in the whey. Mostly, the colloidal citrate is metabolized by the citrate-positive strains of *Lactococcus lactis* subsp. *lactis* and/or *Leuconostoc* spp. to diacetyl, acetate/2,3-butanediol, acetoin, and CO_2. Diacetyl and sometimes acetate contribute to the cheese flavor. The CO_2 produced is responsible for the formation of small eyes in Dutch-type cheese, but gives an undesirable open texture in Cheddar cheese and the floating curd defect in cottage cheese (McSweeney and Fox, 2004).

B. Lipolysis and Catabolism of Fatty Acids

Milk fat is a critical component that determines cheese quality. Milk lipids affect cheese rheology and texture (Yoshida, 1989), influence flavor by acting as a source of fatty acids and their derivatives and by acting as a solvent for sapid compounds produced from lipids and other precursors. They also form the fat−water interface where many important reactions occur (Collins *et al.*, 2004).

Lipolysis during cheese ripening has been reviewed a number of times (Fox and Wallace, 1997; Fox *et al.*, 2000; McSweeney and Sousa, 2000; Collins *et al.*, 2003a; Collins *et al.*, 2004; McSweeney *et al.*, 2006; Wolf *et al.*, 2009). The breakdown of milk fat by lipases/esterases in cheese releases highly flavored short-chain (C_4−C_{10}) fatty acids. Low levels of these fatty acids are important contributors of flavor in many cheese varieties. Lipolysis is limited in most cheeses; even slight excess degradation of fat can cause rancidity or unbalanced flavor. Lipases in cheese possibly originate from milk, rennet paste, starter and non-starter LAB, secondary starter microbes, and exogenous lipase preparations (Fox and Wallace, 1997; McSweeney and Sousa, 2000; Collins *et al.*, 2004; Deeth and Fitz-Gerald, 2006). In varieties such as Cheddar and Gouda, the weakly lipolytic starter and sometimes the non-starter bacteria contribute to the slow and limited production of free fatty acids (Fox *et al.*, 2000; Collins *et al.*, 2003b) and in Swiss cheese, *Propionibacterium freudenreichii*, along with the thermophilic starter, plays this role (McSweeney, 2004; Thierry *et al.*, 2005). Extensive lipolysis is the major pathway to flavor generation in mold-ripened (blue and Camembert), surface bacterially ripened (e.g. Limburger), and some hard Italian cheeses manufactured from raw milk (e.g. Parmigiano-Reggiano, Grana Padano, and Provolone). Potent extracellular lipases are produced by *Penicillium roqueforti* and *P. camemberti* (Spinnler and Gripon, 2004), and the complex Gram-positive surface microflora, especially *Brevibacterium linens* of smear cheeses (Brennan *et al.*, 2004), also produce extracellular lipases. The pregastric esterase contained in rennet paste (e.g. Pecorino varieties, Provolone, and certain traditional Greek cheeses) (Hamosh, 1990; Fox, 2003a) and milk's indigenous lipoprotein lipases in raw milk cheeses (Olivecrona *et al.*, 2003) also possess strong lipolytic activity.

In addition to their direct impact on cheese flavor, the volatile short-chain fatty acids are important precursors in the series of reactions that lead to the production of different fatty acid flavor derivatives (Collins *et al.*, 2003a): ethyl esters (fruity aromatic notes, floral, goaty), thioesters (cheesy, cooked vegetable), branched chain keto acids (pungent cheesy), and unsaturated alcohols and ketones (mushroom-like) (Collomb *et al.*, 1998). *N*-Methyl ketones, produced by partial β-oxidation of fatty acids, are of particular importance to blue cheese flavor.

C. Proteolysis and Amino Acid Catabolism

Proteolysis is perhaps the most important of the three biochemical events that occur during the maturation of most cheese varieties (especially the internal bacterially ripened cheeses such as Cheddar, Swiss, or Gouda) and is definitely the most complex of the events. The subject has been extensively reviewed (Grappin *et al.*, 1985; Rank *et al.*, 1985; Fox, 1989; Fox and Law, 1991; Fox *et al.*, 1994; Fox and McSweeney, 1996, 2006; Fox and Wallace, 1997; Upadhyay *et al.*, 2004a; Mikulec *et al.*, 2010). Proteolysis contributes to: (1) the development of cheese texture through the hydrolysis of the para-casein matrix, by increasing the water binding capacity of the curd (the newly liberated ionized α-carboxyl and α-amino groups can bind water), and indirectly through an increase in pH due to the release of ammonia during amino acid catabolism; and (2) the flavor of cheese through the production of short–medium peptides (these contribute to the brothy background flavor of cheese; some hydrophobic peptides are bitter) and free amino acids, but most importantly, when these amino acids are catabolized to generate many important volatile flavor compounds, and also by the release of sapid compounds from the cheese matrix during mastication. The caseins are extensively proteolyzed by the action of a wide array of proteinases and peptidases that in cheese originate from six sources: the coagulant, milk's indigenous enzymes (mainly plasmin), starter LAB, NSLAB, secondary starter, and exogenous proteinases and peptidases.

1. Coagulant

In cheeses cooked to a temperature of less than 40°C, about 5–30% of rennet activity is retained depending on the enzyme type, pH at draining, and the moisture content of the cheese (McSweeney, 2011; Upadhyay *et al.*, 2004a). For example, Cheddar curd retains ≈ 6% of the added chymosin; the amount increases as the pH is reduced at whey draining (Holmes *et al.*, 1977; Creamer *et al.*, 1985). Rennet is extensively or completely denatured in varieties where the curd cooking temperatures exceed 55°C, as in Emmental, Parmigiano-Reggiano, and Mozzarella cheeses (Singh and Creamer, 1990; Boudjellab *et al.*, 1994; McSweeney, 2011). The coagulant is mainly responsible for primary proteolysis that generates large and intermediate-sized peptides, which are subsequently hydrolyzed by enzymes from the starter and non-starter microflora of the cheese (McSweeney, 2004). Apart from its specificity for the Phe_{105}–Met_{106} bond in κ-casein, chymosin is weakly proteolytic; α_{s1}-, α_{s2}-, and β-caseins are not hydrolyzed during milk coagulation, but may be slowly acted upon during cheese ripening (Upadhyay *et al.*, 2004a). The primary site of chymosin action on α_{s1}-casein is Phe_{23}–Phe_{24}, which is completely hydrolyzed in 4 months in Cheddar and related cheeses (Carles and Dumas, 1985; McSweeney *et al.*, 1993a; McSweeney, 2011). Two other chymosin-susceptible bonds in this protein are Leu_{101}–Lys_{102} and Trp_{164}–Tyr_{165}, both of which are extensively hydrolyzed in mature Cheddar. Chymosin appears to have limited action on α_{s2}-casein; there are not many studies in this area. Although the Leu_{192}–Tyr_{193} bond of β-casein is very susceptible to the action of chymosin, its hydrolysis is strongly inhibited by just 5% NaCl. This is likely to have significance for cheese flavor since the peptide β-CN (f193–209) and related fragments are very bitter.

