



Improving the flavour of cheese

Edited by Bart C. Weimer

Improving the flavour of cheese

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Bart C. Weimer**



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Introduction

The consistent high quality flavor of cheese is an elusive target. Manufacturers have long sought the exact ingredients needed to produce cheeses that are consistently safe and flavorful. The number of steps needed for production of cheese is so large that this task is almost overwhelming. In any case, there are many cheeses on the market today that meet this demand. Production of cheeses with less fat pushed the limit of consistent flavors and led to new advances in the biochemistry and microbiology of cheese.

Long lists of chemicals found in cheese were generated from the 1950s onward, but little progress was made until recently in understanding how these molecules were produced during ripening. The advent of high throughput genome sequencing and metabolite analysis will impact this area substantially. These new tools will lead to specific genes being associated with specific end products that are controlled by specific processing steps during the cheese making process. With these data in hand it becomes possible to model or simulate cheese making so as to produce ‘virtual’ cheese flavor, thereby reducing the development costs of new cheeses or new cultures.

The book begins with an overall view of the compounds found in cheese that are associated with flavor – good and bad. Part I details the specific substrates in cheese and their metabolism to flavor compounds by the microbes associated with milk and cheese, all with an eye to improving the production of well flavored compounds and limiting those that contribute to off-flavors. Part II outlines the processing considerations to improve cheese flavor that impact the microbial metabolism. Part III tackles the issue of measuring flavor – a subjective assessment of flavor and the impartial instrumental analysis for compound identification. Part IV contains specific case studies aimed at providing a basis for improving flavor.

Taken together, this volume provides new tools, techniques, and ideas to control and improve cheese flavor. Merging fundamental genetics, biochemistry, modeling and cheese processing will lead to new varieties and methods of cheese production with high consumer acceptance.

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Cheese manufacture and ripening and their influence on cheese flavour

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1.1 Influence of cheese manufacture on ripening and quality

‘Cheese is made in the vat.’ This traditional, and superficially obvious, saying contains an important element of truth: that the ripening of rennet-coagulated cheeses, and hence their flavour, texture and quality, are largely predetermined by the manufacturing process. The cheesemaker can vary only time, temperature and relative humidity/packaging during ripening, while many important factors which influence cheese ripening such as moisture content, levels of NaCl, pH, the cheese microflora and physical size of the cheese are influenced by manufacture. Indeed, it is difficult, and often impossible, to remedy during the maturation stage any mistakes made during curd manufacture. Hence, it is useful first to discuss cheese manufacture and how this process can influence ripening. Technological and scientific aspects of cheese manufacture have been described by many authors, including Kosikowski and Mistry (1997), Robinson and Wilbey (1998), Fox *et al.* (2000) and Fox and McSweeney (2004).

The manufacture of rennet-coagulated cheeses is essentially a dehydration process in which the fat and casein in the milk are concentrated 6- to 12-fold, depending on the variety. As shown in Fig. 1.1, the principal operations in cheesemaking are preparation of the cheesemilk (usually pasteurization and standardization), acidification by selected strains of lactic acid bacteria (LAB) known as starters, rennet coagulation, syneresis of the coagulum (which is controlled by factors such as cutting, stirring the curds/whey mixture, cooking and pressing), pressing and shaping the curds, and salting, although manufacturing protocols for particular groups of varieties (particularly Cheddar-type cheeses and *pasta-filata* varieties) have other operations such as controlled

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Fig. 1.1 The major processes and events occurring during cheese manufacture and ripening.

acidification and texturization of the curd and/or heating and stretching the curds in hot water.

Cheese manufacture commences with the selection of milk of the highest quality available. Since milk in countries with a developed dairy industry is now usually stored at refrigeration temperatures prior to processing, the microflora of raw milk is usually dominated by psychrotrophic organisms which can produce heat-stable proteinases and lipases (Suhren, 1988; Kroll, 1988) that at high cell counts ($>10^6$ cfu ml⁻¹) may cause a reduction in cheese yield or the development of off-flavours during ripening. Although some varieties continue to be made using raw milk, the milk for most cheeses is now pasteurized, causing changes to the microflora of the milk and to its complement of indigenous enzymes which can influence ripening. It is well known that cheese made from raw milk ripens more quickly and develops a stronger flavour than cheese of the same variety made from pasteurized milk (Fox *et al.*, 1998) and these differences have been ascribed mainly to heat-induced changes to the native microflora of the milk. In addition to all potential pathogens, many organisms that otherwise would grow later to form part of the non-starter microflora are killed by pasteurization, and the biodiversity of cheese made from pasteurized milk is simpler than that made from raw milk (see Chapter 6). Although heat-induced changes to the microflora of the milk are mainly responsible for differences between raw and pasteurized milk cheese, inactivation of certain indigenous enzymes in milk, particularly lipoprotein lipase, may contribute also (see Collins *et al.*, 2004). In commercial practice, the ratio of casein to fat, or increasingly their concentrations, is controlled by standardization which enhances the cheesemaking properties of the milk and improves cheese yield. Directly or indirectly, standardization influences some important compositional parameters of the final cheese which affect ripening (e.g., moisture and moisture-in-non-fat-substances).

Cheese is a fermented dairy product and, hence, the controlled fermentation of lactose to lactate by the starter during manufacture and the early stages of

ripening is important in all varieties. The science and technology of cheese starter cultures have become very complex (see Parente and Cogan, 2004) and starter cultures play a major role in cheese ripening, directly through their many enzymes and indirectly through acidification and reduction of the redox potential. Since lactic acid bacteria (LAB) are auxotrophic for many amino acids, they possess a complex system of proteinases and peptidases that enable them to liberate amino acids from the caseins as they grow in milk. In cheese, the role of starter proteinases and peptidases appears to be principally in the degradation of intermediate-sized peptides and the liberation of free amino acids (see below). LAB also contain intracellular metabolic enzymes which catalyse the catabolism of amino acids and contribute directly to the formation of volatile flavour compounds (see Yvon and Rijnen, 2001; Collins *et al.*, 2004; Curtin and McSweeney, 2004 and Chapter 4). The reduction in pH caused by the metabolism of lactose to lactate has a major indirect effect on ripening, through changes to (1) the retention of coagulant activity in the curd, (2) the rate of syneresis and hence the final level of moisture in the curd, (3) control of the growth of added and native microorganisms in the cheese, and (4) the activity of enzymes involved in ripening.

The milk for rennet-coagulated cheese varieties is coagulated via limited proteolysis of κ -casein at or near Phe₁₀₅-Met₁₀₆, through the action of selected proteinases in rennet preparations, followed by the Ca²⁺-induced aggregation of rennet-altered micelles at a temperature greater than ~18°C (Horne and Banks, 2004). The traditional rennet used for the manufacture of most cheese varieties is a brine extract of the abomasa of milk-fed calves (or the young of other dairy animals), and contains principally chymosin with a low level of pepsin. However, alternatives to traditional calf rennet are now used widely, including fermentation-produced chymosin or enzymes from *Rhizomucor miehei*, *Rhizomucor pusillus* or *Cryphonectria parasitica*. Depending on factors such as enzyme type, pH at whey drainage, cook temperature and the moisture content of the curd, from ~0 to 15% of the rennet activity added to the milk is retained in the curd (Upadhyay *et al.*, 2004) and, as discussed below, is a major proteolytic agent in the ripening of most varieties, catalysing mainly the hydrolysis of α_{s1} -casein.

The gel formed on the rennet-induced coagulation of milk is quite stable if left undisturbed, but if it is cut or broken, it synereses rapidly, expelling the liquid entrapped within the gel as whey. The rate and extent of syneresis, and thus the moisture content of the cheese, are controlled during manufacture by varying factors such as the composition of the milk, size of curd particles, cooking temperature, rate of acidification, rate of stirring of the curds-whey mixture, and time (Fox and McSweeney, 2004). Operations at this stage of manufacture largely control the moisture content of the cheese and have a major indirect effect on ripening since, all else being equal, high-moisture cheeses will ripen more quickly than cheeses with a low moisture content. A high cooking temperature (e.g., during the manufacture of Swiss-type cheeses or Italian Grana-type varieties) also influences ripening since, in addition to promoting

4 Improving the flavour of cheese

syneresis, it inactivates much of the remaining chymosin, while the level of plasmin, a heat-stable enzyme, is increased through the denaturation of plasmin inhibitors and the inhibitors of plasminogen activators (Farkye and Fox, 1990).

The curds for Cheddar and related varieties are texturized after whey drainage. During this 'cheddaring' process, the pH of the curds decreases to ~5.4, which causes dissolution of some colloidal calcium phosphate, thus altering the Ca:protein ratio and modifying the texture of the curd. In traditional practice, cheddaring is performed by the repeated piling and re-piling of curd blocks in the vat. However, this traditional practice serves simply to facilitate removal of a small amount of whey, to keep the curds warm and to allow time for acid to develop, and it has been replaced in industry by towers and belt systems which achieve the same result (see Bennett and Johnston, 2004). The curds for *pasta-filata* cheese (e.g., Mozzarella) are also allowed to develop acid and, after acidification, are heated to 60–65°C in hot water and stretched, which contributes to the desirable functional characteristics of melted Mozzarella used extensively as a pizza topping. The cooking–stretching step of *pasta-filata* cheeses also has a major influence on their ripening through the inactivation of much rennet activity and the killing of the starter bacteria (Kindstedt *et al.*, 2004).

All cheeses are salted either by immersion in brine (most varieties), or by mixing dry salt with milled curd (Cheddar and related cheeses), or by application of dry salt to the surface of the cheese after moulding. The amount of salt added to cheese is characteristic of the variety; a low level of salt is added to Swiss-type cheeses while varieties ripened under brine (e.g., Feta) contain a high level of NaCl. The rate at which salt-in-moisture (S/M) increases is quite slow in brine-salted varieties, since NaCl must diffuse from the surface of the cheese, while uptake is very rapid in dry-salted varieties (e.g., Cheddar) where NaCl is mixed with milled curd (Guinee and Fox, 2004). The level of S/M and the rate at which S/M increases are factors that have a major influence on ripening (see Guinee and Fox, 2004). NaCl at >1.5% (w/w) inhibits acid production by the starter culture and thus, in some varieties (e.g., Cheddar which is dry salted), salt stops acidification and fixes the pH of the cheese for the early stages of ripening. In Swiss-type cheese, the growth of *Propionibacterium freudenreichii* is inhibited by NaCl to an extent determined by strain and pH. The growth of the secondary culture *Penicillium roqueforti* in Blue cheese is stimulated by 1% (w/w) NaCl but inhibited by >3–5% (w/w) NaCl, depending on the strain. Likewise, a low level of NaCl stimulates the growth of *Penicillium camemberti* on white-mould cheeses (see Guinee and Fox, 2004). NaCl also affects ripening through changes in the activity of enzymes. Plasmin, the principal indigenous proteinase in milk, is stimulated by 2% (w/w) NaCl but is inhibited by a high salt concentration (Noomen, 1978).

There is relatively little evidence for a direct effect of salt on microbial enzymes, although most are probably inhibited by moderately high NaCl levels, particularly at low pH (see Guinee and Fox, 2004). At concentrations typically found in cheese, NaCl probably has little direct effect on chymosin action during

ripening. However, salt plays a major indirect role on the hydrolysis of the caseins by chymosin during ripening through its effects on its substrates. The hydrolysis of α_{s1} -casein in dilute solution by chymosin is stimulated by NaCl to an optimum at ~6% (w/w) (Fox and Walley, 1971) but its hydrolysis is retarded by very low levels of salt in cheese (see Guinee and Fox, 2004). Of more significance to cheese quality is the effect of NaCl on the hydrolysis of β -casein. Increasing the ionic strength of cheese through the addition of NaCl promotes interactions between the hydrophobic C-terminal regions of β -casein and inhibits the access of chymosin to its cleavage sites. Decreasing NaCl levels in the cheese facilitates chymosin action on β -casein and thus the production of hydrophobic peptides from the C-terminal of β -casein (e.g., β -CN f193-209) which are extremely bitter (Kelly *et al.*, 1996). In addition to its indirect effects on flavour through its effects on microbial growth and enzyme activity, salt also has direct effects by contributing to the savoury flavour of cheese. Salt has a major effect on the composition of cheese, since ~2 kg H₂O are lost per kg NaCl absorbed (Fox *et al.*, 2000), and thus also influences flavour and textural changes during ripening through its effect on water activity (a_w) and the moisture content of cheese.

The final or penultimate step in the manufacture of curd is moulding and, in the case of low-moisture varieties, pressing. The curds for brine-salted cheeses are moulded prior to salting, while those for dry-salted varieties are moulded and pressed after the addition of salt. The size and shape into which the curds for a particular variety are moulded are often not simply cosmetic. For example, the traditional size and shape for Emmental is a large wheel up to 1 m in diameter and weighing up to ~100 kg. This large size is necessary to trap some CO₂ produced during ripening and to allow it to reach a sufficient partial pressure to form eyes. Also, many surface-ripened varieties (e.g., Camembert or smear-ripened cheeses) are moulded into the form of a small, short cylinder. Since many important events during ripening occur at the surface, the ratio of surface area to volume is very important for these cheeses. If this ratio is too low (i.e., if the cheese is large), the surface may ripen excessively but the core may remain unripe.

1.2 Overview of cheese ripening

Rennet-coagulated cheeses are ripened for a period ranging from about two weeks (e.g., Mozzarella) to two or more years (e.g., Parmigiano-Reggiano or extra-mature Cheddar) to allow the flavour and texture characteristic of the variety to develop. During this time, major microbiological and biochemical changes occur in cheese.

Microorganisms gain entry into cheese curd either by deliberate addition as part of the starter culture, as added adjunct starters, or by being naturally present in the ingredients of cheese, particularly the milk. The composition of the cheese microflora is affected by the type of starter and adjunct used, pasteurization of

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the milk and the cooking temperature used during manufacture and, later, the environment of the cheese (i.e., NaCl and moisture levels, pH, presence of organic acids and nitrate, redox potential and temperature) (Beresford *et al.*, 2001). Cheese ripening is characterized by a number of microbial changes. In most varieties, the starter reaches cell numbers of $\geq 10^8$ cfu g⁻¹ within one day of manufacture, after which they die off, lyse and release their intracellular enzymes into the matrix of the curd (see Beresford and Williams, 2004; Lortal and Chapot-Chartier, 2005). However, some evidence is emerging which demonstrates that the starter culture only partially lyses, but rather remains unculturable yet metabolically active for production of flavour compounds (Stuart *et al.*, 1998; Chou and Weimer, 2001; Ganesan *et al.*, 2004a, b; Ganesan and Weimer, 2004). Non-starter lactic acid bacteria (NSLAB) are adventitious microorganisms, principally facultatively heterofermentative lactobacilli such as *Lactobacillus casei* and *Lactobacillus paracasei*. NSLAB grow in probably all ripened cheeses at a rate largely dependent on temperature, from a very low initial number (typically $< 10^2$ cfu g⁻¹ after manufacture) to $\sim 10^7$ cfu g⁻¹, thus often becoming the dominant viable microorganisms in many cheese varieties later in ripening, and they play a role in ripening (see Fox *et al.*, 1998; Beresford and Williams, 2004).

The ripening of certain varieties is characterized by the growth of a secondary microflora that often dominates the ripening of these cheeses. *Propionibacterium freudenreichii* grow during the ripening of Swiss-type cheeses and are essential for the characteristic eye development (see Fröhlich-Wyder and Bachmann, 2004). After the manufacture of surface mould-ripened cheeses, yeasts including *Kluyveromyces lactis*, *Saccharomyces cerevisiae* and *Debaryomyces hansenii* grow initially, together with the mould *Geotrichum candidum*. After 6 or 7 days of ripening, *Penicillium camemberti* begins to grow and forms the dense white felt characteristic of the surface of Camembert and Brie. Later during the ripening of these cheeses, Gram-positive coryneform bacteria begin to develop (Spinnler and Gripon, 2004). Yeasts also grow in Blue cheeses but the organism which dominates the ripening of these cheeses is the mould *Penicillium roqueforti* (Cantor *et al.*, 2004). Finally, in bacterial surface-ripened, or smear-ripened, varieties yeasts grow initially on the surface and are followed by a very complex Gram-positive bacterial microflora composed of coryneform bacteria (*Arthrobacter*, *Brachybacterium*, *Brevibacterium*, *Corynebacterium* and *Microbacterium* spp.), micrococci and staphylococci (Brennan *et al.*, 2004). This complex bacterial microflora gives these cheeses their characteristic red-orange colour and pungent aroma.

The biochemical changes which occur in cheese during ripening are often grouped into three major categories:

- Metabolism of residual lactose and of lactate and citrate
- Liberation of fatty acids from triacylglycerols (lipolysis) and the subsequent metabolism of fatty acids to various volatile flavour compounds
- Degradation of the casein matrix of the curd to a range of peptides and,

ultimately, to free amino acids (FAA). FAA then act as substrates for a complex series of catabolic reactions which produce many important flavour compounds, especially carboxylic acids and sulfur compounds.

Since cheese is a fermented dairy product, the metabolism of lactose to lactate by the starter LAB is an essential feature of its manufacture. Most of the lactose in milk is lost in the whey as lactose or lactate but cheese curd at the start of ripening contains a low level of lactose. It is important that the residual lactose in cheese curd is completely metabolized to avoid the development of an atypical secondary flora or excessive Maillard browning on heating. The rate of metabolism of residual lactose in cheese early in ripening is determined largely by the S/M level in the curd, since this parameter greatly affects the action of starter bacteria. In Cheddar and related dry-salted varieties, starter activity is stopped very quickly at the end of manufacture, since the S/M level increases rapidly. In large brine-salted cheeses, the S/M level increases slowly as salt diffuses through the cheese. Lactose that remains unfermented by the starter bacteria is probably metabolized by the NSLAB flora (McSweeney and Fox, 2004). Lactose fermentation during the manufacture and the early stages of the ripening of Swiss-type cheeses is complex. The glucose moiety of lactose is metabolized quickly by *Streptococcus thermophilus* as the curd cools and galactose accumulates initially, but this sugar and any remaining lactose are metabolized by the thermophilic *Lactobacillus* present as a component of the starter culture (Turner *et al.*, 1983; Fox *et al.*, 1990).

Lactate is an important substrate for a series of reactions during ripening. In most cheeses, L-lactate is racemized slowly to DL-lactate by the action of the NSLAB flora. Racemization of lactate is not important for the flavour of cheese but it favours the development of Ca-lactate crystals (see McSweeney and Fox, 2004) which develop in cheese during ripening and, although harmless, may cause consumers to reject the cheese as containing foreign bodies or mould. Racemization appears to be important for the formation of Ca-lactate crystals as it lowers the concentration threshold for crystallization; however, racemization is not an absolute requirement for the development of Ca-lactate crystals.

Lactate can be metabolized by some LAB to acetate, ethanol, formate and CO₂ (Fox *et al.*, 2000) or by pediococci (occasionally present in cheese as a component of the NSLAB flora) to acetate and CO₂ (Thomas *et al.*, 1985). However, these pathways are not significant in cheese, particularly varieties ripened in film wrappings, due to the very low level of oxygen in the curd (McSweeney, 2004). A serious defect known as late gas blowing is caused by the anaerobic fermentation of lactate to butyrate and H₂ and CO₂ by *Clostridium tyrobutyricum*. Late gas blowing is of particular concern in brine-salted cheeses since the S/M level increases slowly; the defect may be avoided by strategies aimed at minimizing the numbers of spores in milk, physically removing the spores or preventing spore germination (see McSweeney and Fox, 2004).