The proteolytic action of rennet substitutes and pepsin (commonly found in commercial rennet preparations) differs from that of chymosin and has been extensively studied and reviewed (Hassan *et al.*, 1988; Broome *et al.*, 2006; Dervisolgu *et al.*, 2007; Jacob *et al.*, 2011). For example, fungal rennet substitutes have very different specificity from that of chymosin; the principal cleavage sites of *Rhizomucor miehei* proteinase in α_{s1}-casein in solution are Phe_{23}–Phe_{24}, Met_{123}–Lys_{124}, and Tyr_{165}–Tyr_{166}, and those in β-casein are Glu_{31}–Lys_{32}, Val_{58}–Val_{59}, Met_{93}–Gly_{94}, and Phe_{190}–Leu_{191}. Pepsins have specificity similar to chymosin, but it has not been established precisely; bovine pepsin has quite a rapid action on the Leu_{101}–Lys_{102} bond of α_{s1}-casein, which is relatively slowly hydrolyzed by chymosin.

2. Indigenous Milk Proteases

Of the many indigenous enzymes in milk, plasmin is the most significant in proteolysis during cheese ripening. Ismail and Nielsen (2010) recently reviewed the current knowledge on this protease and its relevance to the dairy industry. Bastian and Brown (1996) have also reviewed the subject. Plasmin is a serine protease derived from blood with pH and temperature optima of ≈ 7.5 and 37°C, respectively (Upadhyay *et al.*, 2004a). Plasminogen, the precursor, plasmin, and plasminogen activator (PA) are all associated with casein micelles and are incorporated into the enzyme-coagulated casein curd; inhibitors of both plasmin and PA are found in milk serum and so are lost in the whey (Bastian and Brown, 1996). Plasmin readily hydrolyzes β-casein to produce γ-caseins [(γ^1-CN (β-CN f29–209),

γ^2-CN (β-CN f106−209), γ^3-CN (β-CN f108−209)] and some proteose peptones [PP5 (β-CN f105/107), PP8 slow (β-CN f29105/107), and PP8 fast (β-CN f1−28)]. In α_{s2}-casein eight peptide bonds are rapidly hydrolyzed by the action of plasmin, but α_{s1}-casein is less susceptible to plasmin, and κ-casein is immune to its action (Bastian and Brown, 1996). Plasmin is mainly responsible for the limited proteolysis of β-casein in internal bacterially ripened cheeses; large and intermediate-sized peptides are the main products of its action. Being heat stable, plasmin plays a particularly important role in pasta filata and high-cook cheeses in which the coagulant is largely denatured by heat (Gobbetti, 2004). Its contribution is also vital in cheeses in which pH increases during ripening, such as Camembert-type (Spinnler and Gripon, 2004) and smear-ripened cheeses (O'Farrell *et al.*, 2002).

Exogenous plasminogen activators, urokinase and streptokinase, were successfully used to accelerate proteolysis in cheese by increasing the activity of plasmin. For example, urokinase was used in the manufacture of Cheddar (Bastian *et al.*, 1997; Barrett *et al.*, 1999) and ultrafiltered Havarti and Saint Paulin (Bastian *et al.*, 1991) cheeses. In addition to an exogenous streptokinase, Upadhyay *et al.* (2004b, 2006) successfully used a streptokinase-producing strain of *Lactococcus* to accelerate proteolysis in Cheddar.

3. Lactic Acid Bacteria

The auxotrophic LAB are weakly proteolytic, but possess a comprehensive proteolytic system which is essential to meet their complex amino acid requirements (Beresford and Williams, 2004; Upadhyay *et al.*, 2004a). Milk contains very low concentrations of these peptides and amino acids. While their enzymes degrade caseins into small peptides and amino acids, the LAB grow to high cell populations [10^9−10^{10} colony-forming units (cfu)/ml] in cheese milk and inadvertently contribute to the flavor of fermented dairy foods (Law and Mulholland, 1995; Steele, 1995, 1996).

Lactococcus and the thermophilic *Lactobacillus* spp. are economically important starters and their proteolytic systems, especially of the former, have been well characterized and extensively reviewed (Mulholland, 1995; Steele, 1995, 1996; Kunji *et al.*, 1996; Law and Haandrikman, 1997; Christensen *et al.*, 1999; Savijoki *et al.*, 2006). Their proteolytic system consists of a serine protease or cell envelope protease (CEP) anchored to the cell membrane and extending out of the cell wall, giving it ready access to extracellular substrates, peptide and amino acid transport systems, intracellular proteinases, and a number of intracellular peptidases (Upadhyay *et al.*, 2004a; McSweeney, 2011). The CEP is mainly responsible for the hydrolysis of the larger peptides produced from α_{s1}-casein and β-casein by chymosin and plasmin, respectively. The intracellular peptidases (aminopeptidases, dipeptidases, and tripeptidases) are responsible for the release of free amino acids after the cells have lysed. Research (Farkye *et al.*, 1990; Lane and Fox, 1997; Broadbent *et al.*, 2002) using CEP negative (Prt^-) and CEP positive (Prt^+) strains of the starter has shown that, although CEP is active in cheese during ripening, it is not essential; some differences in quality were found between Prt^- and Prt^+ cheeses, but Prt^- cheeses were also good. Other lactococcal enzymes or those from the NSLAB serve its function. The starter LAB, both *Lactococcus* and thermophilic *Lactobacillus*, die off rapidly after curd manufacture; following lysis, the dead cells release their intracellular enzymes.

Bitterness is a common problem in cheese, especially in Cheddar, Gouda, and other internal bacterially ripened cheeses. The hydrophobic peptides (molecular mass < 6 kDa and a mean hydrophobicity > 1400 cal per residue) derived from caseins, especially from the C-terminal region of β-casein (β-CN f193−209), are very bitter (Singh *et al.*, 2005; McSweeney, 2007, 2011). Bitterness in cheese develops due to incorrect patterns of proteolysis causing excessive production of bitter peptides, or due to peptidase activity that is insufficient to degrade the hydrophobic peptides to free amino acids (Lemieux and Simard, 1991; HabibiNajafi and Lee, 1996; McSweeney, 1997; Frister *et al.*, 2000). Recent studies (Broadbent *et al.*, 1998, 2002; Pillidge *et al.*, 2003; Broadbent and Steele, 2007b) suggest that both the lactococcal CEP and peptidase systems play a significant role with respect to bitterness and cheese quality in general. Besides, excessive chymosin activity can also cause bitterness, but plasmin is unlikely to produce bitter peptides. Changing the milk coagulant to a more suitable type, using a starter culture or adjunct with high peptidase activity, blending bitter and non-bitter strains, using a *Lactobacillus* adjunct culture with a bitter strain, and ensuring an adequate level of NaCl in cheese are some strategies that can be used to ameliorate bitterness in cheese (McSweeney, 2007, 2011).

The activity of NSLAB appears to supplement the proteolytic activity of the starter LAB. Several cheese studies with controlled microflora (McSweeney *et al.*, 1994; Rehman *et al.*, 2000) suggest that NSLAB are less significant than the starter in flavor development in Cheddar and other similar varieties. The initial numbers of NSLAB in the freshly produced Cheddar curd are very low (< 100 cfu/g); depending on the ripening temperature, they grow to reach 10^7−10^8 cfu/g within about 3 months (Beresford and Williams, 2004; McSweeney, 2011) and thereafter their numbers more or less remain constant, as in Cheddar (Fox *et al.*, 1998; Peterson and Marshall, 1990), or may decline,

as in Swiss cheese (Beuvier *et al.*, 1997). So, the viable microflora of long ripened cheese is dominated by mesophilic lactobacilli during most of its ripening. The NSLAB are more numerous and more diverse in raw milk than in pasteurized milk cheese (McSweeney *et al.*, 1993b; Grappin and Beuvier, 1997; Albenzio *et al.*, 2001; de Angelis *et al.*, 2001; Mannu and Paba, 2002; Dasen *et al.*, 2003) and they contribute to the formation of small peptides and amino acids (Beresford and Williams, 2004). In general, raw milk cheeses are known to ripen more quickly and develop more intense flavor than those made from pasteurized milk (Beuvier and Buchin, 2004; McSweeney, 2011). This is primarily due to the presence of indigenous NSLAB in raw milk which are incorporated into the cheese; heat-induced inactivation of the indigenous lipoprotein lipase is also of significance. For health and safety reasons and for the production of cheese with consistence in quality, it is unlikely that large cheese-making firms will revert to the use of raw milk. Starter and NSLAB adjuncts, usually selected *Lactobacillus* strains, are being used to simulate the flavor characteristics of raw milk cheese in the pasteurized milk product (Law, 2010b). However, adjuncts added to pasteurized milk do not closely simulate the flavor of raw milk cheese, probably because they contain only a few strains of lactobacilli, whereas the NSLAB of raw milk cheese are quite heterogeneous. Development of improved adjuncts is likely in the future as more work on NSLAB is being carried out.

The secondary microflora added to, or encouraged, to grow in a number of cheese types have specific functions and possess proteolytic systems similar to LAB. For example, various strains of *Lactobacillus* are added to Cheddar to improve flavor or accelerate ripening. Proteolysis by *Brevibacterium linens* at the surface of smear-ripened cheeses is due to the extracellular proteinase and aminopeptidase, and a number of intracellular peptidases secreted by the bacterium (Rattray and Fox, 1999). Both *Penicillium roqueforti* and *P. camemberti* produce potent extracellular aspartyl and metalloproteinases and various peptidases that contribute to the extensive proteolysis in blue, Camembert and Brie-type cheeses, respectively (Cantor *et al.*, 2004; Spinnler and Gripon, 2004).

4. Amino Acid Catabolism

Amino acids are the end products of proteolysis, and together with small peptides they contribute directly to the savory background flavor of cheese and to its taste; amino acids may be sweet, bitter, or sour. Research has now made it apparent that the production of amino acids is not the rate-limiting step because accelerating proteolysis does not necessarily accelerate flavor development (Upadhyay and McSweeney, 2003). It is now generally agreed that amino acids in principle serve as precursors for a complex series of reactions that produce a wide range of sapid and aromatic compounds such as amines, acids, carbonyls, ammonia, and sulfur compounds. The catabolism of amino acids was reviewed by Ardo (2006), Curtin and McSweeney (2004), Ganesan and Weimer (2007), and Yvon and Rijnen (2001), and is believed to proceed via two major pathways:

- Transaminase action catalyzed by aminotransferases, in which the amino group of an amino acid (usually aromatic and branched chain amino acids and methionine) is transferred to an acceptor molecule (usually α-ketoglutarate) to produce the corresponding α-keto acid. α-Keto acids are unstable and they degrade to aldehydes, carboxylic acids, hydroxyacids, alcohols, and other compounds.
- Elimination reaction by amino acid lyases (side-chains of amino acids are cleaved), which is particularly important in the production of volatile sulfur compounds from the side-chain of methionine.

In addition, decarboxylases in certain strains of LAB remove the carboxylic group of amino acids and produce amines. Of particular concern are the biogenic amines (amines from His, Trp, and Tyr), which at high levels can bring about adverse physiological reactions in susceptible consumers. The action of deaminases, for example, in surface smear-ripened cheeses, removes the α-amino group of amino acids and results in the formation of carboxylic acids and ammonia.

D. Controlled/Accelerated Cheese Ripening and Cheese Flavor Technology

There is an ever-increasing consumer demand for cheeses with distinctive characteristics and interesting flavors. That an increasing proportion of cheese is made from pasteurized milk does not offer many possibilities to cheese manufacturers for flavor diversification. But, with advances in cheese ripening technologies, it is possible to make cheese both exciting and safe. Flavorful cheeses with characteristic body attributes also have premium prices and need to be ripened at a low temperature for months or even years, so there is considerable industrial interest in technologies to accelerate the ripening process. Some of the options available for the control of cheese ripening and flavor technology are listed below (Farkye, 2004):

- elevated ripening temperatures
- high-pressure processing
- addition of exogenous enzymes
- attenuated starter cultures
- use of culture adjuncts
- GM starters.

It is obvious that any technology that can accelerate ripening and shorten cheese storage time will increase the profit margin and/or offer more competitive pricing to the cheese manufacturer. Temperature control and enzyme technology are the methods of choice to hasten the ripening process; culture-based technology did not meet with much success (Law, 2010b). High-pressure treatment of cheese is known to accelerate proteolysis and has the potential to speed up cheese maturation. These techniques are discussed below.