Lactate metabolism is vital for the ripening of Swiss-type and surface mould-ripened cheeses (e.g., Camembert). In the former group of cheeses, lactate is

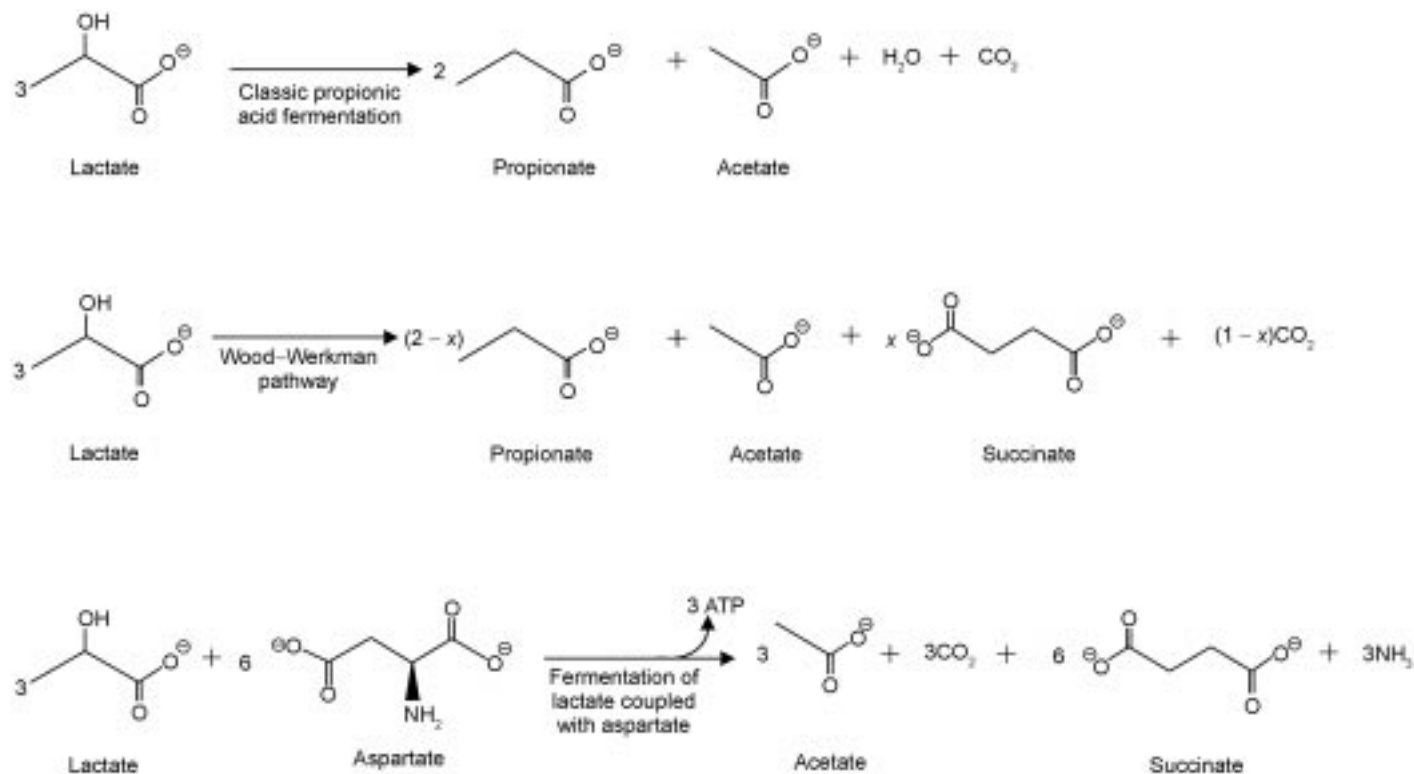


Fig. 1.2 Utilization of lactate by *Propionibacterium freudenreichii* (after Fröhlich-Wyder and Bachmann, 2004).

metabolized by *Propionibacterium freudenreichii* by one of three pathways (Fig. 1.2), although the Wood–Werkman pathway is of minor importance. Strains of *P. freudenreichii* differ in their aspartase activity and hence in their ability to couple the fermentation of lactate with aspartate; strains with low aspartase activity metabolize lactate mainly by the classical propionate fermentation (Fröhlich-Wyder and Bachmann, 2004). The volatile carboxylic acids produced in these biochemical pathways contribute to the flavour of Swiss-type cheeses, while the CO₂ migrates through the curd where some accumulates to form the eyes characteristic of these varieties.

The catabolism of lactate is extensive in surface mould-ripened cheeses (e.g., Camembert and Brie) where it is metabolized oxidatively at the cheese surface by *Penicillium camemberti*. Metabolism of lactate causes deacidification of the cheese surface, resulting in a pH gradient from the core into the exterior. When lactate is exhausted, *Penicillium camemberti* metabolizes proteins, ultimately producing NH₃ that diffuses into the cheese. The concentration of calcium phosphate at the exterior exceeds its solubility at the high pH and it precipitates as Ca₃(PO₄)₂ at the surface, thus causing the migration of calcium phosphate from the centre of the cheese towards its surface (Fig. 1.3). The movement of calcium phosphate from the centre of the cheese and the high pH lead to the characteristic softening of Camembert-type cheeses which become almost liquid on extended storage (McSweeney and Fox, 2004).

Although milk contains a relatively low level of citrate (~8 mmol L⁻¹) and most of it is lost in the whey, citrate is an important precursor for flavour compounds and CO₂ which causes development of the few characteristic eyes in Dutch cheeses (Fox *et al.*, 1990; Parente and Cogan, 2004; McSweeney and Fox, 2004). In Dutch-type cheeses, citrate is metabolized with the production of diacetyl, acetoin, 2,3-butanediol and CO₂ by citrate-positive strains of *Lactococcus*, *Leuconostoc mesenteroides* subsp. *cremoris* or *Leuconostoc lactis*, although citrate may also be metabolized by some facultatively heterofermentative lactobacilli that are common components of the NSLAB flora.

As discussed in detail in Chapter 5 and by McSweeney and Sousa (2000) and Collins *et al.* (2003a, 2004), fat is an important source of many volatile flavour compounds in cheese. Unlike many high fat foods, lipid oxidation does not usually occur in cheese to an appreciable extent due to its low oxidation–reduction potential. However, triacylglycerols in cheese are hydrolysed by indigenous, endogenous and/or exogenous lipases to a range of fatty acids. Lipolytic enzymes in cheese generally originate from the milk, from the rennet preparation (if rennet paste is used) or to a limited extent from the cheese microflora. The indigenous lipoprotein lipase in milk is of most importance for lipolysis in raw milk cheeses, since this enzyme is extensively inactivated by pasteurization. Rennet extract used to coagulate milk for the manufacture of most cheese varieties is free from lipase activity. However, the milk for certain, mainly Italian and Greek, cheese varieties is coagulated using a rennet paste produced by macerating the stomach of the young dairy animal together with its contents. Rennet paste contains a potent lipase, pregastric esterase, which causes

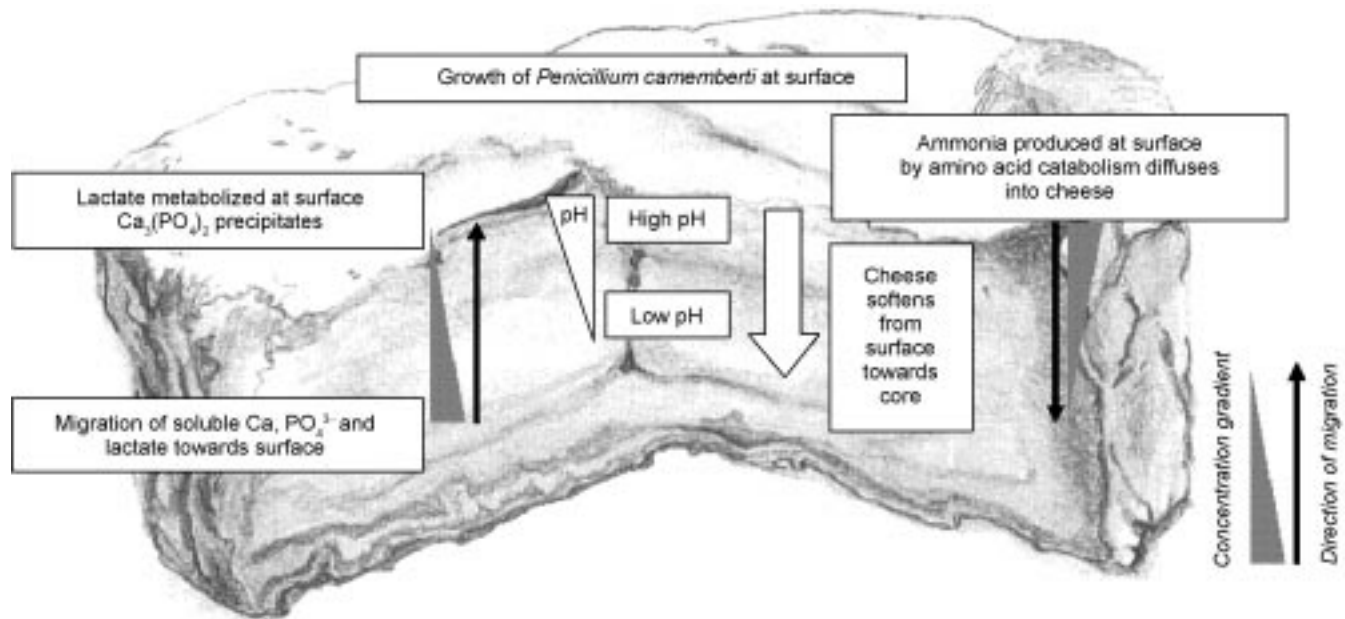


Fig. 1.3 Schematic representation of the changes which occur in Camembert-type cheese during ripening as a consequence of the growth of *Penicillium camemberti* at the cheese surface (modified from McSweeney and Fox, 2004, with permission).

extensive lipolysis in cheeses such as the various Italian Pecorino varieties, Provolone and traditional Greek Feta.

The level of lipolysis in cheese which develops a secondary flora is usually related to the lipolytic activity of that flora. The complex Gram-positive bacterial flora which develops at the surface of smear-ripened varieties includes some organisms which are quite lipolytic and contribute to the liberation of fatty acids in these varieties. *Propionibacterium freudenreichii* is more lipolytic than LAB and contributes to lipolysis in Swiss cheeses during ripening. However, the most lipolytic secondary organisms associated with cheese are *Penicillium* spp. that grow in or on mould-ripened varieties. *Penicillium roqueforti*, enzymes from which catalyse the extensive lipolysis in Blue cheese, is very lipolytic; this organism produces two potent extracellular lipases with pH optima of 7.5–8 and 9–9.5. *Penicillium camemberti*, the characteristic mould growing on Camembert and Brie-type cheeses, produces one extracellular lipase that is optimally active at pH 9 and 35°C (Lamberet and Lenoir, 1976). A low level of lipolysis occurs in internal bacterially-ripened varieties made from pasteurized milk (e.g., Cheddar and Gouda) as a result of the action of enzymes from starter and non-starter LAB which, although weakly lipolytic, are present at high numbers for long periods of time (McSweeney, 2004). Lipolytic enzymes in LAB are intracellular and a relationship between cell lysis and lipolysis has been demonstrated in Cheddar cheese (Collins *et al.*, 2003b).

Ruminant milk fat is rich in short chain fatty acids which, when liberated by lipolysis, contribute directly to the flavour of cheese. Although some lipolysis occurs in all cheeses, it is most extensive in varieties made using rennet paste, cheeses that are ripened for a long period of time or which develop a strongly lipolytic secondary flora (e.g., Blue or smear cheeses). Low levels of free fatty acids develop during the ripening of most other varieties (e.g., Swiss, Gouda); in these cheeses, excessive lipolysis is undesirable and results in rancidity (Collins *et al.*, 2003a).

In addition to their direct contribution to cheese flavour, fatty acids serve as important precursors for many volatile flavour compounds produced through a series of pathways known collectively as fatty acid metabolism (Collins *et al.*, 2003a, 2004; McSweeney, 2004). It was thought that esters were formed by direct reaction of an alcohol (usually ethanol) with a fatty acid. However, recent work (Liu *et al.*, 2003; Holland *et al.*, 2005) has provided evidence that the major pathway for the production of esters may be via a transferase reaction (alcoholysis; $R_1\text{-COO-R}_2 + R_3\text{-OH} \rightarrow R_1\text{-COO-R}_3 + R_2\text{-OH}$). Thioesters are formed by the reaction of a fatty acid with a thiol compound, most commonly CH_3SH , thus producing a range of *S*-methylthioesters (McSweeney and Sousa, 2000; Collins *et al.*, 2003a, 2004). Fatty acid lactones are cyclic compounds formed through the intramolecular esterification of a hydroxyacid; both γ - and δ -lactones have been found in cheese and have five- and six-sided heterocyclic rings, respectively. Recent work by Alewijn *et al.* (2007) has suggested that lactones in Gouda cheese are formed by a one-step non-enzymatic reaction in which a hydroxy fatty acid esterified in a triacylglycerol undergoes an intra-

molecular transesterification to release a lactone directly. However, the metabolism of free fatty acids is most extensive and of most importance in Blue cheeses where fatty acids are metabolized to alkan-2-ones (*n*-methyl ketones; R-CO-CH₃) by *Penicillium roqueforti* via a pathway similar to the early stage of β -oxidation. Approximately 11 alkan-2-ones have been found in cheese, but the most common are pentan-2-one, heptan-2-one and nonan-2-one which give the characteristic pungent aroma to Blue cheese (McSweeney, 2004). Alkan-2-ones may be reduced to their corresponding secondary alcohol (R-COH-CH₃).

Proteolysis is perhaps the most complex biochemical event that occurs in most cheese varieties during ripening and the literature on this topic has been reviewed extensively (Grappin *et al.*, 1985; Rank *et al.*, 1985; Fox, 1989; Fox and Law, 1991; Fox *et al.*, 1993, 1994, 1995a, b, 1996a; Fox and McSweeney, 1996, 1997; Sousa *et al.*, 2001; Upadhyay *et al.*, 2004). Proteinases and peptidases which catalyse proteolysis in cheese originate from six sources: the milk, the coagulant, starter LAB, NSLAB, secondary cultures and, in rare cases, exogenous proteinases (e.g., used to accelerate ripening).

Enzymes from the coagulant (usually chymosin) play a very important role in ripening, acting principally on α_{s1} -casein which is cleaved at a number of sites, forming peptides including α_{s1} -CN (f1-23), (f24-199) and (f102-199). As discussed above, chymosin action on β -casein in cheese is strongly inhibited by the presence of NaCl. α_{s2} -Casein and *para*- κ -casein appear to be relatively resistant to chymosin action in cheese, although the former protein may be hydrolysed slowly by chymosin *in vitro*.

The role of the indigenous milk proteinases in cheese ripening has been the subject of extensive study (see reviews by Kelly and McSweeney, 2003; Upadhyay *et al.*, 2004). The principal proteinase in milk, plasmin, is produced from its inactive precursor, plasminogen, under the control of a complex system of activators and inhibitors. Plasmin acts principally on β -casein during ripening, producing γ_1 -, γ_2 - and γ_3 -caseins and proteose peptones. *In vitro*, α_{s2} -casein is a very good substrate for plasmin and this protein disappears in many cheeses during ripening. Although this phenomenon has not been studied in detail, it is likely that plasmin degrades α_{s2} -casein during ripening. Recently, the role of lysosomal proteinases from somatic cells in cheese ripening has been studied (see Hurley *et al.*, 2000a; Kelly and McSweeney, 2003; Upadhyay *et al.*, 2004). The presence in milk of cathepsins D and B has been confirmed and a role for the former in cheese ripening has been demonstrated, at least under certain circumstances (Hurley *et al.*, 2000b).

Although a portion of the starter culture lyses during ripening, their enzymes, most of which are intracellular, contribute to flavour production via increased access to substrate; intact cells may also remain metabolically active (Ganesan *et al.*, 2006; Stuart *et al.*, 1998). The principal proteinase of most LAB is lactocepin, a serine proteinase loosely attached to the cell surface and the gene for which is plasmid-encoded. When the cell is growing in milk, lactocepin acts to degrade caseins; however, the major role of this enzyme in cheese ripening appears to be in the degradation of intermediate-sized peptides produced from

the caseins by plasmin or chymosin (Upadhyay *et al.*, 2004). Lactococci, and perhaps other genera of LAB, also possess intracellular proteinases, although their role in cheese ripening is unclear. LAB possess a wide range of intracellular peptidases (Fig. 1.4), including oligoendopeptidases, di- and tripeptidases, aminopeptidases and a number of proline-specific peptidases but apparently no carboxypeptidase. Proline-specific peptidases are of particular importance since the caseins are rich in this amino acid and, due to its ring structure, many peptidases are unable to hydrolyse proline-containing peptides. Peptidases of LAB act on short peptides, often releasing free amino acids. The extensive literature on the peptidases of LAB was summarized recently by Upadhyay *et al.* (2004).

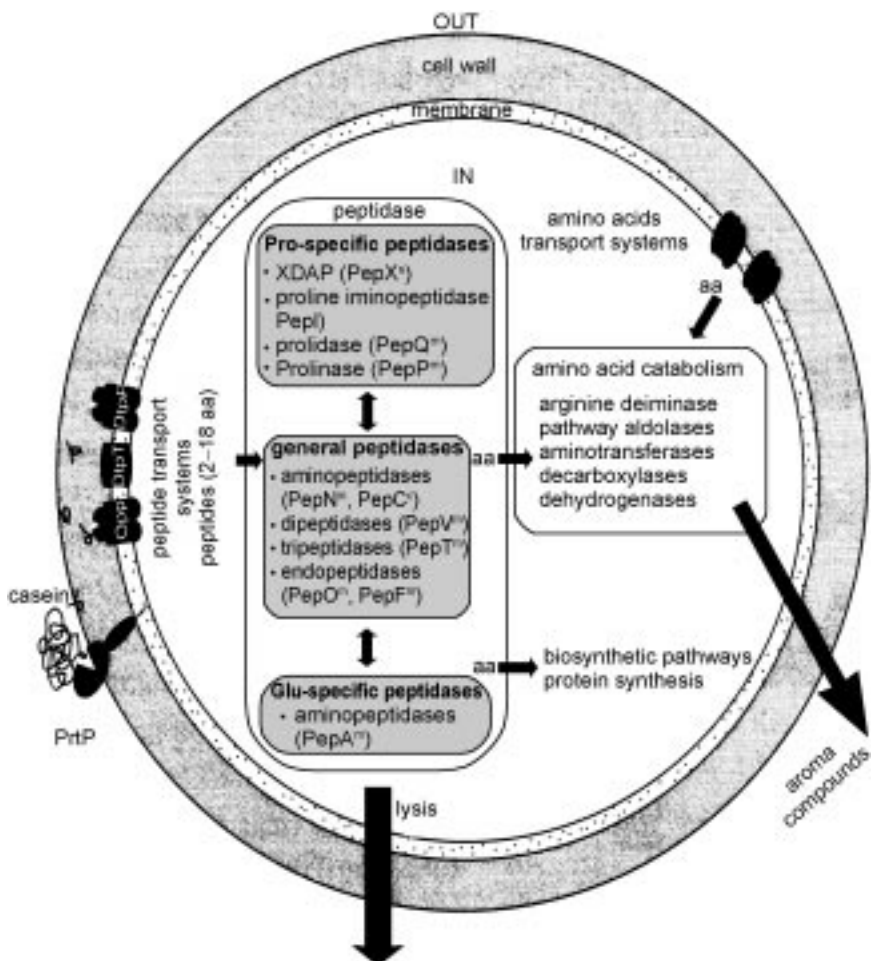


Fig. 1.4 Proteolytic enzymes of *Lactococcus* that contribute to cheese ripening (from Parente and Cogan, 2004, with permission).

The proteinase/peptidase systems of NSLAB appear to be generally similar to those of starter LAB and the proteolytic systems of secondary organisms in cheese often play significant roles in ripening. *Penicillium camemberti* and *Penicillium roqueforti* synthesize aspartyl- and metalloproteinases and an acid carboxypeptidase (Gripon, 1993). *Geotrichum candidum* also produces extracellular and intracellular proteinases but it is thought that its contribution to proteolysis in Camembert is less than that of *Penicillium camemberti* (Gripon, 1993). As discussed above, smear-ripened cheeses are characterized by the growth of a complex Gram-positive bacterial flora. Although organisms from a number of genera are present on the surface of these cheeses, enzymes from *Brevibacterium linens* have been studied in most detail, although some information is available about enzymes from *Arthrobacter nicotianae* and *Micrococcus* spp. *Brevibacterium linens* secretes extracellular proteinases, extracellular aminopeptidases and intracellular proteinases and peptidases (see Rattray and Fox, 1999; Upadhyay *et al.*, 2004). The secondary organism of Swiss-type cheeses, *Propionibacterium freudenreichii*, is weakly proteolytic but does produce active peptidases which contribute to the ripening of these varieties (Gagnaire *et al.*, 1999).