1. Elevated Storage Temperatures

This is a relatively simple and low-cost technology that is frequently applied to accelerate cheese ripening. However, using elevated temperatures poses the risk of stimulating the growth of spoilage and pathogenic microbes (Fox *et al.*, 1996) and should only be applied to cheeses made from pasteurized milk using GMP. The practical choices for forced ripening are hard and semi-hard cheeses such as Cheddar, Gouda, and Edam, which are very stable and have relatively simple LAB microflora (Law, 2010b). Relatively low temperatures of less than 10°C are typically used in cheese stores. Temperature increases up to 12°C decrease the maturation time of Cheddar cheese by about 60% without adversely affecting its body or texture (Law, 2001). Hannon *et al.* (2002, 2004) used combinations and successions of ripening temperature (20°C for 1 week/12°C for 6 weeks, followed by the normal 8°C for 8 months) and gained 2 months' maturation in Cheddar cheese without a loss in flavor balance.

2. High-Pressure Processing

There is an increasing body of evidence (Yokoyoma *et al.*, 1992; Messens *et al.*, 2000, 2001; Saldo *et al.*, 2000, 2002; O'Reilly *et al.*, 2001, 2002) that the use of ultra-high-pressure technology in young cheese speeds up ripening. Very high pressures in the range of 100−1000 MPa are applied for a short span of time. High pressure breaks up the cells of the starter to release the intracellular enzymes, activates these enzymes, and thereby rapidly increases the proteolytic and possibly other flavor-generating reactions (Stewart *et al.*, 2006; Law, 2010b). At present, this technological option is limited to research laboratories, but the food industry already has an interest in this branch. With more studies on its industrial relevance and with the development of the required hardware, high-pressure processing can be extended to factory applications (Stewart *et al.*, 2006; Law, 2010b).

3. Enzyme-Based Cheese Ripening and Flavor Technology

The use of enzyme-modified cheese (EMC) as an ingredient in processed cheese and as food flavoring is well known. However, the application of enzymes to the controlled ripening and flavor development of cheeses meant for direct consumption is a relatively new concept (Collomb *et al.*, 1998; Law, 2010b). The main challenge to this method is to distribute gram quantities of the enzyme within tonnes of cheese; addition to cheese milk is not an option, as 95% of ripening enzymes are lost in the whey. In addition, currently there is a limited availability of approved commercial enzymes. The development of microencapsulation technology makes it possible to physically enmesh the liposome-entrapped enzymes into the curd matrix as it is being formed (Kirby *et al.*, 1987). Owing to the high cost of phospholipids this method is not economically viable for industrial applications, but is suitable for small-scale applications (Law, 2010b). Alternative approaches to proteinase encapsulation exist, for example, the use of food-grade gums (Kailasapathy and Lam, 2005), and these may be an option. In dry-salted varieties such as Cheddar, the enzymes can be granulated together with salt and distributed into the milled curd (Law and Wigmore, 1982, 1983). Based on the research findings on the role of starter lactococci and commercial proteinases in Cheddar cheese ripening (Law and Wigmore, 1982, 1983), the Accelase™ range of enzymes was developed for commercial use by Imperial Biotechnology Limited (IBT, now a part of Danisco).

Added to the ease of incorporation and efficiency in the distribution of enzymes within the curd, a wide range of flavor options is also available in the Accelase series. Accelase contains a mixture of endopeptidases and

exopeptidases (also lipases) which promote a balanced breakdown of caseins into non-bitter peptides and flavor-generating amino acids. The Accelase enzymes are fabricated into a special formulation based on the type of cheese and coagulant, the starter culture regime used, and the market destination for the cheese (Law, 2010b). Unlike the dry-salted varieties, washed curd cheeses such as Gouda and Edam are particularly difficult to ripen with enzymes. Although the enzymes can be introduced into the cheese matrix with wash water or at the soft curd stage, both methods result in curd softening, yield reduction, and loss of enzymes into the wash water (Collomb *et al.*, 1998; Law, 2010b). Mechanical injection of enzymes into the finished cheese may be an option (Wilkinson and Kilcawley, 2005), but this technique has to be expanded to the market stage.

Applications for flavor diversification are mostly culture based and involve the addition of top notes to traditional flavor profiles using selected strains of lactobacilli (*Lactobacillus casei*, *Lb. plantarum*, and *Lb. helveticus*). The methods in use are briefly discussed in the following sections.

4. Attenuated Starter Cultures

Starter LAB function well as cheese-ripening agents, but because of their primary acidification role, it is not possible simply to add a bit more of the culture to enhance flavor. Attenuation or weakening of starter cultures by various means prevents or reduces their acidification function, at the same time retaining their intracellular enzymes to be subsequently released into the cheese matrix during ripening via lysis (Upadhyay and McSweeney, 2003). Petterson and Sjöström (1975) were the pioneers in the use of attenuated starters to accelerate the ripening of a Swedish semi-hard cheese, Svecia; attenuation was by heat treatment of cells. Attenuated bacterial cells can also be prepared by freeze shocking, spray drying, treatment with lysozymes under sublethal conditions, and selection of lactose negative (Lac^{-ve}) mutants (Klein and Lortal, 1999; Azarnia *et al.*, 2006; Law, 2010a, b). Madkor *et al.* (2000) have found that Cheddar cheese made with heat- or freeze-shocked (HS or FS) *Lb. helveticus* strain exhibited greatly enhanced rates of free amino group formation and lipolysis and the highest flavor and aroma scores were obtained for the FS *Lb. helveticus*-treated cheeses. Based on the work of Ardo and Pettersson (1988), a heat-shocked strain of *Lb. helveticus* was made by Medipharm (Sweden) and is commercially sold in Sweden and Finland as Enzobact™. Originally, Enzobact was developed to accelerate ripening in full-fat hard and semi-hard cheeses, but is now mostly used in the ripening of reduced-fat varieties of traditional Swedish hard cheese (Azarnia *et al.*, 2006; Law, 2010b).

Lysozyme is commercially extracted from hen egg white and is a relatively inexpensive enzyme. Although it is a promising tool for starter culture attenuation, its application is limited by the fact that the pretreatment process is laborious and not practical for routine use in cheese production (Law, 2010b). The Lac^{-ve} mutants occur spontaneously in all starter cultures and these natural LAB variants provide the most practical and economically feasible sources of attenuated cultures (Law, 2010a, b). Vindfeldt (1993) has described the technical development and scope for commercial application of these cultures. They are first selected based on their flavor profiles, easily isolated and grown on a glucose medium, packaged, and sold as frozen concentrates in amounts corresponding to their cheese-ripening power per liter of cheese milk or kilogram of cheese.

In 2007, Upadhyay *et al.* demonstrated that high-pressure treatment ($\approx$ 200 MPa) can impair acid production in lactococcal strains without causing cell lysis and can offer a potential means for attenuation of LAB cultures. Smith (2005) developed a method to accelerate cheese ripening using a biological agent (not an attenuated bacterial starter culture; for example, *Brevibacterium linens*, *Kluyveromyces lactis*, *Staphylococcus xylosus*, *Arthrobacter nicotianae*, and *Geotrichum candidum*) that has been treated with a surface active agent such as sodium dodecyl sulfate to kill, incapacitate, or otherwise reduce cell viability (by acting on the cell membranes), but allowing the intracellular enzymes to access the substrate, as well as allowing the release of the reaction products. The treated biological agent is added to the cheese milk along with a starter culture possessing significant aminopeptidase activity.

Flavor Control CR™ is a range of natural non-acidifying *Lactococcus* cultures (selected from starter cultures) developed by Chr. Hansen A/S in Denmark for commercial application as a flavor-enhancing system. The culture enhances the overall flavor intensity of cheese by accentuating all important flavor notes. It enhances balanced, mellow, rounded, and clean flavors, and suppresses unwanted flavors such as sour, bitter, and flat notes. The CR 500 series and CR 213 ripening blends are especially well suited for low-fat Cheddar-type cheese, but can also bring out a special flavor profile in full-fat cheeses (Chr. Hansen, 2008; Law, 2010b). The effectiveness of these CR cultures is most pronounced in low-fat cheese at near to normal salt concentrations ($\approx$ 1.75 g/100 g). CR cultures are priced similarly to quality enzyme blends, but are much easier to apply; they can be used 'off the shelf', can be thawed (sold as frozen concentrates) and added directly to cheese milk along with the primary starter (Law, 2010b). Furthermore, there are no regulatory restrictions on their use as is the case with GM LAB cultures.

5. Non-Starter Adjunct Cultures

Adjuncts are the secondary or non-starter microflora that are deliberately added to cheese milk (at levels $< 0.01\%$) to bring out desired cheese flavor and functionality (Crow *et al.*, 2002). Examples of some traditional adjuncts are *Penicillium*, *Brevibacterium*, *Geotrichum*, yeasts, *Propionibacterium*, and *Leuconostoc*. The flavor control option provided by secondary microflora in Cheddar-type cheeses is based on adding distinctive flavor/aroma top notes (e.g. cooked, fermented, sweet, nutty) to basic flavor profiles. Their use as commercial adjunct flavor cultures, alone or in combination with attenuated starters, therefore provides complete control over flavor intensity and flavor character (Law, 2010b). Unlike attenuated cultures, adjunct technology also has the potential to harness metabolic flavor pathways of viable cells (Broome, 2007). Culture companies carefully select desired strains by screening out many potential defect-forming traits and, based on their ability to compete against adventitious microflora, produce them in quantities, and blend them reproducibly for sale and use in cheese factories (Crow *et al.*, 2002; Law, 2010b). Popular culture adjuncts are mostly NSLAB (*Lb. casei*, *Lb. plantarum*, *Lb. helveticus*), although surface-smear microflora (*Brevibacterium linens*, staphylococci, yeasts) are also available to give additional flavor notes. Cheddar cheese, with its relatively high pH and more solid matrix, has the potential to deliver probiotics (Playne, 2002). Selected strains of the probiotic cultures *Bifidobacterium* (McBrearty *et al.*, 2001), *Enterococcus* (Gardiner *et al.*, 1999), and *Lactobacillus* (Gardiner *et al.*, 1998) can also improve cheese flavor (McBrearty *et al.*, 2001). Inner-Balance is a brand of Cheddar cheese marketed in Australia (Mainland Dairies) and the UK and it contains the probiotic culture adjunct *Lb. rhamnosus* DR20™, which also provides flavor consistency and improves and accelerates flavor quality (Crow *et al.*, 2002). Enterococci, despite some of their negative effects on cheese flavor and some food safety concerns, have a great potential as cheese flavor adjuncts and probiotic cultures and are being tried in dairy products (Crow *et al.*, 2002; El-din *et al.*, 2002; Bulajic and Mijacevic, 2003; Abeijon *et al.*, 2006; Bhardwaj *et al.*, 2008).

In the case of mold cultures, the challenge is to keep a fine balance between mold growth and the development of flavor notes via the metabolism of living organisms; there is less scope to control the flavor and aroma of mold-ripened cheeses (Law, 2010b). Still, Chr. Hansen has developed the commercial SWING™ range of mold cultures by selecting from the inherent variability of enzyme and pigment production and growth rate/metabolic ratios within available cultures.

6. Genetically Modified Lactic Acid Bacteria

Over the past two decades, much research knowledge has accumulated in relation to the biochemical basis of the vital functions of dairy LAB, including acid production, flavor/aroma production, protein utilization, extracellular polysaccharide secretion, and bacteriophage resistance; this advanced knowledge base forms the basis of the many GM LAB that the culture companies now provide for industrial trials (Law, 2010b). The USA and the European Union require that the GM strains comply fully with all the federal/national regulations covering GM organisms for food applications; the GM LAB are constructed using food-grade cloning systems.

Three general strategies are used for the genetic modification of LAB to be applied to cheese-ripening and cheese flavor technology (Law, 2010b): (1) altered lactose metabolism; (2) increased peptidase production capacity; and (3) increased starter cell lysis in the matrix of young cheese. Mutated strains of *Lactococcus lactis* can be produced with altered metabolic routes from lactose to the key intermediates, α-acetolactate (precursor of diacetyl, which contributes to the buttery aroma) and acetate; the result is an accumulation of aroma compounds at higher than normal concentrations (de Vos, 1996; Swindell *et al.*, 1996). Such GM lactococcal cultures are now commercially available. Commercial strains of *L. lactis* spp. with genetically enhanced production of two general aminopeptidases have been developed and these cultures are now being used to increase the flavor quality and intensity in Cheddar and Dutch-type cheese (Law, 2010b). Peptidases prevent the accumulation of bitter peptides that may cause flavor defects. Lastly, starter cultures can be genetically altered to lyse quickly in cheese. It is now established that the quality and pace of cheese flavor development are positively correlated with the rate and extent of starter cell lysis and subsequent release of enzymes into young cheese (Crow *et al.*, 1995). Based on the original work of Gasson's group in the UK, lactococcal variants of commercial starters containing the gene for the bacteriophage lysin are constructed in a way that the gene expression is controllable by external stimuli such as changes in pH, salt concentration, and temperature (Law, 2010b). The NIZO food research company in the Netherlands adopted a similar approach and developed and patented the NICE system, in which microgram quantities of Nisin are added to trigger the cloned phage lysin. The latter technology is based on the research findings of de Ruyter *et al.* (1997).