Proteolysis contributes to the development of cheese texture directly through hydrolysis of the casein matrix of cheese and by reducing the a_w of cheese through changes to water binding by the COO^- and NH_3^+ groups liberated on hydrolysis of a peptide bond and, indirectly, via an increase in pH caused by the liberation of NH_3 from amino acids produced by proteolysis. Proteolysis also contributes to the flavour and off-flavour of cheese by producing short peptides and amino acids, some of which have flavours, and by facilitating the release of sapid compounds from the cheese matrix during mastication (Upadhyay *et al.*, 2004). However, in recent years, it has become apparent that the most important role of proteolysis in the biogenesis of cheese flavour is through the production of free amino acids which are catabolized to a wide range of volatile flavour compounds. Amino acid catabolism has been an active area of research in recent years and the topic has been reviewed (McSweeney and Sousa, 2000; Yvon and Rijnen, 2001; Smit *et al.*, 2002; Curtin and McSweeney, 2004; Ardö, 2006) and is discussed in detail in Chapter 4.

Amino acids in cheese appear to be catabolized by two major pathways (Yvon and Rijnen, 2001) initiated by the action of an aminotransferase or an amino acid lyase. Other pathways including decarboxylation and deamination also occur; the latter may be quite important in certain varieties (e.g., Gruyère) in which much NH_3 is produced during ripening. The details of catabolic pathways remain to be elucidated fully, and work to date has concentrated on branched-chain and aromatic amino acids and methionine. These investigations have provided insight into the production of many flavour compounds in cheese. As an example, the catabolic pathways for leucine are shown in Fig. 1.5.

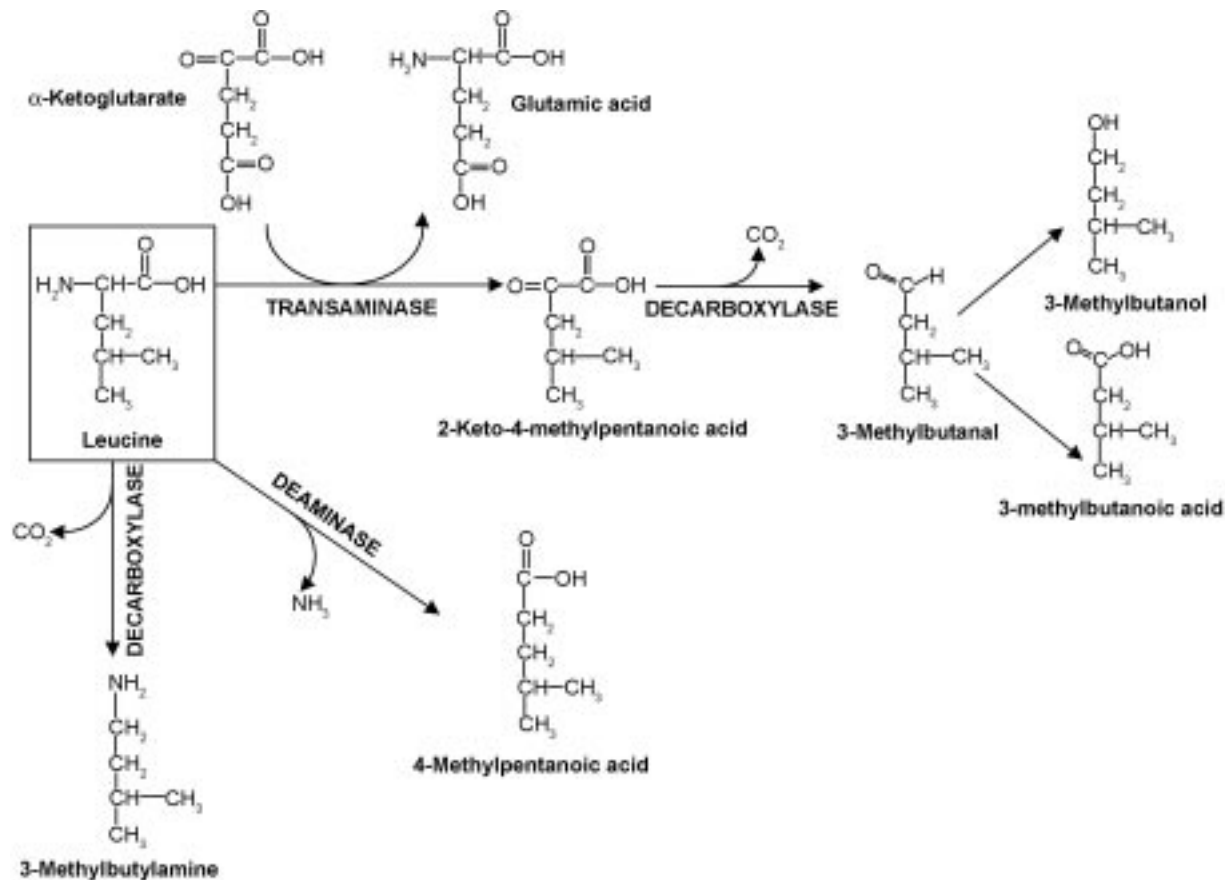


Fig. 1.5 Pathways for the catabolism of leucine; similar catabolic pathways also exist for the other branched-chain amino acids (from McSweeney, 2004, with permission).

1.3 Bitterness

Bitterness is a taste sensation perceived towards the back of the tongue and is a taste defect associated with dairy products including cheese, fermented milks and casein hydrolysates. Literature on the bitter defect was reviewed by Lemieux and Simard (1991, 1992), McSweeney (1997) and McSweeney *et al.* (1997). Although numerous compounds in cheese have a bitter taste at sufficient concentration, the bitter defect in cheese results from the excessive accumulation of hydrophobic peptides. The mean hydrophobicity (Q) of a peptide is an important factor in determining the bitterness of a peptide (McSweeney, 1997):

$$Q = \frac{\sum \Delta f_i}{n}$$

where Δf_i is the hydrophobicity of the amino acid side chain (free energy of transfer) and n is the number of amino acid residues in the peptide. However, the distribution of hydrophobic amino acid residues along the peptide chain also influences bitterness (Adler-Nissen, 1986). Therefore, degradation of proteins with a high mean hydrophobicity is likely to produce bitter peptides; this is of particular significance for cheese as the caseins are relatively hydrophobic proteins. Peptides of molecular mass *ca.* 0.1 to 6 kDa with $Q > 1400$ cal residue⁻¹ are often bitter. Thus, bitterness is principally associated with short, hydrophobic peptides; larger peptides, even if they are relatively hydrophobic, are perceived as being less bitter than a short peptide of the same value of Q . Likewise, the degradation of a short peptide into its constituent amino acids residues through the action of exopeptidases reduces the intensity of bitterness.

The bitter defect in cheese occurs when hydrophobic short peptides accumulate to an excessive extent either due to overproduction or to inadequate degradation due to lack of peptidase activity. Certain starter strains are associated with the development of bitterness either because their proteinases (particularly lactocepin) produce bitter peptides directly from precursor polypeptides or, more likely, because they lack sufficient peptidase activity necessary to degrade bitter peptides produced by other enzymes (e.g., chymosin).

The specificity of different proteinases on a given protein substrate is also of significance to the development of bitterness. Plasmin, which cleaves β -casein towards its N-terminus and at the centre of the molecule, produces much less bitterness than chymosin which cleaves at the very hydrophobic C-terminal region of this protein and can produce very bitter peptides (e.g., β -CN f193-209) directly. Indeed, the action of chymosin is of great significance to the development of bitterness. Hence, factors which influence the retention of rennet activity in the curd (e.g., type and quantity of rennet used, drain pH, cooking temperature) can influence the development of bitterness.

The pH of cheese also influences the development of bitterness by affecting the activity of chymosin and other enzymes. The level of salt in cheese has a

major role in the development of bitterness, since NaCl concentration is a major factor influencing the ionic strength (μ) of the aqueous phase of cheese. The development of bitterness in cheese is dependent on ionic strength, since a high μ favours the hydrophobic association of the C-terminal region of β -casein and perhaps other large, hydrophobic but non-bitter polypeptides, thus inhibiting chymosin action (Fox and Walley, 1971). Hence, cheese with a low salt content is very prone to bitterness (Stadhouders and Hup, 1975; Stadhouders *et al.*, 1983; Visser *et al.*, 1983; Kelly *et al.*, 1996). NaCl also inhibits lactocepin (Exterkate, 1990) and affects the porosity of the starter cell wall (and thus the release of intracellular peptidases; Visser *et al.*, 1983).

The use of exogenous proteinases (e.g., to accelerate ripening; see Fox 1988/89, or in the manufacture of enzyme-modified cheese, see Kilcawley *et al.*, 1998) often causes the development of bitterness, as does the use of coagulants with an excessive ratio of general proteolysis to milk coagulating activity (e.g., certain plant enzymes). Bitterness is also often encountered during the ripening of reduced fat cheese (Banks *et al.*, 1992), perhaps due to reduced opportunity for hydrophobic peptides to partition into the lipid phase. Cheese made from milk containing high levels of proteinases produced by psychrotrophic bacteria may also develop bitterness (Hicks *et al.*, 1986) or factors that reduce starter culture numbers (e.g., bacteriophage or antibiotics) and thus reduce the level of LAB peptidases in the cheese matrix.

Bitterness is also of significance to casein hydrolysates and various strategies have been adopted to ameliorate this defect, including adsorption of hydrophobic peptides onto activated charcoal, their removal using hydrophobic interaction chromatography, solvent extraction or isoelectric precipitation, the use of cyclodextrins as masking agents or hydrolysis of bitter peptides using exopeptidases (see McSweeney *et al.*, 1997). In cases where bitterness develops unavoidably in cheese, the most useful debittering strategy involves the use of starters, adjuncts or enzyme preparations with high exopeptidase activities. Koka and Weimer (2000) also found that bitter peptides found in Cheddar cheese could be hydrolysed by the purified proteinase of *Pseudomonas fluorescens* RO98.

1.4 Acceleration of cheese ripening

Cheese ripening is a slow, and thus expensive, process. Costs involved in cheese ripening stem primarily from the inventory cost of delaying the sale of a large proportion of a year's production, the capital cost associated with ripening rooms and the need to control temperature and, often, relative humidity. The cost of ripening a hard cheese such as Cheddar has been estimated at approximately €500–800 (US\$640–1025) per tonne of cheese matured for nine months (Upadhyay and McSweeney, 2003). Hence, methods to accelerate cheese ripening have received considerable attention in the scientific literature and have been reviewed by Fox (1988/89), El Soda and Pandian (1991),

Wilkinson (1993), Fox *et al.* (1996b) and Upadhyay and McSweeney (2003). Various approaches used to accelerate cheese ripening include:

- Addition of exogenous enzymes
- Increasing the level of LAB enzymes through the use of attenuated starters or an increased rate of lysis
- Use of adjunct cultures
- Genetic modification of starter bacteria
- The use of high hydrostatic pressures
- Elevated ripening temperature.

The addition of free or encapsulated enzymes to cheese at various stages of manufacture has been studied by numerous authors but with limited success (see Upadhyay and McSweeney, 2003). Apart from the fact that much of the enzyme added to the milk may be lost in the whey, the addition of single enzymes will accelerate only one step in the complex series of biochemical events that constitutes cheese ripening, often leading to an unbalanced flavour. The use of a mixture of enzymes has the major advantage of accelerating multiple ripening steps and a number of such preparations, usually containing proteinases, peptidases and, often, lipases are available commercially (see Upadhyay and McSweeney, 2003).

Another approach to increase the enzyme complement in cheese is to add enzymes naturally encapsulated within attenuated cells (Klein and Lortal, 1999). Attenuated starters are LAB which are unable to produce acid during cheese manufacture but which can provide enzymes that contribute to ripening. LAB cells may be attenuated by heat-shock, freezing/thawing, freeze or spray drying, lysozyme treatment, use of solvents or through natural (e.g., by the removal of a plasmid to produce lactase-negative mutants) or induced genetic modification. The advantages of attenuated starters include that they contain a wide range of enzymes, are subject to few legal barriers and are largely retained in the cheese curd. Lysis of LAB cells occurs during ripening at a rate dependent on the strain and liberates many enzymes important for ripening into the matrix of the curd. Acceleration of lysis through the use of bacteriophage, bacteriocins or bacteriocin-producing cultures may influence ripening (see Upadhyay and McSweeney, 2003). However, the correct balance between intact and lysed cells is important for ripening, as the former may be metabolically active and can catalyse co-factor dependent reactions more easily (Crow *et al.*, 1995).

The use of adjunct cultures to accelerate ripening or to modify cheese flavour has shown considerable promise. Adjunct starters are organisms which are added to cheesemilk or encouraged to grow in or on cheese but which do not contribute to acidification. Many cheeses are made using secondary cultures (e.g., *Propionibacterium freudenreichii* in Swiss-type cheeses, *Penicillium camemberti* or *Penicillium roqueforti* in mould-ripened cheeses, or a complex Gram-positive bacterial surface flora on smear-ripened cheeses). Indeed, Cheddar is amongst the few varieties which are not traditionally made using secondary cultures. Hence, much research has been focused on the effects of

NSLAB and thermophilic lactobacilli as adjuncts on the ripening of full-fat and reduced-fat Cheddar and most success has been achieved with the latter adjuncts (e.g., Tobin, 1999; Hannon *et al.*, 2003). Use of surface ripening cultures as adjuncts has also met with some success to accelerate ripening of reduced-fat Cheddar cheese (Weimer *et al.*, 1997).

Despite substantial research and highly successful mutation strategies for genetic modification in LAB, approaches to accelerating cheese ripening involving genetic modification (GM) of starter bacteria have achieved little commercial success, mostly due to regulatory and consumer concerns related to the use of GM organisms in foods and partly because the enzymes targeted (mainly proteinases and peptidases) play a relatively minor direct role in the development of cheese flavour (see Upadhyay and McSweeney, 2003). However, recent research on amino acid catabolic enzymes (for reviews see Yvon and Rijnen, 2001; Curtin and McSweeney, 2004; Ardö, 2006) and other enzymes which are important for the production of volatile flavour compounds may give impetus to the construction of GM starters with improved cheesemaking potential. High hydrostatic pressures have been investigated as a means of accelerating ripening (see Trujillo *et al.*, 2000; O'Reilly *et al.*, 2001; Huppertz *et al.*, 2002; Upadhyay and McSweeney, 2003). However, despite an early report of the usefulness of this technique (Yokoyama *et al.*, 1992), recent research has shown only modest acceleration of ripening, probably as a consequence of increased lysis due to the pressure treatment (Upadhyay and McSweeney, 2003).

Probably the most efficient and simplest method for accelerating the ripening of hard cheeses is the use of an elevated ripening temperature. Increasing the ripening temperature accelerates proteolysis (e.g., Aston *et al.*, 1983a, b; Fedrick *et al.*, 1983; Folkertsma *et al.*, 1996), lipolysis (Folkertsma *et al.*, 1996; O'Mahony *et al.*, 2006) and influences cheese microflora (Cromie *et al.*, 1987; Folkertsma *et al.*, 1996). However, elevated temperatures may cause texture defects during ripening (e.g., changes to the shape of the cheese or exudation of liquid fat at the surface). In addition, elevated temperatures may increase the risk of microbial spoilage or the development of an unbalanced or poor flavour, and hence cheese ripened at a high temperature should be monitored very closely. Nevertheless, it appears that a ripening temperature of up to *ca.* 15°C (Fedrick, 1987; Folkertsma *et al.*, 1996) is a potentially viable approach to accelerate the ripening of Cheddar cheese made in large factories with good hygiene and adequate monitoring systems.

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Compounds associated with cheese flavor

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2.1 Introduction

‘Say cheese’, calls the photographer in any part of the world when a photograph is to be taken. This is the extent to which the taste of cheese has influenced people such that it brings a smile upon a mention. However, cheese flavor is a very complex phenomenon. Even though there is a unique flavor for every cheese, there is a large range of specific compounds that have varying quantities in each cheese type to constitute cheese flavor. Years of research have not yielded a single unique compound that contributes to any cheese flavor in isolation (Manning, 1979a, b). Hence, the Component Balance Theory of Cheese Flavor (Müllder, 1952), that attributes cheese flavor to a delicate balance among a multitude of compounds, is widely accepted.

It is commonly accepted that bacterial metabolism is the key to the development of cheese flavor for acid production in the vat and complex compound production during ripening (Law *et al.*, 1976). Many compound classes are present in cheese, a majority of which originate from bacterial metabolism, either direct products of metabolic pathways (e.g. volatile sulfur compounds and fatty acids), or chemical combinations of different product classes such as methional and S-methyl-thioesters (Cuer *et al.*, 1979a, b; Lamberet *et al.*, 1997; Lee *et al.*, 1997; Weimer and Dias, 2005). Large libraries of such compounds are extensively studied, but only generalities about the compounds and their production have been yielded (Urbach, 1993, 1995). The role of selected peptides in bitterness of cheese is widely accepted (Broadbent *et al.*, 1998; Edwards and Kosikowski, 1983; Kai, 1996). However, the role of a specific compound or a class of compounds to dominate the preferential organoleptic properties has yet to be demonstrated beyond volatile sulfur, methyl ketones and fatty acids in Cheddar cheese (Weimer

and Dias, 2005; Weimer *et al.*, 1999). Recent research efforts have focused on the ability to understand the roles of particular substrates available in cheese to act as flavor precursors for these compounds (Avsar *et al.*, 2004; Fox and Wallace, 1997; Gallardo-Escamilla *et al.*, 2005; Smit *et al.*, 2004a, b).

Fat, proteins, peptides, amino acids, volatile sulfur compounds, alcohols, aldehydes, ketones and volatile fatty acids are some of the classes of compounds that contribute to cheese flavor (Urbach, 1993). Volatile sulfur compounds play a major role in many cheese types (Aston and Douglas, 1983; Ferchichi *et al.*, 1985; Hemme *et al.*, 1982; Law and Sharpe, 1978; Manning, 1979b; Weimer *et al.*, 1999); but they alone do not lead to the total flavor perception of any cheese type. All attempts to simulate Cheddar cheese flavor have not been successful in producing real cheese flavor (Manning, 1979a). This suggests that the major components do not play a single-handed role and the role of minor components must be considered. Eventually, a scheme to define the chemical basis of flavor beyond the Component Balance Theory is necessary to objectively control and modify cheese flavor. This chapter attempts to assess and understand the role of some of the classes of cheese flavor compounds. The association of bacteria in generating these compounds will also be briefly discussed.

2.2 Bacteria and cheese flavor

Addition of bacteria is an essential step in cheese-making towards a good-flavored cheese. Cheese without bacteria fails to develop flavor during ripening (Aston and Douglas, 1983; Law and Sharpe, 1975). The flavor profile of cheeses depends on bacteria involved in cheese ripening and their catabolic capabilities leading to the different flavor compounds present in different cheeses (Fox and Wallace, 1997).

Bacteria are involved in all steps of cheese making. In the initial stages of Cheddar cheese ripening, lactic acid bacteria (LAB) catabolize lactose to lactic acid. Lactose is reduced to undetectable levels after 30 days (Crow *et al.*, 1993). Proteolysis by bacteria slowly degrades the casein matrix over time. The peptides and amino acids are transported and utilized by bacteria in the matrix (Christensen *et al.*, 1999). Peptides are directly related to bitterness in Cheddar cheese (Broadbent *et al.*, 1998). Amino acids are catabolized to flavor compounds involved in positive Cheddar flavor in culture and cheese slurries (Dias and Weimer, 1999; Harper and Wang, 1980a, b). The type of bacteria added modulates flavor production in cheese during ripening.