VIII. YOGURT

A. Introduction to Fermented Dairy Foods

Cheese and yogurt are the most popular dairy products derived from fermented milk. Apart from these, cultured buttermilk (used as an ingredient in baking; uses *Lactococcus lactis* subsp. *cremoris* or *diacetylactis*), acidophilus milk (therapeutically benefits the gastrointestinal tract; uses *Lactobacillus acidophilus*), and sour cream (cultured cream; uses *L. lactis* subsp. *cremoris* or *diacetylactis*) are also consumed in North America and Europe. Other fermented dairy products enjoy regional popularity in certain parts of the world, for example kefir (an alcoholic fermented milk drink that uses kefir grains that contain a combination of bacteria and yeasts capable of producing kefiran, a ropy polysaccharide), kumiss (produced from mare's milk; uses a liquid culture of bacteria and yeasts), filmjölk (a Nordic dairy product similar to cultured buttermilk; uses *L. lactis* and *Leuconostoc mesenteroides*), långfil (an elastic variant of filmjölk containing ropy LAB that produce exopolysaccharides), and viili (also similar to filmjölk, but includes *Geotrichum candidum* and sometimes ropy LAB). For further information, the reader may refer to Robinson and Tamime (1990) and Tamime and Robinson (1999b).

The set-style, stirred, and drinking forms of yogurt are the most common; the first type is incubated and cooled in the final package and is characterized by a firm, gel-like structure, whereas in the latter two forms, the final coagulum is broken by stirring (stirred type) or by homogenizing to a low-viscosity drink (drinkable yogurt) before cooling and packing. Another popular American type is the frozen yogurt, which first originated in New England. Physically, it resembles ice cream but is characterized by the sharp acidic taste of yogurt, and is available in three categories: soft, hard, and mousse. Frozen yogurts contain high levels of sugar and stabilizers to maintain the air-bubble structure during freezing. Concentrated/strained yogurt (e.g. Eastern Mediterranean labneh, Egyptian laban, Icelandic skyr, Indian chakka, and shrikhand from buffalo milk) is produced by straining cold/unsweetened yogurt by application of pressure in a cloth bag (Berge system), centrifugation, or ultrafiltration. Finally, a powder form of dried yogurt is also produced and finds different applications in the food industry (e.g. baked goods and confectionery) (see Tamine and Robinson (1999b).

B. Yogurt Manufacture

Yogurt making dates back thousands of years and is an ancient craft, but still today the commercial production of yogurt is a complex process and combines both art and science. The manufacturing process of yogurt was well described by Tamine and Robinson (1999a) and is also briefly discussed by Goff on the University of Guelph's Dairy Science and Technology Education website. When refrigerated milk arrives at the plant, the first step is to modify its composition to suit yogurt specifications. This process involves clarification of milk into cream and skim milk, followed by standardization to the desired fat and solids-not-fat (SNF) content. While the average fat content of milk ranges from 3.7% to 4.2% (w/w), the fat content of commercial yogurt can range anywhere from 0.1% to 10%. The Pearson's square method (Tamine and Robinson, 1999a) is a very convenient way to calculate the components required to standardize milk. To increase the SNF content, the industry most commonly adds milk powder (whole or skimmed, 3−4% is recommended) to the yogurt mixture, but one or more other ingredients may also be used: concentrated milk, high-protein milk powders, buttermilk powder, whey powder/concentrate, casein powder, and even non-milk proteins (e.g. proteins from soy, legumes, or sweet potato). Added solids improve yogurt viscosity/consistency, reduce syneresis, and impart a better mouth-feel, besides contributing to specific functionalities related to the material added (e.g. phospholipids from buttermilk possess emulsifying properties).

The yogurt milk base is usually also fortified with non-milk solids such as stabilizers/emulsifiers, and sweetening agents. Stabilizers are mostly gums/hydrocolloids which are added (at a level of 0.1−0.5%) as single compounds or as a blend of either natural (gelatin, pectin, guar and locust bean gums, cereal starches, alginates, and carrageenans) or modified natural/semi-synthetic (carboxymethyl cellulose, xanthan, low-methoxy pectin, and modified starches) gums. The hydrocolloids in yogurt function as gelling/thickening and stabilizing agents by binding water as water of hydration, and by reacting with and stabilizing milk protein molecules in the form of a network that retards free water movement (Tamine and Robinson, 1999a). Sweeteners are normally added to fruit and flavored yogurts and can be sugars such as sucrose, invert sugar, glucose, and fructose, or non-caloric high-intensity sweeteners such as aspartame and saccharin.

Following standardization and fortification of the base milk with a cocktail of milk and non-milk solids, the mixture is pasteurized or heat treated using a continuous plate heat exchanger for 30 minutes at 85°C or 10 minutes at 95°C. The higher heat treatments that are used not only achieve the destruction or elimination of pathogens and

spoilage microbes, but also encourage production of factors capable of stimulating or inhibiting starter culture activity, and most importantly bring about very favorable changes in the physicochemical properties of milk through denaturation of whey proteins and their attachment to the surfaces of casein micelles (refer to Section IV).

The pasteurized mix (at 65−70°C) is then homogenized, usually by a single-stage homogenizer at pressures ranging from 15 to 20 MPa. Besides uniformly blending in all the ingredients, this process effectively reduces fat globule size, increases fat surface area, and coats the surface with mainly proteins; casein micelles cover nearly 25% of the surface. In effect, the homogenized fat globules act as large casein micelles and participate in acid precipitation reactions. Yogurt made from homogenized milk is firmer, smoother, and more stable (reduced creaming and wheying off) during storage.