Previous studies focused on identifying the best flora type, either singly or in combination, to produce an acceptable Cheddar cheese flavor (Aston and Creamer, 1986; Baankreis, 1992; Banks *et al.*, 1989; Bhowmik *et al.*, 1990; Broadbent *et al.*, 1998; Christensen *et al.*, 1999; Crow *et al.*, 1993; Desmazeaud and Cogan, 1996; Dias and Weimer, 1999; Fox and Wallace, 1997; Khalid and Marth, 1990; Yvon *et al.*, 1999). The focus is often on nonstarter lactic acid bacteria (NSLAB), predominantly lactobacilli, as the causal agents of flavor on

the basis of their population in later stages of cheese ripening (Dias and Weimer, 1998; Laleye *et al.*, 1990; Muehlenkamp-Ulate and Warthesen, 1999; Peterson and Marshall, 1990; Trepanier *et al.*, 1991). The role of different genera in Cheddar cheese flavor continues to be elusive and the cumulative interpretations from numerous studies add to the controversy. Nonstarter lactobacilli intensify cheese flavor and have been adopted for addition as starter culture in hard cheeses (Fox *et al.*, 1996; Laleye *et al.*, 1990; Reiter *et al.*, 1967). However, lactococci have a unique causative role in cheese flavor, and consequently remain the heart of the starter culture (Parra *et al.*, 2000).

Total bacterial counts are related to flavor development. The lower the number of starter lactococci, the more intense the Cheddar flavor; when lactococcal plate counts of cheese are $>10^9$ cfu g⁻¹, cheese is bitter (Lowrie *et al.*, 1974). Recently, a number of authors found that lactococci enter a state of no growth, but they remain metabolically active (Kunji *et al.*, 1993; Rallu *et al.*, 1996; Stuart *et al.*, 1999; Thomas and Batt, 1969). This may lead to a limited population of lactococci detected by bacterial plate counts during cheese ripening. However, lactococcal starters continue to remain metabolically active and play a causative role in cheese flavor.

2.3 Cheese flavor

Different cheese types have groups of flavor compounds that are responsible for their unique flavors (Table 2.1) (Kristoffersen, 1975; Vedamuthu *et al.*, 1966). But the knowledge of flavor impact is limited to a few groups only (Urbach, 1993). Compounds contribute specific flavor attributes based on their physico-chemical properties (Urbach, 1993). Some compounds represent typical flavors of certain cheeses, acting as impact compounds for that flavor but not for the total flavor perception (Table 2.1). Multiple lists of flavor compounds are available in the literature (Fox and Wallace, 1997; Fox *et al.*, 1996; Urbach, 1993, 1995).

Flavor compounds are classified by at least four systematic schemes as organoleptic, chemical classes, cheese type, and originating substrate. While a sharp, nutty flavor is typical of aged Cheddar from some parts of the world, a similar profile in Mozzarella or Gouda cheeses is totally unacceptable. Several fractions of cheese have been analyzed for flavor dominators and all of them, neutral, basic, acidic, aqueous- or organic-partitioning, are known to contribute to the flavor and aroma of cheese (Aston and Creamer, 1986; Libbey and Day, 1964; McGugan *et al.*, 1979; Preininger *et al.*, 1994). This sparks debates as to which class of compounds plays the most important role, and begs the question of the appropriate ratios that are needed for the best flavor.

Hydrophobic fractions contain organic compounds and peptides from cheese that are related to flavor (Broadbent *et al.*, 1998; Kai, 1996). But perception of flavors is primarily attributed to water-soluble components. Tests for taste panel selection typically involve identifying a particular flavor type, beneficial or

Table 2.1 Flavor compounds formed during cheese ripening (adapted from El Soda, 1993; Urbach, 1993)

Type of cheese	Associated flavor compounds	Impact compounds
Cheddar	Lactic acid, acetic acid, amino acids, sulfur compounds, ammonia	Methanethiol
Swiss-type	Lactic acid, propionic acid, acetic acid, amino acids (proline), sulfur compounds, alkyl pyrazines	3-Methyl butyric acid
Blue-veined	Volatile fatty acids, ketones, amino acids, lactones, aromatic hydrocarbons, methyl ketones, secondary alcohols	Heptan-2-one
Italian	Volatile fatty acids, amino acids, alcohol, ketones	<i>n</i> -Butyric acid
Gouda	Amino acids, fatty acids	
Tilsit	Methanethiol, methyl thioacetate and thiopropionate, hydrogen sulfide	

otherwise, from aqueous solutions (Baldwin *et al.*, 1973). Compounds in the aqueous fraction alone are capable of volatilizing, hence providing flavor and aroma perception (Brennand *et al.*, 1989). Bacterial metabolism occurs in the aqueous phase. Collectively, these observations lead us to believe that the free water in cheese may facilitate flavor generation, while some partitioning across the aqueous and lipid phase occurs with increasing concentrations of compounds.

Flavor compounds are generated from substrates available during cheese ripening. The metabolism of sugar, protein, and lipids in cheese by bacteria releases the different flavor compounds (Gallardo-Escamilla *et al.*, 2005; Lin *et al.*, 1979). Both beneficial and off-flavor components arise from metabolism by bacteria found in cheese (Reiter and Sharpe, 1971; Steele and Unlu, 1992; Urbach, 1995). The aromatic amino acid-related rosy, fruity or putrid flavors result from metabolism of tyrosine, tryptophan and phenylalanine (Carunchia-Whetstine *et al.*, 2005; Ummadi and Weimer, 2001; Yvon *et al.*, 2000). Some putrefactive flavors are attributable to the metabolism of arginine (Crow and Thomas, 1982). Pyruvate and lactic acid act as substrates for many flavor components (De Vos, 1996; London, 1990). Metabolism of sulfur amino acids produces volatile sulfurs, which are beneficial at low concentrations but lead to off-flavors such as putrid, rotten-egg, cabbage-, cauliflower- and garlic-like at higher concentrations (Weimer *et al.*, 1999). Notably these products arise from amino acids, and hence from the proteins and peptides of milk during the course of cheese ripening.

The direct role of amino acids and peptides in cheese flavor is limited (Aston and Creamer, 1986; Engels and Visser, 1994) to contribution to base cheese flavor (Sandine and Elliker, 1970) and acting as substrates for enzymatic modification reactions (Urbach, 1995). Volatile fatty acids, aldehydes, ketones, lactones, esters and volatile sulfur compounds are some of the major classes that

correlate with flavor development during cheese ripening. Volatile fatty acids exist both alone and in combination with volatile sulfur compounds as thioesters. Volatile sulfur compounds are one of the major classes of flavor compounds that correlate with good Cheddar cheese flavor (Dias and Weimer, 1999; Manning *et al.*, 1976; Weimer *et al.*, 1999).

2.3.1 Sulfur compounds in cheese

Volatile sulfur compounds (VSCs) (Table 2.2), especially methanethiol, correlate to positive flavor and aroma development in Cheddar cheese (Manning *et al.*, 1976; Pereira *et al.*, 1966; Weimer *et al.*, 1999). VSCs are identified in cheese as flavor precursors that provide impact at low concentrations, i.e. ppb to ppm (Weimer *et al.*, 1999). Methanethiol is the principal component identified to be responsible for Cheddar aroma. Other compounds such as hydrogen sulfide, methyl and dimethyl di- and trisulfides are also found in cheese, but their role in cheese flavor is not well defined (Dias, 1998).

VSCs are highly reactive and may also react with other products of bacterial catabolism. Methanethiol can be easily oxidized to form dimethyl disulfide and dimethyl trisulfide (Burbank and Qian, 2005; Fang and Qian, 2005) as well as other reaction products. For example, S-methyl-thioesters, the products of reactions between methanethiol and volatile fatty acids, impact cheese flavor subsequent to the enzyme production of both partner compounds (Table 2.2). While microbial mechanisms of thioester production exist, they are yet to be characterized for contribution to cheese flavor (Cuer *et al.*, 1979a, b).

Table 2.2 Detection thresholds and properties of sulfur compounds found in cheese

Compound	Detection threshold (ppb)	Medium	Flavor/aroma description	Precursor
Hydrogen sulfide	0.18	Air	Rotten eggs	Cysteine
Carbonyl sulfide				Cysteine
Methanethiol	2	Water	Cooked cabbage	Methionine
Methional	21	Skim milk	Boiled potato	Methionine
Dimethyl sulfide	20	Milk	Cabbage	
Dimethyl disulfide	20	Skim milk	Cauliflower	Methanethiol
	1.2	Water	Garlic	
Dimethyl trisulfide	0.01	Water		Methanethiol, methanethiol + formaldehyde
S-methyl thioacetate	5	Liquid cheese	Cooking cauliflower	Methanethiol + acetic acid
S-methyl thiopropionate	100	Liquid cheese	Cheesy	Methanethiol + propionic acid
S-methyl thiobutyrate	100	Liquid cheese	Chives	Methanethiol + butyric acid

VSCs are products from metabolism of sulfur-containing amino acids, cysteine and methionine (Weimer *et al.*, 1999). Brevibacteria can cleave the terminal thio-group from methionine to produce methanethiol in a single step reaction (Di Franco *et al.*, 2002). Dimethyl disulfide is inversely related to flavor development (Wei *et al.*, 2001). The mechanisms of production of dimethyl and trimethyl sulfides and other such polymeric VSCs are still not clear beyond the defined reaction compounds route. However, based on the reactivity of VSCs, it would be reasonable to suppose that these are formed from methanethiol oxidative reactions. Additionally, hydrogen sulfide and methional are also found in cheese. Methional is produced by Strecker degradation of methionine, the reaction capable of light induction (Wei *et al.*, 2001). Methional in combination with diacetyl and *n*-butyric acid is reported to form good Cheddar aroma (Dacremont and Vickers, 1994).

Recent reports indicate that LAB and brevibacteria are capable of fixing inorganic sulfate from media to VSCs (Ghosh, 2004). However, fixing of sulfur into protein was not conclusively identified. Methionine is needed for protein synthesis as the initial amino acid. The ability of bacteria associated with cheese to fix sulfur instigates a curiosity into the source of this action, as caseins, while devoid of cysteine, contains methionine that are released by proteolysis into the whey, leaving only a small amount for metabolism during cheese ripening (Banks and Dagleish, 1990; Chapman and Sharpe, 1990). Genomic analysis also suggests that the LAB do not share a pathway commonly found in other proteobacteria, but contain a yet uncharacterized mechanism unique to lactococci (Ghosh, 2004).

VSCs alone do not allow Cheddar flavor production. Volatile fatty acids and carbonyl compounds are also necessary to provide ideal Cheddar flavor (Liebich *et al.*, 1970). The balance between VSCs and volatile fatty acids may play a role in ideal cheese flavor. At least H₂S is correlated to quantities of volatile fatty acids in Cheddar cheese flavor (Table 2.3). Hence, the role of volatile fatty acids in cheese flavor is important.

2.3.2 Fatty acids, esters, and carbonyls in cheese

Fatty acids in cheese

During cheese ripening fatty acids (FAs) are produced from lipolysis, amino acid catabolism and lactose fermentation. Both esterase and lipase enzymes have

Table 2.3 Relationship of flavor character of commercial Cheddar cheese to ratio of fatty acids (FA) to hydrogen sulfide (H₂S) concentration (Kristoffersen, 1967)

Number of cheese lots	Flavor character	Ratio of meq FA (g) to mM H ₂ S (g)
5	Balanced	14.2 : 1
4	Sulfide	7.2 : 1
5	Fermented	27.9 : 1

lipolytic activities and hydrolyze milk lipids to form 'free' fatty acids (FFA). Most FAs, with carbon chain lengths from C4 to C20, arise from hydrolysis of triglycerides during cheese aging. However, some FAs are products of lactose fermentation (via pyruvate) and amino acid degradation (see Chapter 4 for a detailed review). Lactose fermentation produces acetic, propionic and butyric acids, but lactose is depleted quickly during ripening, so it is likely that only a small amount of FA arise from lactose. Amino acid degradation, including catalytic deamination, can generate branched-chain fatty acids such as isobutyric and isovaleric acids (Ganesan *et al.*, 2006; Kuzdzal-Savoie, 1980). Conversion of amino acids to fatty acids also occurs via complex metabolic pathways in lactococci, lactobacilli, and brevibacteria (Ganesan *et al.*, 2004a, b, 2006; Ganesan and Weimer, 2004). A small amount of FAs can also be generated from the oxidation of alcohols, aldehydes, ketones, and esters (Molimard and Spinnler, 1996).

Methods for free fatty acid analysis

FFAs are measured with several methods. The acid degree value was widely used to monitor fatty acid liberation in milk, cream and cheese (Ikens *et al.*, 1988; Lin and Jeon, 1987; Richardson, 1985). However, this titration method measures the total amount of liberated fatty acids instead of individual fatty acids. It is important to know the concentrations of individual fatty acids because each fatty acid will have different aroma attributes and sensory detection thresholds. Individual FAs can be accurately determined by gas chromatography (GC) with or without derivatization; however, they must first be separated from triglycerides, prior to analysis. Among many methods used to isolate free fatty acids, the alkaline arrestant silicic acid column method was widely used in early work (McCarthy and Duthie, 1962; Woo and Lindsay, 1980). However, not only is the preparation of the KOH-silicic acid column and 'sample cap' tedious, the column performance also lacks reproducibility. Potential water leakage from the sample cap to the KOH column, in combination with prolonged fat contact with the strongly basic column, can greatly increase the possibility of fat hydrolysis, causing analysis variability.

Other techniques involved with free fatty acid isolation include alumina column chromatography (de Jong and Badings, 1990; Deeth *et al.*, 1983), strong anion exchange (Needs *et al.*, 1983; Spangelo *et al.*, 1986) followed by methylation with methyl iodide, or direct soap formation with tetramethylammonium hydroxide (Chavarri *et al.*, 1997; Martin-Hernandez *et al.*, 1988; Martinez-Castro *et al.*, 1986; Metcalfe and Wang, 1981). All of these methods have limited success. On the other hand, aminopropyl weak anion exchange columns have been successfully used to isolate FAs from lipid extracts (Chavarri *et al.*, 1997; de Jong and Badings, 1990; Qian and Reineccius, 2002). The FAs can then be directly analyzed by gas chromatography without derivatization. This method is simple and quick, and nearly 100% recoveries have been achieved with most free fatty acids. Hydrolysis of triglycerides and lactic acid contamination were not observed with this method and all FAs can be analyzed with good repeatability.

Aroma contribution of free fatty acids

The pH of cheese exerts a considerable influence on the flavor impact of FAs (Bills and Day, 1964); at pH 5.2 (typical pH for cheeses) a considerable portion of free fatty acids exist in their salt form, which reduces their aroma impact. This is important to note because only the protonated forms of fatty acids can contribute to cheese flavor due to their odor-active nature (Pinho *et al.*, 2002); if the fatty acids remain attached to the glyceride 'backbone', they will remain odorless. With regards to the overall aroma, it is the shorter chain FAs that play the greatest role where those with even-numbered carbon chains are considered to be the major contributors to the quality and flavor potency of aged Italian cheeses (Barbieri *et al.*, 1994; Ha and Lindsay, 1991; Qian and Reineccius, 2002; Virgili *et al.*, 1994; Woo and Lindsay, 1984). These fatty acids normally have odor thresholds of a few parts per million (ppm, or mg kg^{-1}). The sensory detection thresholds of FAs have been investigated in both water and oil by several authors (Brennand *et al.*, 1989; Patton and Josephson, 1957; Siek *et al.*, 1969; Urbach *et al.*, 1972), although the values can vary widely due to different methods used in the studies (Table 2.4).

Short-chain FAs have higher threshold values in water than in oil, possibly due to their higher solubility in water, corresponding to a lower vapor pressure in aqueous media than in an oil, or lipid, matrix. The opposite is true when a lipid matrix, such as that of cheese, is evaluated, where short-chain FAs will have relatively higher vapor pressures and therefore lower sensory thresholds. From butyric acid to capric acid, the sensory thresholds in a lipid phase generally increase with increasing chain length. Longer-chain fatty acids, especially those with more than 12 carbon atoms, exhibit even higher thresholds in lipid matrices, presumably because of very low vapor pressures, and will therefore have very little impact on the overall aroma. In Parmesan, the concentrations of butyric, caproic, and capric acids are much greater than their sensory thresholds, thus these compounds can contribute to the overall aroma of the cheese (Qian and Reineccius, 2002, 2003).

Fatty acids (FAs) are present in all cheeses at varying concentrations. Their role is not limited to being direct flavor components alone. They are also substrates for reduction reactions mediated by short-chain dehydrogenases of bacteria to produce aldehydes, ketones, alcohols, and lactones (Fox *et al.*, 1995). Some FAs potentially are esterified by bacteria to produce esters or are converted to aldehydes, ketones or alcohols (Fenster *et al.*, 2003b; Holland and Coolbear, 1996; Lamberet *et al.*, 1997). Many compounds in these classes are important flavor components of cheeses (Urbach, 1993). In addition, these compounds can react and form other important aroma compounds.

The typical flavors of FAs in isolation are often considered negative at higher concentrations (Table 2.4). Except for *n*-valeric acid, none of these compounds possesses a flavor that resembles cheese flavor. However, in low concentrations and in combination with other groups of compounds, FAs contribute desirable flavors to cheese (Paulsen *et al.*, 1980). Therefore, FAs are considered important in cheese flavor (Liebich *et al.*, 1970; Sandine and Elliker, 1970) and are also included in synthetic flavor formulations (Law, 1983).

Table 2.4 Fatty acids and their related flavors in cheese

Fatty acid (IUPAC name)	Trivial name	Flavor threshold (ppm)	Aroma or flavor attribute
Ethanoic	Acetic	22–100 water 0.12–7 oil	Pungent, sweaty, vinegar, sour
Propionic	Propionic	20–40 water	Acid, sharp, sour
<i>n</i> -Butyric	Butyric	0.3–6.2 water 0.14–3.0 oil	Rancid, sharp, acid, cheesy
<i>n</i> -Pentanoic	Valeric	1.1–6.5 water	Nutty, cheese-like, sour, meaty, sweaty
<i>n</i> -Hexanoic	Caproic	0.29–27.0 water 2.5–10.0 oil	Acidic, sweaty, cheesy, sharp, goaty
<i>n</i> -Heptanoic		0.28–10.4 water	Soapy, fatty acid-like, goaty rancid
<i>n</i> -Octanoic	Caprylic	3–19 water 10–350 oil	Goaty, waxy, soapy, cheesy, sweaty
<i>n</i> -Nonanoic		2.4–8.8 water	Fatty, soapy, waxy, green
<i>n</i> -Decanoic	Capric	1.4–10.0 water 5–200 oil	Soapy, waxy
2-Methyl propionic	Isobutyric	0.05–8.1 water	Sweaty, fatty acid-like, cheesy, rancid, caramel
2-Methyl butyric	Isovaleric	0.07 water 0.02 oil 0.07	Sweet, fruity, waxy, cheesy, rancid, sour, sweaty Sharp, sweaty, sweet, fruity
3-Methyl butyric			Fruity, pleasant
2-Ethyl butyric			Fruity, pleasant
4-Methyl octanoic		0.6	Goaty, muttoney
4-Ethyl octanoic		0.006	Characteristic goaty
9-Decenoic		4.3	Sweet, fatty
Undecanoic		0.1	Soapy, waxy
10-Undecanoic		2.3	Soapy, sweet
Dodecanoic	Lauric	2.2–16 water 700 oil	Soapy, metallic

The role of FAs in cheese flavor depends on their concentration. FAs are found in typical aged, good-flavored Cheddar cheese at a concentration of ~1000 ppm and in varying amounts in other cheeses (Table 2.5) (Woo *et al.*, 1984). Most even straight-chain fatty acids and branched chain fatty acids (BCFAs) are at concentrations higher than their reported flavor and aroma thresholds (Attaie and Richter, 1996). The concentrations of acetic acid, *n*-butyric acid and *n*-caproic acid increase during cheese ripening, with a concomitant improvement in flavor (Barlow *et al.*, 1989; Chin and Rosenberg, 1997; Kristoffersen, 1967). Above these levels, FAs lead to off-flavor in cheeses (Law and Wigmore, 1984). Hence, FAs also contribute to off-flavors as the concentrations rise.