The homogenized mixture is cooled to the incubation temperature (40−45°C) and pumped into jacketed fermentation tanks. The starter culture (≈ 3% w/w of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* in a 1:1 ratio) is directly metered into the mix while pumping, or added to the fermentation tanks. The actual fermentation can take place either in retail containers (set-style yogurt) or in bulk tanks (stirred yogurt). A temperature of typically 42°C is maintained for 2−2.5 hours under quiescent conditions; pH and/or titratable acidity (TA) are carefully monitored during this period. When pH reaches 4.6 (TA of 0.85−0.90%), i.e. when a continuous solid mass of gel is formed, the yogurt is immediately cooled to nearly 5°C, at which it is stored, or processed further to produce other yogurt forms. Cooling starts when the pH of yogurt is relatively high and the rate is carefully controlled so that the final product has the desired level of acidity and gel structure. While slow cooling can increase yogurt acidity, very rapid cooling may lead to whey separation, possibly due to excessive contraction of the protein matrix (Rasic and Kurmann, 1978).

Fruit and/or flavored yogurts, available in a great variety of forms, are the most popular types, especially in North America, Europe, and Australia. Before the 1950s, yogurt was virtually unknown outside the Middle East and Balkan region, but the addition of sweetening agents and fruits greatly increased its popularity and acceptability worldwide. Various flavoring agents (fruits, natural, and/or synthetic flavors) are added to yogurt. In set-style yogurt these are normally added to the mix before incubation, but in the stirred type they are often incorporated into the formed gel.

C. Yogurt Starter Cultures

Yogurt is made with live and active cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, and the US Food and Drug Administration (FDA) requires that these two specific LAB be present in a product for it to be called yogurt (Van de Water and Naiyanetr, 2008). Although other cultures may be added to yogurt, they are not required. In the milk environment, *Lb. bulgaricus* and *S. thermophilus* coexist and interact beneficially in a stable associative relationship also known as protocooperation (Liu *et al.*, 2009). Protocooperation, previously defined as biochemical mutualism, involves the exchange of metabolites and/or stimulatory factors (Pette and Lolkema, 1950). Most strains of *S. thermophilus* have fewer nutritional requirements and hence grow preferentially in milk. In fact, during the first exponential growth of *S. thermophilus*, no growth of *Lb. bulgaricus* is observed. In the second phase, as the pH of milk begins to drop, growth of *S. thermophilus* (less acid tolerant) slows down and it provides several growth factors such as formate, pyruvate, folate, CO_2, and even some long-chain fatty acids that stimulate *Lb. bulgaricus* (more acid tolerant) to grow exponentially. The latter, in turn, releases cell wall proteases and cytoplasm peptidases that hydrolyze caseins into peptides, subsequently broken down to amino acids. Since *S. thermophilus* strains lack extracellular proteases, their growth is strongly stimulated in cocultures with *Lb. bulgaricus* strains serving as a source of these amino acids able to support a second exponential growth phase for *S. thermophilus*. The growth of *Lb. bulgaricus* continues in the third growth phase. (Please refer to the work of Sieuwerts *et al.*, 2008.)

D. Bioyogurt

The dairy sector is the largest (33%) functional food market and dairy products are the main vehicle for probiotic supplementation (Leatherhead Food International, 2006). Added to the fact that many consumers associate yogurt with good health, supplementation with probiotics has drastically increased yogurt consumption in recent years (Hekmat and Reid, 2006). As defined by FAO/WHO (2001), probiotics are 'live microorganisms which when administered in adequate amounts confer a health benefit on the host'. Some commonly used dairy probiotics include specific strains of LAB such as *Lactobacillus acidophilus*, *L. casei*, *L. rhamnosus*, *Bifidobacterium bifidum*, *B. animalis*, *B. longum*, and *B. infantis* (Granato *et al.*, 2010). Other LAB such as *Enterococcus faecalis* and

E. faecium, also known to possess probiotic properties, and *Propionibacterium freudenreichii* and *Saccharomyces boulardii*, are non-lactic probiotics (Granato *et al.*, 2010). Some examples of probiotic yogurt products available on the world market are acidophilus bifidus/AB yogurt (A + B + yogurt culture; A = *L. acidophilus*, B = *Bifidobacteria*), Bifidus yogurt/Biobest (*B. bifidum* or *B. longum* + yogurt culture), Bioghurt/ABT yogurt (A + B + *S. thermophilus*), Bifilak/Cultura/Biomild (A + B), and BA Bifidus active (*B. longum* + yogurt culture) (Lourens-Hattingh and Viljoen, 2001). In recent years, the health enhancement effects of dairy probiotics have been attributed to the metabolic release of physiologically active peptides during milk fermentation. The bioactive peptides may be released during food processing or after degradation by digestive enzymes and have been shown to possess opiate, antithrombotic, antihypertensive, immunomodulating, antibacterial, antigastric, and mineral carrier properties (Vinderola *et al.*, 2008). Some documented physiological/health benefits of probiotics (Van de Water and Naiyanetr, 2008; Granato *et al.*, 2010) are:

- enhancement of the immune system (of the gastrointestinal tract, and possibly the respiratory tract)
- treatment of gastrointestinal disturbances: primary or secondary lactose maldigestion, diarrhea caused by antibiotics, *Clostridium difficile*, or food allergies, infant diarrhea due to rotavirus enteritis, traveler's diarrhea, and possibly diarrhea from *Helicobacter pylori* infections, inflammatory bowel disease, and colon cancer
- regulation of gut motility (constipation, irritable bowel syndrome)
- improved absorption of nutrients, e.g. iron, calcium, and important B vitamins
- lowering of serum cholesterol (prevention of atherosclerosis) and hypertension
- detoxification of carcinogens (prevention of cancer and suppression of tumors) and reduction of catabolic products eliminated by the liver and kidney (prevention of urogenital infections).

The key challenge to making any probiotic food is to improve/maintain culture viability in the product during processing, storage, and transit through the stomach and small intestine, besides finding cost-effective ways to produce these foods (Prado *et al.*, 2008; Figueroa-Gonzalez *et al.*, 2011). In the USA, the National Yogurt Association (NYA) specifies a therapeutic minimum of 10^8 cfu/g of LAB (at the time of manufacture) as a prerequisite to using the NYA 'Live and Active Culture' logo on the product's containers. A dairy probiotic product must not only contain a minimum number of cells to confer health effects, but must also have good sensory properties to be acceptable by consumers. Several factors influence the survival of probiotic bacteria in fermented dairy bioproducts: the strains used, culture conditions, inoculation levels and interspecies interactions, the fermentation medium's composition, acidity, presence of nutrients/growth promoters/inhibitors, osmotic pressure, dissolved oxygen levels, and the temperature and time of incubation and storage (Lourens-Hattingh and Viljoen, 2001).