The levels of FAs change in relation to age of cheese, ripening temperature and cheese composition (Chin and Rosenberg, 1997). While FA concentrations are associated with desirable flavor, individual FAs modulate the flavor profile of cheeses (Table 2.6) because of their low flavor thresholds and distinctive

Table 2.5 Typical concentrations of total fatty acids (TFA) in cheese varieties

Variety	TFAs (ppm)	Variety	TFAs (ppm)
Sapsago	211	Gjetost	1,658
Edam	356	Provolone	2,118
Mozzarella	363	Brick	2,150
Colby	550	Limburger	4,187
Camembert	681	Goats' milk	4,558
Port Salut	700	Parmesan	4,993
Monterey Jack	736	Romano	6,754
Cheddar	1,028	Roquefort	32,543
Gruyère	1,481	Blue (US)	32,230

flavors. Propionic acid, *n*-butyric acid, *n*-valeric acid, *n*-caprylic acid, and thio-esters of these FAs have aroma properties compatible with participation in cheese flavor development (Law, 1984). FAs are vital for development of typical flavor of blue-veined cheese, both alone and as substrates for oxidation to methyl ketones (Law, 1984). Italian hard cheeses like Romano, Provolone and Parmesan cheeses contain FAs that are attributed to small amounts of deliberately added lipase (Law, 1984), consisting of a large number of BCFAs (Ha and Lindsay, 1993). *n*-Valeric acid, and BCFAs like 4-methylvaleric acid, 2-ethylcaproic acid and 6-methylheptanoic acid, have cheese-like, nutty flavor at concentrations as low as 2.5 ppm (Brennand *et al.*, 1989) but are not found in milk fat. Occurrence of these highlights microbial metabolism in cheese.

n-Butyric, *n*-caproic, *n*-capric and 3-methylbutyric acids are the FAs among the flavor compounds in cheeses (Chin and Rosenberg, 1997; Urbach, 1993). Lauric, myristic, palmitic and stearic acids are present in cheese (Vandeweghe and Reineccius, 1990) but are not implicated in flavor. *n*-Butyric acid at concentrations of 45–50 ppm and *n*-caproic acid at 20–25 ppm are associated with optimum Cheddar flavor (Barlow *et al.*, 1989). However, isovaleric acid

Table 2.6 Fatty acids present in water-soluble fractions of eight cheese types (adapted from Engels and Visser, 1997)

Cheese type	Fatty acid				
	Acetic	Propionic	<i>n</i> -Butyric	<i>n</i> -Valeric	<i>n</i> -Caproic
Gouda 20+	✓				✓
Gouda	✓				✓
Proosdij	✓		✓		✓
Gruyère	✓	✓	✓	✓	✓
Maasdam	✓	✓	✓		✓
Edam	✓		✓		✓
Parmesan	✓		✓		✓
Cheddar		✓	✓		✓

and *n*-valeric acid were also reported to be absent from commercial Cheddar cheeses in early work (Bills and Day, 1964; Peterson *et al.*, 1949), possibly due to analytical sensitivity.

Esters and carbonyls in cheese

FAs along with their own individual flavor also exist as ketones, esters, and lactones in the reduced conditions of cheese (Law, 1983, 1984; Law *et al.*, 1976). Additionally, fatty acid ethyl esters, especially of caproic and caprylic acids, are involved in cheese flavor (Fox and Wallace, 1997). The esterases of bacteria have also been recently scrutinized, but are not causatively linked to cheese flavor (Fenster *et al.*, 2003b; Fernandez *et al.*, 2000; Holland and Coolbear, 1996).

Aldehydes and ketones are known to be impact compounds for some cheeses (Table 2.1). Straight-chain aldehydes and alcohols are products of FAs that arise from reduction reactions (Avsar *et al.*, 2004). The reactions are attributable to aldehyde and alcohol dehydrogenases from bacteria, respectively (Lees and Jago, 1976). Acetaldehyde and ethanol are derived from sugar metabolism (Lees and Jago, 1976), while longer-chain products arise from either longer-chain FAs or amino acid metabolism (Gallardo-Escamilla *et al.*, 2005). The branched-chain aldehydes and alcohols that are abundant in goats' milk cheeses are also derived from goats' milk lipids; while in cows' milk cheeses, typically Cheddar cheese, branched-chain FAs, aldehydes and alcohols are derived from branched-chain amino acid catabolism (Smit *et al.*, 2004a, b). Strecker degradation is also involved in generating flavor-promoting aldehydes (Keeney and Day, 1957).

Diacetyl, acetoin and 2,3-butane-diol are produced from pyruvate metabolism and are involved in the flavor of fresh cheese curd (Marugg *et al.*, 1994; Verhue and Tjan, 1991). These compounds are the carbonyls that provide the sweet, nutty and fresh flavors of the curd (Gallardo-Escamilla *et al.*, 2005). While these compounds are also present in aging cheese, a definite role in cheese flavor is yet to be established. Ketones such as acetone, butanone, 2,4-pentane-dione and some methyl ketones are also identified as impact compounds in certain cheeses (Qian *et al.* 2002; Sable and Cottenceau, 1999). Methyl ketones are generated from β -oxidations of fatty acids followed by decarboxylation; the low redox potential and micro-aerophilic conditions of the cheese matrix may play a role in facilitating the chemical reactions. Microbial metabolism that leads to these ketones is yet to be demonstrated.

2.3.3 Sources of flavor components

Carbohydrates

Lactose is the primary energy source in milk for LAB, which utilize sugars as an energy source during growth and acid production. The amount of residual lactose available to bacteria in cheese depends upon starter activity, washing of curd, and mode of salting of cheese (Desmazeaud and Cogan, 1996). Lactose is reduced to <0.01% after a week of ripening of hard cheeses (Crow *et al.*, 1993).

Hence, it does not account for FAs beyond the initial week of cheese ripening. Therefore, lactose has little influence on FAs produced during cheese ripening. However, the residual lactic acid in cheese curd is still available to form the more important and flavorful lactones and other carbonyls.

Carbohydrates are the primary carbon and energy source for LAB during curd formation, and hence are implicated in flavor generation. During the initial stages of ripening, some genera utilize carbohydrates to produce FAs. For example, *brevibacteria* convert glucose to FAs near pH 7, and galactose to FAs near pH 8, but their ability to produce FAs from carbohydrates at pH 5.2 is limited (Hosono, 1968a, b).

In other genera, FAs are not directly derived from carbohydrates but from products of carbohydrate catabolism (i.e. pyruvate, lactic acid, citrate, and acetyl-CoA) (Lawrence *et al.*, 1976). *Propionibacteria* convert lactic acid to acetic and propionic acids, which are important in typical flavor of Swiss cheese (Turner and Martley, 1983). NSLAB utilize citrate for energy in absence of carbohydrates and produce formate by the citrate–formate pathway (Peterson and Marshall, 1990). Citrate is reduced to diacetyl and further to acetoin, 2,3-butanediol and 2-butanone, which is important in cheese flavor (Langsrud and Reinbold, 1973). *Leuconostoc* and *Lactococcus lactis* ssp. *diacetylactis* convert lactic acid to diacetyl and acetaldehyde, products commonly found in Gouda and other fresh milk cheeses. However, FAs are not formed from citrate and its metabolites. Acetyl-CoA is converted to straight, even-chain fatty acids of length C-2 to C-20 (Harper *et al.*, 1978) but not BCFAs. There are, therefore, multiple mechanisms that lead to straight-chain FAs, aldehydes, and ketones from carbohydrates by microbial metabolism, including lipase–esterase activity and central carbon metabolism of starter culture, adjunct culture, and NSLAB.

Lipolysis

Lipolysis is the reaction that cleaves the glycerides of milk fat to produce FAs. Rise in specific FAs is associated with rancid flavor in milk (Banks and Dagleish, 1990). Lipolysis is one source of FA generation in cheese (Fox *et al.*, 1993, 1995, 1996; Fox and Stepaniak, 1993; Fox and Wallace, 1997) and is very important in Blue and Italian type cheeses wherein *Penicillium roqueforti* spp. and pregastric esterase, respectively, are the principal lipolytic agents (Fox *et al.*, 1996). The resultant FAs may subsequently be converted to reduced or oxidized to carbonyl compounds. However, lipase from starter cultures was not found to be critical for FA production.

Fresh raw milk contains ~0.1% FAs prior to lipolysis (Kurtz, 1974); hence milk itself is not a major contributor to FAs in cheese. FAs liberated from lipolytic reactions correspond to the FAs attached to milk fat as glyceryl esters. The initial FA composition of milk fat is affected by season and milk source (Reiter *et al.*, 1967) and is defined after collection and processing of milk prior to cheese making. *n*-Butyric acid, *n*-caproic acid, *n*-heptanoic acid, *n*-caprylic acid, *n*-nonanoic acid, *n*-capric acid, 9-decenoic acid and higher chain-length BCFAs are found in milk fat (Ha and Lindsay, 1990). Hence, some of the

smaller even-chain FAs also arise from milk fat. But this does not account for the levels of smaller BCFAs like isobutyric and isovaleric acids in cheese. This raises the question of factors involved in lipolysis of milk fat.

Lipases from bacterial and fungal sources are involved directly in fat hydrolysis and cheese flavor as noted by cheese variety and culture addition. Short-chain FAs on the glyceride are preferred over BCFAs by microbial fungal lipases (Ha and Lindsay, 1993). This directs the FA profile of mold-ripened cheeses; but fungal lipases may not be present in regular Cheddar cheese as molds are considered contaminants in Cheddar cheeses.

Lactococcal lipase and esterases are capable of hydrolyzing mono- and diglycerides more readily than triglycerides (Lawrence *et al.*, 1976); but the levels of mono- and diglycerides are <0.5% of milk fat (Banks and Dalgleish, 1990; Kurtz, 1974). Even if we consider that some FAs in cheese are likely to arise from mono- and diglycerides by lactococcal lipolysis, it amounts to ~0.75 ppm. This does not contribute to FA quantities in cheese significantly in addition to considering triglyceride hydrolysis. Also, intracellular lipase–esterase activity of bacteria does not positively correlate with Cheddar cheese flavor (Weimer *et al.*, 1997). The known lipase–esterases of lactococci (Bolotin *et al.*, 2001) (http://www.jgi.doe.gov/JGI_microbial/html/index.html), and other LAB (El Soda *et al.*, 1986; Fenster *et al.*, 2003a, b; Khalid *et al.*, 1990) possess only esterase activity (Nardi *et al.*, 2002). Therefore, the role of fat hydrolysis is questionable and may be less important than amino acid catabolism in generation of FAs in Cheddar cheese.

Milk contains native lipoprotein lipase and lipases contributed by raw milk microflora. Native milk lipase is destroyed by pasteurization (Andrews *et al.*, 1987; Stadhouders and Mulder, 1960) and is not active at the salt content and pH of cheese (Khalid and Marth, 1990). Lipases from Gram-negative bacteria in raw milk such as *Pseudomonas* spp. and *Achromobacteriaceae* possess thermal resistance and cause lipolysis in cheese milk (Stadhouders and Mulder, 1960). The addition of external lipolytic enzymes from similar raw milk microflora has a negative effect on flavor quality in Cheddar cheese (Fox *et al.*, 1996). Intracellular lipase–esterase activity of starter bacteria does not positively correlate with Cheddar cheese flavor (Weimer *et al.*, 1997). This suggests that Cheddar cheese FAs develop due to neither native milk lipases nor addition of any other external lipase.

FA generation by lipolysis depends on FA distribution on the glycerol moiety. FAs in milk fat are stereospecifically distributed among sn-1,2,3 positions of glycerol. The distribution varies inversely with chain length at sn-3 position and directly at sn-1 position. The variation is greater at sn-1 and sn-3 positions than at sn-2, and is relatively stable with milk production season (Ha and Lindsay, 1993; Parodi, 1979).

Attempts were made at identifying FA quantities and associated formulae to identify FA levels with good cheese flavor. One such study calculated the extent of lipolysis from the C-16 value (which is described as 22.3% of butterfat) using the formula (Eq. 2.1) (Urbach, 1993):

$$\text{Extent of lipolysis} = \frac{\%C-16}{22.3} \times \frac{100}{1} \times \frac{100}{3.3} = \%C-16 \times 13.6 \quad 2.1$$

The study defined good quality cheese to have <0.52% lipolysis at 0 months to <1.6% at 20 months. Cheeses with a higher extent of lipolysis have off-flavors like soapy, oniony, metallic and vomit (Urbach, 1993). Hence, the level of FAs is important in contributing to a balanced cheese flavor. But this assumes FA production by lipolysis only. FAs are not solely derivable from fat. They have other potential sources of origin like lactose and amino acids. This deepens the dogma in importance of milk fat in FA generation in Cheddar cheese.

A theoretical estimate of amounts of short-chain FAs that are also derived from milk fat represents the maximum possible FAs that can be produced. Calculating the amount of short-chain FAs ($\leq C-6$) that can be attributed to lipolysis, from FA distribution in milk fat (Parodi, 1979), the maximum possible FAs is only ~150 ppm, which is only ~15% of the total FAs found in Cheddar cheese and even lower than in many other cheeses. The above calculation neglects action of lactococcal lipases. Also, this includes only *n*-butyric and *n*-caproic acid which are only straight-chain FAs.

The contribution of fat to FA production in cheese or cheeselike conditions is also experimentally elucidated by other studies. Their approach is either to remove fat from milk and study skim-milk cheeses or alternatively to substitute milk fat with vegetable lipids (Wijesundera and Drury, 1999). Acetic acid concentration is similar in whole-milk cheese and skim-milk cheese; but the concentration of higher FAs is reduced in skim-milk cheese. This difference is attributed to factors like higher moisture, lower fat and higher salt in fat-free or fat-substituted cheeses (Dulley and Grieve, 1974). Cheddar and Romano cheese slurries from skim milk contain very low concentrations of short-chain FAs, which is attributed to abnormal ripening conditions (Harper *et al.*, 1978). While *n*-butyric acid arises in milk from lipolysis, the amount of *n*-butyric acid in Cheddar cheese is twice that of esterified *n*-butyric acid in milk fat (Bills and Day, 1964). This preempts the conclusion that fat is the sole source of FAs in Cheddar cheese.

In vegetable fat slurries, FAs less than C-10 are detected in traces versus milk fat cheese slurries (Harper *et al.*, 1978). These FAs do not represent any contribution of vegetable lipid fatty acids in fresh cheese curd. Aging produces short-chain FAs in vegetable lipid cheese but not FAs greater than C-12; however, short-chain FAs are at concentrations lower than in milk fat cheese (Harper *et al.*, 1978). Other mechanisms are needed to produce FAs that are not found in milk fat.

Proteolysis

Milk proteins are rich in several amino acids essential for bacterial growth (Banks and Dagleish, 1990). Amino acids support survival of bacteria for at least four years upon exhaustion of sugars in media (Ganesan *et al.*, 2006). The abundant proteins of milk potentially supply the carbon requirement of the

carbohydrate-starved bacteria. Proteolysis, hence, becomes relevant to bacterial survival to provide amino acids as carbon and nitrogen sources, and leads to flavor compounds from metabolic activity of organisms in the cheese matrix.

Protein degradation by bacteria leads to compounds that are both part of acceptable and unacceptable cheese flavor (Aston *et al.*, 1983b; Law *et al.*, 1992; Muehlenkamp-Ulate and Warthesen, 1999). A few decades of cheese flavor characterization are narrowly focused on extensive development of technologies to characterize proteolytic degradation of caseins (Aston *et al.*, 1983a, b; Fenelon and Guinee, 2000; Law *et al.*, 1992; O'Keeffe *et al.*, 1976). Fractionation and identification of specific peptides, finding the starter and adjunct cultures that produce them, and finding the phases into which the peptides partition, have been among the focal points (Fox *et al.*, 1996; Law *et al.*, 1992; O'Keeffe *et al.*, 1976). Alternatively, genetic characterization and engineering of lactococci that produce or alternatively degrade several other peptides, the biochemistry of peptidolytic enzymes or peptidases, and the identification of proteases to produce oligopeptides are extensively characterized (Baankreis, 1992; Bhowmik and Marth, 1989; Christensen *et al.*, 1999). The sheer volume of literature over the decades (more than 3000 papers) aptly pays tribute to the importance of proteolysis in flavor development.

The main focus on proteolysis arose with the need to avoid bitterness defects in large lots of commercial cheese manufacture (Kai, 1996). Several starter culture technologies, including use of adjunct and NSLAB, were adopted to alleviate bitterness (Khalid and Marth, 1990; Lynch *et al.*, 1999). Further developments in protein analysis and fractionation identified the two most widely accepted components – α_s -casein_{1–9} and β_s -casein_{193–209} as the main contributors of bitterness (Broadbent *et al.*, 1998). This eventually led into bacterial identification for ability to hydrolyze bitter peptides. Beyond this, the development of these techniques allowed extensive characterization of the bacterial proteolytic system involving some proteases and a large section of peptidases (Christensen *et al.*, 1995, 1999; Foucaud *et al.*, 1995). Bitterness is related to slow acid development during the initial processing of cheese curd (Edwards and Kosikowski, 1983) but is preventable by high aminopeptidase activity of the starter or adjunct culture. However, the search for peptides has not yielded any that are involved in flavor beyond providing substrates for flavor development, essentially amino acids and background flavors.

Amino acids

The level of *n*-butyric acid in cheese is higher than the total amount of *n*-butyric acid found as glyceryl esters with milk fat (Bills and Day, 1964). Hence, other potential sources that can yield *n*-butyric acid by microbial catabolism may have a role during cheese ripening. One such source is milk protein by the conversion of amino acids to flavor compounds (Hemme *et al.*, 1982; Nakae and Elliott, 1965a, b).

Leucine, glutamate, phenyl alanine, valine and lysine are the principal amino acids in Cheddar cheese (Engels and Visser, 1994; Wood *et al.*, 1985). Multiple

mechanisms for production of flavor compounds from amino acids are postulated. Both enzymatic and non-enzymatic degradation of amino acids in cheese yield flavor compounds (Visser, 1993). Deamination of amino acids in cheese produces ammonia and α -keto acids, and specific FAs. Isovaleric and 3-methylbutyric acids found in Livarot and Pont l'Évêque cheeses are produced from leucine and isoleucine, respectively (Stark and Adda, 1971). Transamination and Strecker degradation yield aldehydes (Fox and Wallace, 1997).

A theoretical estimate of maximum possible FAs from amino acids can be made from data available for amino acid composition of casein (Banks and Dalgleish, 1990). The maximum possible total FAs from this calculation is ~41,000 ppm, considering that only known precursors are involved in the contribution and also that only ~30% of protein in cheese is broken down by proteolysis (Chapman and Sharpe, 1990), whereas the actual total FA content in Cheddar cheese is around 1028 ppm (Fox *et al.*, 1993). The lower amounts of FAs may be due to the various stress conditions in cheese that slow down metabolic processes and the structural modifications caseins undergo during proteolysis. All peptides that result from casein degradation may not be totally converted to amino acids, and further to FAs.

The degradation of proteins also leads to release of methionine, in addition to fixing of sulfur by bacteria. VSCs are produced by lactococci and brevibacteria from sulfate (Ghosh, 2004). It is also known that methionine concentrations increase during ripening (Dias and Weimer, 1999; Stuart *et al.*, 1999).

In conclusion, several mechanisms and diverse bacterial types are involved in cheese flavor production. A larger spectrum of compounds has been identified in the past. However, a direct role for many classes, while known in other foods, is yet to be established for beneficial cheese flavor. The availability of high-throughput technologies such as gas chromatography and liquid chromatography in combination with mass spectrometry is likely to help identify more components. Future work lies in assigning roles of the multitudinal combinations of flavor components to yield the best cheese.

2.4 Future trends

Multidimensional chemical separation technology such as isolation, fractionation and chromatography has, in the past, extensively helped cheese flavor research in categorizing groups of impact compounds. The pathways that these compounds arise from and their precursor organisms have been the subject of active debate over several decades of metabolism research. In the recent past, novel high-throughput technologies for metabolic prediction such as genomics, proteomics, and metabolomics have become accessible and cost-effective per unit of information derived from complex sample mixtures. Cases in point are the abilities for simultaneous measurement of transcriptional activity of several thousand genes and identification of over 4000 small molecules (molecular weight <1000 Da) in a single chromatographic run of less than 10 minutes. The

power of metabolomics will allow rapid profiling of flavor components and further extensive identification of flavor profiles related to both beneficial and off-flavors of cheese. Genomics will allow the characterization of metabolic capabilities of existing strains in the cheese industry used for fast acid production and phage resistance to functionally contribute to flavor generation. The flavor metabolism research may extensively benefit from these high throughput capabilities for producing the cheese that brings a smile onto your face.

2.5 Sources of further information and advice

Numerous reviews on flavor production and compounds exist. Work done by Ghosh, Harper, Reineccius, Schormüller, Urbach and others has defined a long list of compounds associated with cheese. Specifically, a review by Schormüller (1968) is particularly useful as a starting point for these compounds. The challenge remains to associate specific compounds at specific concentrations with 'good' cheese flavor:

SCHORMÜLLER, J. (1968). 'The chemistry and biochemistry of cheese ripening'. *Adv. Food Res.* **16**: 231.

2.6 References

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Part I

Microbial physiology and the development of cheese flavour

3

Carbohydrate metabolism and cheese flavour development

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3.1 Carbohydrate compounds present in milk

Lactose is the principal carbohydrate component in the milk of all mammals; its concentration varies widely between species, ranging from ~ 0 to 100 g L^{-1} (Fox *et al.*, 2000). Bovine milk usually contains more lactose than any other solid constituent, with a concentration of $45\text{--}50 \text{ g L}^{-1}$ (Walstra, 1984). The lactose content of bovine milk varies with breed and stage of lactation. Generally, the concentration of lactose progressively decreases during lactation (Fox *et al.*, 2000).

The carbohydrate composition of bovine milk consists of lactose ($\sim 4.8\%$ w/v), citrate ($\sim 0.18\%$ w/v) and trace amounts of glucose, galactose, glucosamine, fructose, galactosamine, neuramic acid, neutral and acidic oligosaccharides, nucleotides and nucleic acids (Walstra, 1984; Walstra *et al.*, 1999; Gopal and Gill, 2000; Schlimme *et al.*, 2000; Adamberg *et al.*, 2005). The origins of these trace sugars include carbohydrates released from glycomacropeptide of κ -casein and glycoproteins and glycolipids contained in the milk fat globule membrane (MFGM) (Adamberg *et al.*, 2005). MFGM contains protein, phospholipids, cerebrosides, glycoproteins, glycolipids, phospholipids, enzymes and other polar materials. A heavily glycosylated mucin glycoprotein forms part of MFGM and may play a role in resistance of MFGM to protease attack or resistance to microbial infection by prevention of adhesion to epithelial cells (Schroten *et al.*, 1992).

Lactose is a reducing disaccharide composed of D-glucose and D-galactose linked by a $\beta 1\text{--}4\text{--}o$ -glycosidic bond. Lactose is approximately one-third as sweet as sucrose and its hydrolysis to component sugars yields a sweeter taste than

lactose. As lactose is a reducing sugar, it participates in the Maillard reaction, with potentially undesirable consequences for cheese flavour and functionality. Of particular importance in milk is citrate, present as an ionic salt in a colloidal state at a level of $\sim 1.8 \text{ g L}^{-1}$ (Fox and Wallace, 1997).

Bovine milk contains nucleotides, nucleosides and nucleobases (Schlimme *et al.*, 2000). The ribonucleosides, adenosine, cytidine, inosine, uridine, guanosine and two derivatives of adenosine were monitored in milk at various stages of lactation by Raezke and Schlimme (1990). During the colostrum phase the level of ribonucleosides decreases to reach a constant level three weeks postpartum. Generally, the levels of ribonucleosides in bovine milk decrease in the order uridine $\gg$ cytidine $>$ adenosine, with guanosine and inosine having comparable levels throughout lactation. The origin of ribonucleosides in milk is as yet unknown, but they may originate either from transit from blood across the blood–milk barrier or from metabolism and secretion in the mammary gland. In milk the concentration of pyrimidine ribonucleosides ($\sim 17 \mu\text{mol L}^{-1}$) exceeds that of purine ribonucleosides ($\sim 3 \mu\text{mol L}^{-1}$) by some five-fold and appears to mirror the requirement for uracil ribonucleosides for lactose synthesis in the mammary gland.

A number of ribonucleotides such as adenosine-5'-monophosphate (5'-AMP), cytidine-5'-monophosphate (5'-CMP) and uridine-5'-monophosphate (5'-UMP) were detected in bovine milk over a two-month lactation period (Gil and Sanchez-Medina, 1981a; Schlimme *et al.*, 2000). Ribonucleotide levels were highest one day postpartum and decreased gradually thereafter. Levels of 5'-UMP ribonucleotide reached a maximum two days postpartum ($390 \mu\text{mol L}^{-1}$) and were undetectable 10 days postpartum. Generally, levels of 5'-CMP were comparable to those of 5'-UMP and increased marginally up to 10 days postpartum ($\sim 45 \mu\text{mol L}^{-1}$), decreasing to $\sim 20 \mu\text{mol L}^{-1}$ two months postpartum. The ribonucleotides 5'-AMP and 5'-CMP are present in milk over lactation, while 5'-guanosine-monophosphate (5'-GMP) appears to be absent from milk. Orotic acid is the principal nucleotide-related compound in milk and increases in concentration over lactation to reach levels of $400 \mu\text{mol L}^{-1}$ six months postpartum (Gil and Sanchez-Medina, 1981b; Schlimme *et al.*, 2000).

Oligosaccharides contain 3–10 monosaccharide residues in bovine colostrum and milk. Oligosaccharides can be classed as either neutral or acidic with the latter type containing residues of *N*-acetylneuramic acid (sialic acid). Ten sialyl-containing oligosaccharides and eight neutral oligosaccharides are present in bovine milk that contains colostrum and decline rapidly postpartum (Gopal and Gill, 2000). The major neutral oligosaccharide is *N*-acetyl-lactosamine, present in early colostrum but absent beyond seven days postpartum (Saito *et al.*, 1984). The predominant acidic oligosaccharide in colostrum is 3-sialyl-lactose, with sialyl-lactosamine, 6-sialyl-lactose and disialyl-lactose also present.

Bovine milk also contains a range of compounds referred to as glyco-conjugates where carbohydrates are covalently attached to either protein or lipid compounds; these include gangliosides, neutral glycolipids, glycoproteins and glycopeptides (Neeser *et al.*, 1990). The overall role of oligosaccharides and

glycoconjugates is not entirely clear but they may provide a source of energy and protection against bacterial infection for the newborn animal.

3.2 Cheese manufacture and ripening

Most natural cheese manufacture involves heat treatment of raw milk, addition of starter cultures, coagulation (enzymatic and/or acidification), whey drainage, salting (dry or brine), pressing and ripening.

3.2.1 Milk pre-treatment

Modification of the lactose content in milk for cheesemaking has been examined extensively for its effects on the biochemistry and flavour profile of Cheddar cheese. Huffman and Kristoffersen (1984) manufactured Cheddar cheese with depleted or increased lactose contents through either curd washing or the addition of lactose powder to milk, respectively. For lactose-enriched cheese, an increase in lactose content over the control cheese was noted in draining whey (0.41% compared with 0.27%) with little effect noted on lactate production, acidification rate or curd moisture content. Sensory analysis of the cheeses found a sharper flavour note in lactose-enriched cheeses after nine months of ripening compared with the control. More recently, Ur-Rehman *et al.* (2004) produced Cheddar cheese with a post-pressing lactose content of 0.61% (control), 0.25% (low) or 2.20% (high). This was achieved through either washing the curd or addition of lactose powder. Modification of lactose content did not affect cheese composition, survival of starter culture or NSLAB growth during ripening. Proteolysis, as measured by water-soluble nitrogen levels, was similar during ripening for all cheeses. Descriptive sensory analysis of these cheeses by a 14-member sensory panel found no significant differences in flavour between cheeses after 120 days of ripening. However, control cheeses had the highest scores for 'intensity' flavour descriptor, while low-lactose cheese had the lowest score for this descriptor. For the 'sour/acid' descriptor, cheeses with low lactose content scored lowest, with control or high-lactose cheeses having comparable scores. It would appear that a reduction in lactose content in cheese impacts negatively on the perception of acidity and flavour intensity in cheese, while increasing the lactose content over normal values does not enhance either of these attributes in Cheddar cheese.

Hydrolysis of the lactose component of milk for cheese manufacture using lactase or β -galactosidase pre-treatment was undertaken in an effort to accelerate the ripening of cheese by generating glucose or galactose moieties that omit the energy and enzymatic requirement for the early stages of the glycolytic pathway in starter LAB. In general, a stimulation of lactococcal growth was reported, with shortened lag periods, a reduction in manufacturing time and no increase in cell numbers over untreated milks (Gilliland *et al.*, 1972). A dramatic acceleration in ripening time and an improvement in flavour was reported on

addition of Maxilact (a commercial lactase enzyme preparation derived from *Kluyveromyces lactis*) to Cheddar cheese milk with increased proteolysis over control cheeses (Marschke and Dulley, 1978; Marschke *et al.*, 1980). Hemme *et al.* (1979) found that Maxilact treatment of milk for Gruyère cheese manufacture did not stimulate acid production by *Lactobacillus helveticus*, *Lactococcus lactis* or *Lactobacillus bulgaricus*. Maxilact appeared to stimulate acid production by *Streptococcus thermophilus* through production of proteolytic end products rather than lactose hydrolysis. Grieve *et al.* (1983) demonstrated that the presence of contaminating acid endopeptidase activity in the Maxilact preparation could be responsible for the increased proteolysis and flavour development. However, Gooda *et al.* (1983) claimed that Maxilact contained very low levels of contaminating proteolytic activity and that increased proteolysis in Cheddar cheese resulted from increased populations of starter culture and NSLAB peptidases due to a stimulation in growth of lactococci and lactobacilli to 10 or six times that of controls, respectively.

Manipulation of the lactose content during cheese manufacture through the replacement of whey with water is commonly practised in the manufacture of Dutch, Swiss and Italian-type cheeses. The replacement of whey with hot water during the manufacture of these cheese types is used to control curd pH and moisture content. Typically, during Gouda-type cheese manufacture residual lactose is metabolized to lactic acid within 12 hours of manufacture (van den Berg *et al.*, 2004).

3.2.2 Effect of heat treatment on carbohydrates in cheese milk

When milk is heated, lactose may isomerize to lactulose, converting glucose to a fructose moiety. The glucose moiety can also convert to epilactose, but this isomerization event is quite rare. Indeed, the level of lactulose in heated milk is used as an indicator of the intensity of heat treatment which it receives. Heat treatment of milk can have a major impact on cheese flavour due to the Maillard browning reaction where an α -amino acid (principally the ϵ -amino group of lysine) and lactose initiate a complex series of reactions resulting in the formation of a range of cyclized compounds and H_2S , which can contribute to the formation of adverse flavours in the milk for cheese manufacture (Boelrijk *et al.*, 2003). The extent of the Maillard reaction in milk is directly related to the severity of heat treatment. Raw milk for cheesemaking is typically heat treated using a high temperature short time (HTST) pasteurization (72°C for 15 s), which is a relatively mild treatment with minimal impact on off-flavour development. However, it is evident that process variations resulting in increased pasteurization temperatures can result in the development of off-flavours by the Maillard browning reaction; this is especially evident in UHT treated dairy products where this reaction causes both flavour and nutritional defects (Boelrijk *et al.*, 2003).

Ribonucleosides including cytidine, guanosine and inosine show an increase in concentration in thermized or pasteurized milk, while adenosine concentra-

tion decreases on heat treatment of raw milk. These changes in ribonucleoside content in heated milks have been attributed to enzymatic activity in raw milk from enzymes such as adenosine deaminase and alkaline phosphatase during the heating-up phase prior to milk processing (Martin *et al.*, 1997; Schlimme *et al.*, 1998).

3.2.3 Coagulation

Rennet coagulation of milk occurs in two phases: the hydrolysis of κ -casein and the formation of para- κ -casein and glycomacropeptides. The glycomacropeptide (GMP) from κ -casein carries carbohydrate groups including *N*-acetyl-galactosamine (NGA), *N*-acetyl-neuraminic acid (NANA) and galactose. During cheese manufacture, GMP as well as a portion of the nucleotides, oligosaccharides and glycoconjugates principally partition with the whey (Schlimme *et al.*, 2000; Gopal and Gill, 2000). However, a substantial amount of these carbohydrates is sometimes retained in the cheese curd during manufacture and appears to have an impact on NSLAB growth during cheese ripening.

3.2.4 Metabolism of carbohydrate during acidification by starter culture lactic acid bacteria in cheese manufacture

Starter culture LAB grow and acidify in cheese milk and subsequently contribute to cheese flavour, mainly via their proteolytic enzyme complement. These microorganisms generate lactic acid, a major contributor to the flavour of acid-coagulated and immature rennet-coagulated cheeses. The production of lactic acid, together with its buffering capacity, determines the final pH of the cheese and so influences the microflora and enzymatic reactions responsible for cheese flavour.

Metabolism of carbohydrate by starter culture LAB can be broadly classified as homofermentation or heterofermentation. Homofermenters such as *Lactococcus lactis* utilize the glycolytic pathway involving the phospho- β -galactosidase enzyme to exclusively generate the L-isomer of lactic acid, while homofermenters such as *Lactobacillus helveticus* or *Streptococcus thermophilus* generate the D- or DL-isomers of lactic acid, respectively. In the case of heterofermenters, the lactose component in cheese milk is converted to glucose and galactose or galactose-6-phosphate, using the Embden–Myerhof pathway; glucose may then be oxidized to pyruvate that forms the substrate for subsequent conversion to lactate and compounds such as diacetyl, acetoin, acetaldehyde and ethanol (Cogan and Hill, 1993; Monnet *et al.*, 1995).

In Cheddar cheese, the main product of starter carbohydrate metabolism that contributes to the flavour profile is L-lactic acid, particularly early in the maturation process (McSweeney and Sousa, 2000). After manufacture, the levels of lactic acid in Camembert, Swiss, Cheddar and Dutch-type cheeses are ~1.0, 1.4, 1.5 and 1.2%, respectively. In the case of citrate, most is lost in the whey at drainage (~94%). However, growth of citrate utilizing LAB in the

presence of fermentable carbohydrate, including mixed D-, DL- and L-type starter cultures containing citrate positive (Cit⁺) lactococci or *Leuconostoc* spp., results in the production of a number of important flavour compounds including acetate, diacetyl, acetoin, butanediol and CO₂ (Monnet *et al.*, 1995).

The oligosaccharide components in milk may act as pre-biotic compounds to stimulate the growth of beneficial bacteria including bifidobacteria. However, little is known regarding the effect of naturally occurring pre-biotics in milk on the growth and metabolism of the microbial flora and on cheese flavour.

3.2.5 Metabolism of residual carbohydrate during ripening

During Cheddar cheese manufacture most of the lactose (~98%) is removed in the whey at drainage; however, 0.7–1.5% lactose remains in the curd after manufacture. Normally, residual lactose is metabolized to L-lactic acid by starter culture within the first few weeks of ripening. However, in cheeses with high salt-in-moisture (S/M) levels (6–7%) lactose can persist for up to six months. High levels of residual lactose in cheese curd may encourage the development of off-flavours during ripening by allowing NSLAB or spoilage organisms to grow to high numbers (at least 10^7 – 10^8 cfu g⁻¹). During Cheddar cheese ripening, L-lactic acid is also racemized by NSLAB to D-lactic acid or oxidized to acetic acid. Mesophilic NSLAB in cheeses can also metabolize residual lactose to lactic acid, ethanol and CO₂. Ethanol may contribute directly to cheese flavour or become esterified with free fatty acids to produce ethyl-esters responsible for fruity-type flavours in cheese. Racemization of L-lactic acid by NSLAB does not appear significant from a flavour point of view, but may contribute to textural or nutritional defects in the ripened cheese. Huffman and Kristoffersen (1984) suggested a relationship between residual lactose levels and thiol group (–SH) development in Cheddar cheese.

Residual lactose fermentation is more important in Swiss and Dutch varieties than in Cheddar cheese. Dutch-type varieties contain 1.4% lactose at pressing, most of which is converted to L-lactic acid within 24 hours. Swiss varieties contain 1.7% lactose after moulding but this is completely metabolized to galactose and L-lactic acid by *Streptococcus thermophilus* starter culture within 8–10 hours. *Streptococcus thermophilus* is unable to metabolize galactose, which is converted by the lactobacilli in the starter culture to a mixture of L- and D-lactic acid. However, if the lactobacilli are galactose negative, galactose accumulates in the curd, causing Maillard browning upon heating with consequent undesirable effects on flavour and appearance. It is generally understood that manufacture of premium quality Swiss-type cheese should involve the removal of all residual galactose from the curd (Fröhlich-Wyder, 2003).

Galactose-negative starter cultures are specifically used in the production of Mozzarella or *pasta-filata* type cheeses used as toppings for pizza where it is desirable for browning to occur upon cooking. Interestingly, directly acidified Mozzarella cheese does not display the same degree of browning on cooking and this has been attributed to the absence of proteolytic end-products, which

participate in the Maillard reaction. Conversely, starter culture acidified Mozzarella shows an increase in browning properties during ripening (Oberg *et al.*, 1991, 1992; Guinee and Kilcawley, 2004).

During Swiss-type cheese ripening, transfer to the hot room allows the rapid growth of propionibacteria that metabolize lactic acid, especially the L-isomer, to propionic acid, acetic acid and CO₂, contributing to eye development and flavour. Propionic acid is the main compound responsible for the distinctive sweet-type flavours of Swiss-style cheese varieties and can reach a level of 6 g kg⁻¹ of cheese. Evolution of glycolytic end-products of LAB metabolism during the ripening of Emmental cheese has been monitored in the aqueous phase of cheese (i.e. cheese juice) by Salvat-Brunaud *et al.* (1995). Cheese juice was expressed at four stages of ripening: post-brining, pre- and post-hot room and at the end of ripening. Lactose, glucose and galactose were not detected in the juice at any of the sampling points during ripening. Post-brining, the level of lactic acid corresponded to 90% of the lactose produced by LAB growth. The L-isomer of lactic acid constituted 60–70% of the lactate ion present during the initial two sampling points of ripening, but as ripening progressed this reduced to ~47% of the residual lactic acid. Propionic acid appeared in the juice after the hot room and reached 4.9 g acid kg⁻¹ cheese, reducing marginally to 4.1 g acid kg⁻¹ at the end of ripening. Similar to propionate, acetate was detected only after the hot room and was present at 5.8 g acid kg⁻¹, reducing to 5.0 g acid kg⁻¹ at the end of ripening. In contrast, citrate was present at 3.7 g kg⁻¹ up to the hot room and was not detected thereafter.

Overall, there are a number of pathways by which lactate can be transformed during cheese ripening: racemization to D-lactic acid by NSLAB; metabolism by propionibacteria to propionate, acetate and CO₂; metabolism by secondary microflora in mould- and smear-ripened cheeses to CO₂ and H₂O; oxidation by NSLAB to acetate, ethanol, formate and CO₂; and anaerobic metabolism through the germination and outgrowth of *Clostridium tyrobutyricum* spores to form butyric acid and H₂. The latter transformation is highly undesirable in cheese and leads to a late gas blowing event, principally in brine-salted varieties, with adverse textural, flavour and public health implications.

Cheddar cheese contains 0.2–0.5% citrate (w/w), which is metabolized by NSLAB (Fox and Wallace, 1997). Typically in Cheddar cheese no residual carbohydrate remains after four weeks of ripening and citrate provides the principal substrate for growth by Cit⁺ starter cultures and NSLAB (Turner and Thomas, 1980). However, metabolism of residual citrate may lead to problems with open texture in Cheddar cheese through the production of ~16 mmol CO₂ kg⁻¹ (Martley and Crow, 1996; Fox and Wallace, 1997).