Besides having beneficial effects on human health, an ideal probiotic strain is able to withstand food processing and storage conditions, has good growth characteristics, is compatible with the starter cultures, is resistant to acid and bile, attaches to the human gut epithelial cells, colonizes in the human intestine, and produces antimicrobial substances such as bacteriocins (Granato *et al.*, 2010). Several selected strains of *Lactobacillus* and *Bifidobacterium* cultures are available commercially. *Lactobacillus acidophilus* is more acid tolerant (can tolerate pH 4.6) than bifidobacteria (growth is retarded at pH < 5.0) (Lankaputhra *et al.*, 1996), so for practical applications, the pH of the final product must be maintained above 4.6. GMP, use of starter *L. bulgaricus* cultures with reduced 'over-acidification' behavior, or use of ABT-yogurt starter cultures (*L. acidophilus*, *B. bifidum*, and *S. thermophilus*) can aid in pH control (Lourens-Hattingh and Viljoen, 2001). Other ways to prevent acid stress include previous exposure of a strain to a lower pH for a short time to induce acid tolerance (Sanz, 2007), applying heat shock (58°C for 5 minutes) to yogurt before the addition of probiotic culture (Marshall, 1992), storage at temperatures less than 3−4°C, addition of whey protein concentrate to increase the buffering capacity of yogurt (Kailasapathy and Rybka, 1997), and addition of sodium citrate or calcium carbonate to neutralize the lactic acid produced during fermentation (Zhao and Li, 2008). Probiotic bacteria prefer an anaerobic/microaerobic environment; oxidative stress can be minimized with active packaging (Miller *et al.*, 2003), addition of ascorbic acid (Dave and Shah, 1998; Zhao and Li, 2008), electroreduction of milk (Bolduc *et al.*, 2006), use of glucose oxidase (Cruz *et al.*, 2010), and microencapsulation (Talwalkar and Kailasapathy, 2003). Addition of prebiotics (selectively fermented ingredients that allow specific changes in the composition and/or activity of the gastrointestinal microbiota) (Roberfroid, 2007) such as chicory inulin and oligosaccharides (Figueroa-Gonzalez *et al.*, 2011) and growth-promoting substances such as casein hydrolysate, whey protein concentrate, cysteine, and tryptone can improve the growth and viability of probiotic bacteria (Lourens-Hattingh and Viljoen, 2001). A higher level of inoculum (10−20% recommended) and the use of concentrated or freeze-dried direct-to-vat cultures containing a minimum of 5×10^9 cfu/g (IDF, 1996) will ensure a high cell count at the end of incubation, during storage, and until consumption (Lourens-Hattingh and Viljoen, 2001).

E. Yogurt Flavor

Yogurt's popularity as food largely depends on its sensory characteristics, with aroma and taste being the most important. Yogurt is well liked for its delicate and low intense acidic flavor. So far, more than 90 flavor compounds have been identified in yogurt including carbohydrates, alcohols, aldehydes, ketones, acids, esters, lactones, sulfur compounds, pyrazines, and furan derivatives (Ott *et al.*, 1997). But the flavor of yogurt is mainly attributed to the group of carbonyl compounds, acetaldehyde, diacetyl, acetoin, and acetone, which are present in relatively high concentrations (in decreasing order) (Imhof *et al.*, 1994; Kaminarides *et al.*, 2007). Of these, acetaldehyde is suggested as the indispensable component of the typical yogurt flavor; good flavored yogurt requires about 23−40 mg/kg (at least 8−10 mg/kg) of acetaldehyde to be present (Gaafar, 1992; Kneifel *et al.*, 1992; Georgala *et al.*, 1995). Acetaldehyde imparts to yogurt its characteristic green apple or nutty flavor (Bodyfelt *et al.*, 1988). Production of acetaldehyde takes place via several pathways which use different compounds as precursors, such as glucose, catechol, glyceraldehydes, acetylene, threonine, glycine, and even DNA (Zourari and Desmazeaud, 1991; Chaves *et al.*, 2002; Tamime and Robinson, 2007). Breakdown of threonine to acetaldehyde and glycine is reported as the major pathway and the reaction is catalyzed by the enzyme threonine aldolase, present in both *Lb. bulgaricus* and *S. thermophilus*. At the higher temperatures (40−45°C) used in yogurt manufacture, *Lb. bulgaricus* is the main contributor of the enzyme (Zourari and Desmazeaud, 1991) as the aldolase is inactivated in *S. thermophilus* at 30−42°C (Wilkins *et al.*, 1986).

Diacetyl is the other major volatile compound that contributes to the buttery notes in yogurt aroma; the typical concentration in yogurt ranges from 0.2 to 3.0 mg/kg (Cheng, 2010). When present with acetaldehyde, it is reported to contribute to the well-rounded delicate flavor of yogurt; overproduction of acetaldehyde in relation to diacetyl can result in harsh flavors. There are different views on the ideal ratio of acetaldehyde to diacetyl that may be required to result in the desired fullness in yogurt flavor (Zourari and Desmazeaud, 1991; Panagiotidis and Tzia, 2001; Boelrijk *et al.*, 2003). The diketone is derived from the fermentation of citrate in milk (Vedamuthu, 2006) or, according to Nilsson (2008), from both lactose and citrate. Acetoin is readily produced from diacetyl by the enzyme diacetyl reductase (Collins, 1972); about 1.2−28.2 mg/kg of acetoin is typically present in milk (Beshkova *et al.*, 1998; Alonso and Fraga, 2001). Diacetyl and acetoin together impart the mild, pleasant, and buttery taste, and their combination is critical for the rich flavor perception of yogurt (Cheng, 2010). Two other volatile components of minor importance are acetone and 2-butanone, both of which contribute to the sweet, fruity aroma of yogurt (Carcoba *et al.*, 2000; Gallardo-Escamilla *et al.*, 2005).

Acetaldehyde, diacetyl, and the other volatiles described above are the most important volatile flavor compounds present in yogurt, but are not the only flavor components. The overall flavor of yogurt comes from lactic acid and a complex and well-balanced mixture of several aromatic and taste components, which include volatiles and non-volatiles (e.g. lactic, pyruvic, acetic, and formic acids) already present in milk and specific compounds produced during fermentation (Imhof *et al.*, 1994; Ott *et al.*, 1997). Factors such as the source of milk (cow, sheep, or goat), processing techniques, added components (stabilizers, fruits, flavorings, probiotics, and prebiotics), packaging materials, and storage conditions all have an impact on the final taste and aroma of yogurt (Routray and Mishra, 2011). Further, undesired odorants can be produced during the storage of yogurt, and as in any other fat-rich dairy product, lipid oxidation is the major contributor to these off-flavors.

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