In Dutch-type cheese, metabolism of citrate results in the production of a number of compounds important in the flavour of the cheese. The principal flavour compounds produced from citrate metabolism by LAB and NSLAB include acetate, diacetyl, acetoin, 2,3-butanediol and 2-butanone. The latter two compounds contribute to buttery or acetone-type odours in cheese, respectively (Sable and Cottenceau, 1999). Diacetyl is significant in the aroma and flavour of

Cottage cheese and Quarg. Diacetyl and its derivative 2-butanone contribute to the Cheddar flavour profile as well (Dimos *et al.*, 1996).

In some artisanal Swiss cheese manufacture, facultative heterofermentative non-starter lactobacilli are used as adjunct cultures to retard the propionic acid fermentation by fermenting hexoses to lactic acid. These cultures, which include *Lactobacillus casei* and *Lactobacillus rhamnosus*, grow during ripening by complete metabolism of the citrate present in fresh curd (9 mmol kg^{-1}) to generate formic acid, acetic acid and CO_2 (Fröhlich-Wyder, 2003).

Metabolism of lactic acid occurs extensively in surface mould-ripened varieties such as Camembert and Brie. The concentration of lactic acid in these cheeses is around 1% at one day. The lactic acid is produced exclusively by the mesophilic starter culture and hence is mainly L-lactate. Secondary organisms such as, initially, *Geotrichum candidum* and other yeasts, followed by *Penicillium camemberti*, and in traditional manufacture by *Brevibacterium linens* and other coryneform bacteria, quickly colonize and dominate the surface of these cheeses (Fox *et al.*, 2000). The pH of the cheese surface increases as the yeasts and moulds rapidly metabolize lactate to CO_2 and H_2O (McSweeney and Sousa, 2000). When the lactate has been exhausted, *Penicillium camemberti* metabolizes amino acids released from the caseins with the production of NH_3 (Cogan and Hill, 1993). Ripening of Camembert and Brie results in a softening of the texture from the surface towards the centre. This occurs because of the pH increase and the diffusion of $\text{Ca}_3(\text{PO}_4)_2$. Deacidification also has a major effect on the development of cheese texture, since Ca^{2+} precipitates at the surface as $\text{Ca}_3(\text{PO}_4)_2$, causing a Ca^{2+} gradient to arise within the cheese. Deacidification is essential for the growth of coryneform bacteria; *Brevibacterium linens* does not grow below pH 5.5. The growth of these particular bacteria, which dominate the smear during ripening, are primarily responsible for the characteristic flavour of such cheeses (McSweeney and Sousa, 2000).

Residual GMP present in cheese provides substrates for the growth of NSLAB in Danish semi-hard cheese types. *Lactobacillus paracasei* metabolizes most of the carbohydrates available in GMP with the exception of NANA (Adamberg *et al.*, 2005). The significance of GMP metabolism by NSLAB to cheese flavour is unknown, but it may provide a competitive advantage for certain NSLAB with which to colonize cheese and influence flavour.

The relationship between starter culture non-viability and NSLAB growth was first demonstrated by Thomas (1987) where strains of *Lactobacillus plantarum*, *L. casei*, *L. brevis* and *Pediococcus pentosaceus* grew to maximal cell densities when inoculated into washed suspensions of various starter cultures. Indeed, the maximal cell densities attained by the various NSLAB were shown to be starter culture related, with the highest cell density reached by *Lactobacillus brevis* of $\sim 10^7 \text{ cfu ml}^{-1}$. Addition of ribose to NSLAB and starter culture suspensions during incubation further increased growth of NSLAB, with *Pediococcus pentosaceus* increasing 20-fold. Thomas (1987) attributed NSLAB growth to the products released during lysis of the starter culture, including nitrogenous compounds and carbohydrates such as ribose and N-acetyl amino

sugars. In particular ribose, which is metabolized by many NSLAB strains, appeared to be released from lysing starter LAB in quantities sufficient to support the growth of NSLAB to densities of more than 10^7 cfu ml⁻¹. The implications of this study for cheese flavour are unclear, as only trace amounts of acetate, L(+)- and D(-)-lactate were formed on growth of NSLAB within this growth medium. Williams *et al.* (2000) showed that in addition to ribose, NSLAB strains grew on NAG and sialic acid, which may have originated from nucleic acid or casein deglycosylation. Adamberg *et al.* (2005) calculated the theoretical amount of bound carbohydrates in cheese as being <500 mg kg⁻¹, consisting of 400 mg kg⁻¹ derived from MFGM proteins, <30 µg kg⁻¹ derived from casein and 50 mg kg⁻¹ ribose. This concentration of carbohydrate could potentially support the growth of NSLAB to densities of 5×10^7 cfu g⁻¹.

The influence of differential carbohydrate metabolism on the growth kinetics of NSLAB in cheese is currently unknown. However, Williams *et al.* (2000) and Adamberg *et al.* (2005) noted inter-species or inter-strain differences in the growth of NSLAB on various carbohydrate substances in synthetic media. If these trends are mirrored in cheese then the dominance of a particular NSLAB strain/species in a particular cheese variety is directly influenced by the type and level of residual carbohydrate. In terms of cheese flavour, the contribution of NSLAB to flavour is generally recognized as being variable and dependent on the strain/species present in the cheese in addition to their density and proteolytic enzyme systems. Commercially, NSLAB strains are selected as adjunct cultures to enhance or accelerate the flavour of cheese (Klein and Lortal, 1999; Beresford *et al.*, 2001). In the light of the recent developments in NSLAB metabolism, a more targeted approach to selection of NSLAB adjuncts may be warranted to ensure strains are provided with their preferred substrates such as carbohydrates from GMP and MFGM or ribose or nucleotides from autolysed starter cultures.

3.3 Carbohydrate metabolism and flavour formation from amino acid catabolism

Amino acid catabolism is now recognized as a major pathway for flavour formation in cheese. However, this switch to a new nutrient source requires that lactose utilization stops before nitrogen metabolism for flavour formation can begin (Stuart *et al.*, 1999). Some flavours arising from protein metabolism require a number of major catabolic pathways, some that are initiated by amino acid transamination and result in the formation of α -keto acids, which are subsequently catabolized to different flavour and aroma compounds (Ganesan *et al.*, 2004; Tanous *et al.*, 2005). Aminotransferases are pyridoxal-5'phosphate dependent enzymes that are located inside the bacterial cell. Initially, the amino group of an amino acid is transferred to pyridoxal-5'phosphate to yield an α -keto acid and an enzyme-bound pyridoxamine-5'phosphate. The amino group is then transferred from pyridoxamine-5'phosphate to an α -keto acid to regenerate an amino acid and pyridoxal-5'phosphate (Curtin and McSweeney, 2004).

α -Ketoglutaric acid is the most studied α -keto acid acceptor for amino acid transamination by *Lactococcus lactis* (Tanous *et al.*, 2005). Yvon *et al.* (1998) suggested that the conversion of amino acids to aroma compounds by LAB is limited in St-Paulin cheese by the lack of available α -keto acids (i.e. amine receptors) and factors involved in their subsequent catabolism. Other studies also found that the inclusion of exogenous α -ketoglutaric acid or through genetically modified *Lactococcus lactis* producing excessive amounts of α -ketoglutaric acid has enhanced the aroma compounds from amino acids in Cheddar and model cheeses (Rijnen *et al.*, 2000; Banks *et al.*, 2001). Pyruvate is also used, but its aminotransferase activity was found to be considerably lower than that of α -ketoglutaric acid for lactococcal strains (Yvon *et al.*, 2000). Conversely, Ganesan *et al.* (2004) found that a number of different keto acids, including pyruvate, are used by LAB for the conversion of amino acids to flavour and aroma compounds with no preference by lactococci or lactobacilli. Amarita *et al.* (2001) found that pyruvate appeared to be as good an acceptor as α -ketoglutaric acid for two lactobacilli that primarily contained methionine aminotransferase activity, which was increased in the presence of glucose. Even though pyruvate appeared to have the greatest impact on amino acid catabolism under experimental conditions, its long-term impact over ripening may be less than that of α -ketoglutaric acid as it disappears rapidly because it is a substrate for other enzymes. The impact of this pathway in cheese ripening may also be limited by the fact that strains with citrate permease activity would quickly utilize available citrate in curd during the early stages of ripening (Tanous *et al.*, 2005).

α -Ketoglutaric acid is produced by two main pathways by LAB: the glutamate dehydrogenase pathway, which produces α -ketoglutaric acid by oxidative deamination of glutamate (Tanous *et al.*, 2002; Helinck *et al.*, 2004), and the production of α -ketoglutaric acid from citrate and glutamate by the successive action of citrate permease, citrate lyase and aspartate aminotransferase. This second pathway was successfully demonstrated in two strains of *Lactococcus lactis* ssp. *diacetylactis* (Tanous *et al.*, 2005).

3.3.1 Flavour generation from ribonucleotides

Milk contains a range of nucleotides and other nucleic acids which have potential flavour-enhancing or *umami*-type attributes. *Umami* is the fifth basic taste sensation, together with sweet, sour, salt and bitter. It is generally described as 'deliciousness' or in Western terms as 'savouriness'. Although many food-stuffs naturally contain components that impart this taste, others are formed during cooking, curing, ageing or fermentation (Marcus, 2005). The chemical compounds responsible for *umami* taste have been isolated and are monosodium glutamate (MSG), the naturally occurring sodium salt of glutamic acid, and the ribonucleotides 5'-inosine monophosphate (5'-IMP) and 5'-GMP (Kawamura, 1990). The nucleotides consist of pentose, phosphoric acid and a base of either purine or pyrimidine. Both IMP and GMP have three isomers that depend upon

the esterification position of the phosphate group on the pentose ring (position 2', 3' or 5'). Only the 5' isomers have *umami* characteristics. One of the most common sources of *umami* flavour is yeast extract, which is typically produced from a yeast containing a high amount of nucleic acids, such as *Torula* (>17% ribonucleic acid). The yeast cells are degraded by a cell wall lysing enzyme, or endo- or exo-proteinases, and the RNA is extracted and treated with an endo-ribonuclease 5'phosphodiesterase that acts on ester bonds to produce 5'AMP, 5'CMP, 5'GMP and 5'uridine monophosphate (5'UMP). Neither 5'CMP nor 5'UMP has *umami* characteristics, but the 5'AMP can be converted to 5'IMP by adenylic deaminase (Nagodawithana, 1992). Both 5'GMP and 5'IMP interact synergistically with MSG to potentiate the *umami* characteristic. 5'GMP is over twice as effective as a flavour enhancer compared with 5'IMP (Yamaguchi, 1991).

Yeast is widely used to develop flavour in soft mould cheeses, such as Camembert, but can also be present in semi-hard varieties such as Cheddar (Prentice and Brown, 1984; Welthagen and Viljoen, 1999). Yeast possesses proteolytic and lipolytic enzymes that act in similar ways to LAB. Some yeast, like bacteria, are successfully used as adjuncts to accelerate ripening of Cheddar cheese (Ferreira and Viljoen, 2003; Ur-Rehman *et al.*, 2003; Osthoff *et al.*, 2004). In addition, nucleic acids and ribonucleases are present as components derived from the cheese microflora along with MSG (Thomas, 1987; Adamberg *et al.*, 2005; Lanciotti *et al.*, 2005) and may provide important sensory characteristics to certain varieties. It is thought that one of the reasons why Parmesan and Gruyère-type cheeses are widely used as seasonings on dishes is that they have very high concentrations of free glutamate due to their extended ripening times (Marcus, 2005).

3.4 Future trends

Many of the fundamentals regarding the fate of the principal carbohydrates present in milk and the contribution to cheese flavour of the end-products of their metabolism by LAB and NSLAB are known. However, despite this, a number of knowledge gaps exist concerning the biochemical pathways involved in the degradation of the lesser carbohydrate components and their effects on the microbial flora and cheese flavour. The effect of lactose hydrolysis of cheese milk on starter culture LAB and NSLAB populations and cheese flavour has not been unequivocally resolved and the use of purified lactase enzymes to revisit this topic appears warranted. The interactions between mucin carbohydrate in MFGM, starter culture and NSLAB growth, the products of starter culture autolysis (especially ribose) and their effects on cheese flavour are still largely unknown but may prove important in the control of flavour. Understanding and enhancing the biotransformation of nucleotides and nucleotides in milk and the cheese matrix to savoury flavour compounds may provide avenues to enhance or accelerate cheese ripening. This may require selection or biotechnological

construction of strains having the particular enzymatic complement required for these pathways. Recent work on amino acid catabolism is unearthing the complex interactions that exist between products of LAB carbohydrate and nitrogen metabolism and their role in cheese flavour, and demonstrates the requirement for a greater understanding of the role of carbohydrate metabolism in cheese flavour.

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4

Amino acid metabolism in relationship to cheese flavor development

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4.1 Introduction

The flavor of each cheese is a product of the delicate balance among multiple compound classes (Müllder, 1952). The unique flavor of every cheese and the sets of different compounds, in varying quantities in each cheese type, are solely attributable to bacterial metabolism (Reiter *et al.*, 1967). The generation of cheese flavor is a complex web of metabolic processes among milk enzymes, added enzymes, the native milk microflora, and the starter culture. Lactose, casein and milk fat are the primary sources of carbon metabolism for bacteria in the cheese matrix during the ripening process.

The milk sugar lactose is metabolized to lactic acid during cheese manufacture. Residual lactose in the curd is utilized by bacteria within a week of cheese manufacture (Fox *et al.*, 1993). By 30 days lactose is exhausted from the cheese matrix and is not available for bacteria to generate glycolytic products. In the absence of lactose, bacteria utilize proteins in cheese as a source of ATP, carbon, sulfur and nitrogen. The caseins are metabolized to peptides and amino acids, which form the metabolic precursors of volatile sulfur compounds, aldehydes, ketones and fatty acids. All these compounds contribute to cheese flavor (Urbach, 1993).

Volatile sulfur compounds along with other classes of compounds commonly found in cheese play a major role in overall flavor perception (Aston and Douglas, 1983; Ferchichi *et al.*, 1985; Hemme *et al.*, 1982; Law and Sharpe, 1978; Manning, 1979; Weimer *et al.*, 1999). While lipolysis is the mechanism involved in generating the lipolytic flavor of Italian cheeses and flavor defects in milk and other dairy products (Law, 1984; Seitz, 1990), volatile sulfur compounds, alcohols, aldehydes and fatty acids involved in flavor arise from

amino acid metabolism. The lipolytic process is highlighted by the limited lipolytic capability of the starter culture and non-starter bacteria used in the manufacture of hard cheeses (El Soda *et al.*, 1986; Medina *et al.*, 2004). Fatty acids play a role in cheese flavor (Sandine and Elliker, 1970), and are produced by lactic acid bacteria (LAB) via glycolysis, lipolysis and proteolysis by amino acid metabolism.

The stressful environment of low temperature, high acid, no lactose, and high salt in the moisture leads to unusual metabolic processes during ripening. Hence, alternate mechanisms for flavor compound production by LAB are active during cheese ripening, as compared to metabolism in pure culture with laboratory media. Protein metabolism and amino acid metabolism dominate the later phase of ripening (Christensen *et al.*, 1999; Tammam *et al.*, 2000). The catabolic mechanisms that are active during flavor generation will depend on the genera and their physiological state and specific metabolic processes in place (Ganesan *et al.*, 2004a, b; Nakae and Elliott, 1965a; Stuart *et al.*, 1999). Sugar starvation and pH activate the metabolic mechanisms in lactococci that prevail during cheese ripening for amino acid metabolism (Ganesan, 2005). This chapter reviews the role of amino acid metabolism among LAB to yield flavor compounds in cheeses. The role of bacterial physiology in flavor production will also be discussed.

4.2 Compounds associated with cheese flavor

Cheese flavor is a combined effect of various compounds at different concentrations and their interactions (Müllder, 1952). Each cheese has a specific group of flavor compounds that are responsible for its flavors (Table 4.1). Multiple classes of organic compounds are implicated in cheese flavor (Kristoffersen, 1975; Vedamuthu *et al.*, 1966). But knowledge of the impact is limited to a few groups (Urbach, 1993). Compounds contribute specific flavor attributes based on their physico-chemical properties (Urbach, 1993). Some compounds represent typical flavors of certain cheeses, acting as impact compounds for that flavor, but not for the total flavor perception (Table 4.1). Multiple lists of flavor compounds are available in the literature (Fox and Wallace, 1997; Fox *et al.*, 1996; Urbach, 1993, 1995).

Flavor compounds are generated from substrates available during cheese ripening. The direct role of amino acids and peptides in cheese flavor is limited (Aston and Creamer, 1986; Engels and Visser, 1994) to contribution to base cheese flavor (Sandine and Elliker, 1970) and acting as substrates for enzymatic modification reactions (Urbach, 1995). Fatty acids and volatile sulfur compounds (VSCs) correlate with flavor development during cheese ripening. VSCs are one of the major classes of flavor compounds that correlate with good Cheddar cheese flavor (Dias and Weimer, 1999; Manning *et al.*, 1976; Weimer *et al.*, 1999).

Fatty acids exist both alone and in combinations with VSCs as thioesters. While microbial mechanisms of thioester production exist, they are yet to be

Table 4.1 Precursors for fatty acid production

Precursor	Keto acid	Fatty acid	Aroma threshold (ppm)
Alanine, glycine, serine	Pyruvate	Acetic acid	40.3
Alanine, aspartic acid, threonine, valine	Pyruvate, ketobutyrate, ketoisovalerate	Propionic acid	
Pyruvate, lipolysis		Butyric acid	0.3 at pH 5.2
		Isobutyric acid	5.3 at pH 2.0
		4-methyl pentanoic acid	0.61 at pH 2.0
		6-methyl pentanoic acid	0.84 at pH 2.0
Isoleucine	Ketoisocaproate	Isovaleric acid	3.2 at pH 2.0
Isoleucine, leucine	4-Methyl-2-ketovalerate	Isovaleric acid	6.5 at pH 5.2
	Ketoisocaproate	<i>n</i> -Valeric acid	1.1 at pH 2.0
	Ketoisocaproate	Isocaproic acid	—

characterized for their contribution to cheese flavor (Cuer *et al.*, 1979a, b). Fatty acids along with their own individual flavor are also converted to ketones, esters, and lactones in the reduced conditions of cheese (Law, 1983, 1984; Law *et al.*, 1976). Additionally, fatty acid ethyl esters, especially of caproic and caprylic acids, are involved in cheese flavor (Fox and Wallace, 1997). The actual sources of these fatty acids, especially branched chain fatty acids (BCFAs), in cheese are potentially branched chain amino acids (BCAA) and their metabolism (Ganesan, 2005).

4.3 Proteolysis in cheese

The gradual breakdown of proteins in the firm, hard cheese curd to smaller-sized peptides and amino acids leads to a cheese with softer texture and better flavor than the original rennet curd (Fox and Wallace, 1997; Fox *et al.*, 1996; Law *et al.*, 1992; Wallace and Fox, 1997). Hence, proteolysis is important in hard cheeses for flavor and texture development (Fox *et al.*, 1996). It provides substrates for flavor development by further protein breakdown and indirectly affects the mouth-feel and flavor release during mastication (Fox and Wallace, 1997).

Starter proteinases act on the residual products of casein breakdown by rennet and plasmin (Law *et al.*, 1992). The oligopeptides are then hydrolyzed to yield smaller peptides and amino acids by intracellular peptidases in LAB (Christensen *et al.*, 1999). It is estimated that ~25% of the proteins in cheese are hydrolyzed to peptides and amino acids in ripening (Chapman and Sharpe, 1990). Amino acid catabolism leads to production of flavor compounds. The general scheme of proteolysis and peptide transport is represented in Fig. 4.1.

Proteolysis in milk and cheese is important to release free amino acids and peptides (Fox *et al.*, 1993, 1995; Fox and Wallace, 1997; Law *et al.*, 1992;

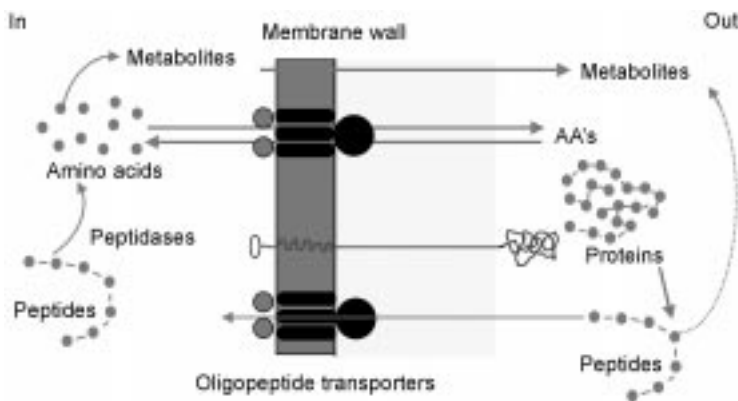


Fig. 4.1 General scheme of proteolysis and peptide and amino acid transport to form flavor-related metabolites by lactic acid bacteria.

McGarry *et al.*, 1994; O'Keeffe *et al.*, 1976; Wilkinson *et al.*, 1994). However, it directly contributes to bitterness (Broadbent *et al.*, 1998) and background flavors. Hence, amino acid catabolism is important for beneficial flavor development in cheese. The di-, tri- and oligo-peptides generated from extra-cellular proteolysis are acquired by the peptide transport systems and broken down to their constituent amino acids prior to catabolism (Siezen, 1999). In cheese, which is a very complex medium, post-carbohydrate exhaustion, amino acids are the simplest molecules available for non-lipolytic bacteria to generate ATP for cellular processes, which in this effort produce compounds that impact flavor. Hence, the conversion of amino acids to fatty acids serves dual purposes in cheese. It aids both survival of bacteria and flavor development in the product.

4.4 Amino acid metabolism

Casein yields flavor compounds by the conversion of amino acids to volatile end products (Hemme *et al.*, 1982; Nakae and Elliott, 1965a, b). Several mechanisms for production of flavor compounds from amino acids are detailed in the literature, that include both enzymatic and non-enzymatic degradation of amino acids in cheese (Visser, 1993). The importance of amino acid metabolism lies in cellular necessity for surviving LAB to acquire energy in the form of ATP, and carbon, sulfur and nitrogen for physiological maintenance.

4.4.1 Amino acid transport and utilization

Amino acids are essential for bacterial growth. Glutamate, valine, methionine, leucine, isoleucine and histidine are essential for LAB. Amino acids also enhance bacterial survival in the absence of other substrates in *Lactococcus lactis* ssp. *lactis* (Thomas and Batt, 1968); arginine extends LAB metabolism

Table 4.2 Amino acid composition of Cheddar cheese and basal chemically defined medium (CDM)

Amino acid	Concentration (ppm)	
	Cheese	CDM
Alanine	286.84	241.4
Arginine	1001.36	172.92
Asparagine	0	91.28
Aspartic acid	1328.1	0
Cysteine	38.517	83.28
Glutamine	0	89.67
Glutamic acid	2769.52	269.01
Glycine	235.98	153.9
Histidine	0	41.13
Isoleucine	404.898	90.48
Leucine	2396.59	90.48
Lysine	1006.98	180.74
Methionine	386.745	91.77
Phenylalanine	1326.84	176.52
Proline	286.445	252.46
Serine	348	252.3
Threonine	382.158	171.87
Tryptophan	0	93.1
Tyrosine	611.625	48.93
Valine	938.477	89.19
Total AA	13749.1	2680.4

and survival during carbohydrate starvation (Chou, 2001; Stuart *et al.*, 1999; Thomas and Batt, 1968). A comparison of the amino acid profiles of a basal chemically defined medium (Jensen and Hammer, 1993) and six-month-old Cheddar cheese (Table 4.2) shows that Cheddar cheese contains almost all amino acids in excess or in comparable amounts to that of the sterile medium. Hence, it may be deduced that if bacteria can maintain viability within such a minimal medium (Stuart *et al.*, 1999), they will remain active in milk and Cheddar cheese and contribute to cheese flavor.

Amino acids are transported by lactococci via three different mechanisms. Alanine, serine, the branched chain amino acids (BCAAs) (leucine, isoleucine and valine), and lysine are transported by a proton-motive-force-driven mechanism. Arginine is transported inside the cell by an ornithine antiporter system. Similar antiporter systems also exist for histidine–histamine, tyrosine–tyramine, and aspartic acid–alanine couples (Poolman, 1993). Glutamate, glutamine, aspartate and asparagine are transported by a phosphate bond-driven transport system (Driessen *et al.*, 1989), likely to be ATP-driven (Bolotin *et al.*, 2001). Hence, transport of most amino acids will require either a gradient (i.e. proton or positive ion) or energy, although permeases also play a role for specific compounds, like amino acids. This may exist only if cells continue to

remain viable and to actively metabolize substrates like amino acids for survival.

It has long been believed that lactococci are alive only in the early steps of cheese-making and produce acid (Arora *et al.*, 1995; Crow *et al.*, 1995; O'Keefe *et al.*, 1976); subsequently they act as 'bags of enzymes' that die and lyse to release metabolic enzymes that are active during cheese ripening (Buist *et al.*, 1998; Ostlie *et al.*, 1995; Wilkinson *et al.*, 1994). However, increasing research into firmicute metabolic states shows that a minor proportion of the lactococcal population may die; however, a larger population continues to exist in a nonculturable state (Ganesan, 2005). The nonculturable population consists of cells with intact cell walls, that remain capable of amino acid and peptide transport and metabolism (Ganesan, 2005). The presence of intact cells reinforces the importance of transport systems that provide alternate substrates such as amino acids for catabolism during sugar starvation and cheese ripening to produce flavor-related compounds.

4.4.2 Amino acid catabolic pathways

Amino acids are either bidirectionally transaminated or deaminated by bacteria to produce α -keto acids and specific flavor compounds (Table 4.1). The keto acids are subsequently metabolized to yield fatty acids, alcohols, and aldehydes. Several unique pathways exist in LAB that are being discovered due to genomics, whereby amino acids are converted to acetyl-CoA, which is interconvertible across the different amino acids and flavor compounds via pyruvate metabolism. Acetyl-CoA and pyruvate are important cellular intermediates for both energy and production of intracellular intermediates and connect to both sugar and amino acid metabolism (Fig. 4.2). A number of amino acid pathways in LAB serve the same or similar purposes, specifically ATP generation by substrate level phosphorylation, pH regulation, redox potential maintenance and balancing nutritional requirements (Fig. 4.3) (Harwood and Canale-Parola, 1981; Zhang *et al.*, 1999). This section reviews metabolism of specific amino acids that yield energy, flavor compounds, and modulate the redox potential.

Arginine catabolism

At the onset of carbohydrate starvation, arginine is utilized by LAB to increase the extracellular pH and produce ATP via the arginine deiminase (ADI) pathway (Fig. 4.4) (Chou, 2001; Thomas and Batt, 1969b). Arginine is co-metabolized with lactose, and is important only during the onset of starvation, as it is catabolized immediately in lactose limitation or lower pH (Chou, 2001). Arginine residues are present in limited number in casein in comparison to other energy-yielding substrates such as BCAAs (Banks and Dalgleish, 1990; Gordon and Kalan, 1974; Jenness and Patton, 1959; Johnson, 1974). Arginine is synthesized from glutamate by LAB (Fig. 4.5) (<http://biocyc.org/META/server.html>). The ADI gene cluster is characterized in lactococci (Chou, 2001) and lactobacilli (Zuniga *et al.*, 1998) but not directly linked to beneficial cheese flavor;

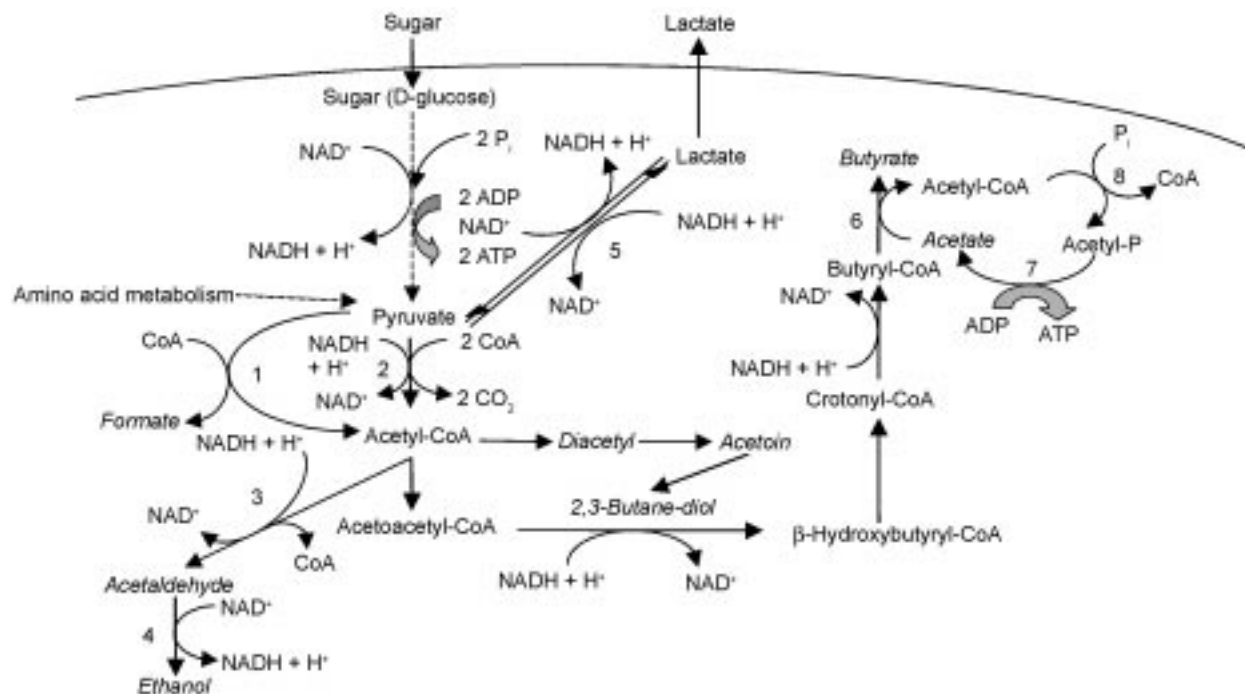


Fig. 4.2 Carbohydrate catabolism to fatty acids and other flavor compounds. Dotted lines represent catabolic steps to pyruvate via glycolysis. Known enzymes are: 1, pyruvate-formate lyase; 2, pyruvate dehydrogenase; 3, aldehyde dehydrogenase; 4, alcohol dehydrogenase; 5, lactate dehydrogenase; 6, acyl kinase; 7, acetyl kinase; 8, phosphotransacetylase. Note that none of the BCFAs are produced here. Amino acid metabolism feeds into pyruvate and acetyl-CoA.

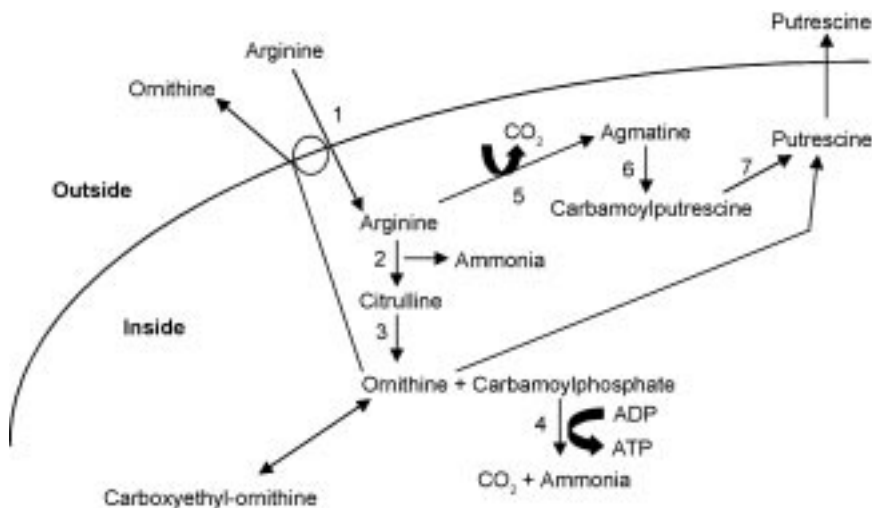


Fig. 4.4 Metabolic fate of arginine in bacteria by arginine deiminase (ADI) or arginine decarboxylase pathway. Enzymes are: (1) arginine/ornithine antiporter, (2) arginine deiminase, (3) ornithine transcarbamoylase, (4) carbamate kinase, (5) arginine decarboxylase, (6) agmatine deiminase, (7) *N*-carbamoylputrescine hydrolase. Steps 1–4 define the ADI pathway; steps 5–7 indicate the arginine decarboxylase pathway. (Data adapted from references Cunin *et al.*, 1986; Poolman and Konings, 1993; Thompson and Miller, 1991.)

however, it produces ATP and modulates pH by the production of ammonia and carbon dioxide. The production of ornithine by this pathway serves to transport arginine via the arginine–ornithine antiporter system, while citrulline acts via the arginine cycle to yield either aspartate or fumarate and arginine (Chou, 2001).

The genes for arginine utilization by the ADI pathway are located within a cluster in lactococci (Fig. 4.4). While other organisms that contain the ADI pathway have the genes organized in a single operon, conversely some of the genes contain their own promoter regions upstream and are regulated separately in lactococci (Fig. 4.4). The genes also have several *cis*-regulatory element sites in their intergenic regions, suggesting the involvement of the pleiotropic carbon regulator CcpA in the regulation of this pathway (Chou, 2001; Gaudu *et al.*, 2003; Kunji *et al.*, 1993). This also points to the interplay between arginine and sugar metabolism observed physiologically by Weimer's group (Chou *et al.*, 2001). Stress conditions and the presence of different substrates stimulated transcription of the ADI gene cluster in lactococci (Fig. 4.4). These results also pointed to the interplay between stress and sugar starvation, along with interconnected pathways for amino acid catabolism.

Lesser-known pathways for LAB arginine metabolism are being identified using biochemical testing in specific strains. One pathway produces nitric oxide (Morita *et al.*, 1997) while another pathway decarboxylates arginine to ultimately produce putrescine (<http://biocyc.org/server.html>), which is identified with the smell of rotting flesh, and hence unacceptable flavor at higher

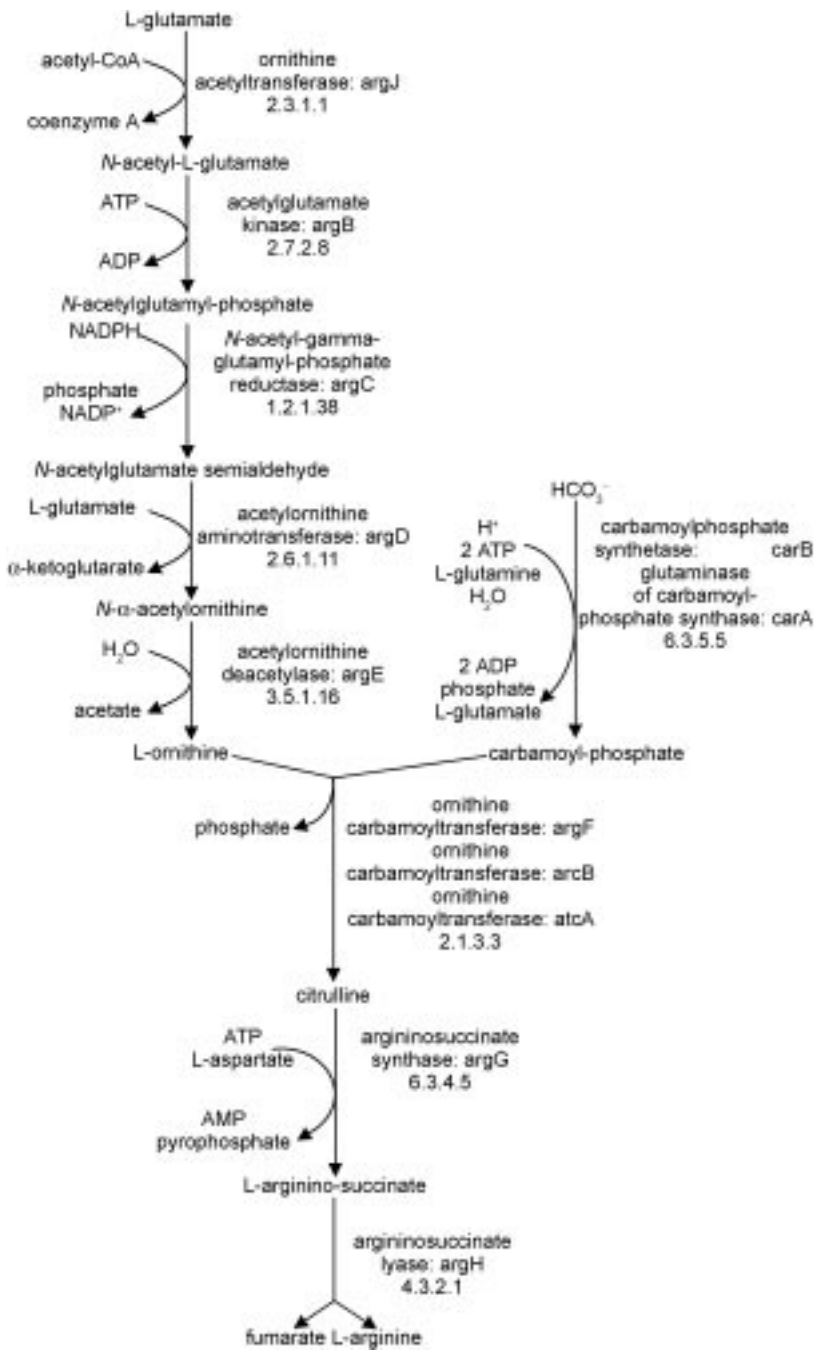


Fig. 4.5 Biosynthesis of arginine from glutamate degradation in *Lactococcus lactis* IL1403. Fig. produced from <http://biocyc.org/META/server.html> based on genomic reconstruction of metabolic pathways.

concentrations (Christensen *et al.*, 1999). The physiological relevance of these pathways is also unknown, but is probably involved with intracellular pH regulation and energy production.

Catabolism of alanine, aspartate and asparagine

Alanine is predominantly a biosynthetic amino acid that forms part of cell wall structures in its D-form (Steen *et al.*, 2005). However, L-alanine is metabolized and produced during carbohydrate starvation and cheese ripening (Harper and Wang, 1980; Harper *et al.*, 1980). Alanine is also produced from aspartate by decarboxylation (<http://biocyc.org/server.html>), but the diversity of this mechanism is not fully defined in LAB. Alanine is either transaminated or deaminated to yield pyruvate (Fig. 4.3), which can be metabolized to lactic acid or to other intermediates and amino acids via the partial Krebs cycle in LAB (Wang *et al.*, 2000). Alanine is an initial substrate that may yield important flavor compound precursors such as pyruvate and oxaloacetate, which are converted to aldehydes and ketones such as acetaldehyde, acetone, 2,3-butanediol and diacetyl during cheese ripening (Figs 4.1 and 4.3).

Aspartate is produced and consumed by lactococci in various environmental conditions. Aspartate biosynthesis occurs in lactococci via an aminotransferase (ATase) (Dudley and Steele, 2001). Another gene for a different ATase also exists in lactococci, but is not known to be involved in biosynthesis (Dudley and Steele, 2001). Transamination or deamination of aspartate produces oxaloacetate, which participates in the partial Krebs cycle of LAB. Asparagine in turn is synthesized from aspartate via asparagine synthetase or synthase reactions, which are bidirectional. These reactions also serve as initiating reactions for asparagine catabolism, which proceeds via aspartate (Fig. 4.3). Sugar-starved lactococci at pH 5.2 produce acetic, propionic, isobutyric, and caproic acids from aspartate, while lactobacilli produce only acetic and propionic acid (Ganesan *et al.*, 2004a). These short-chain fatty acids are flavor components of various cheeses (see Chapter 2). These fatty acids are also produced in cheese by LAB from aspartate and asparagine, apart from providing oxaloacetate for diacetyl and acetaldehyde.

Catabolism of histidine

Histidine is catabolized by LAB to produce metabolic energy and also used to regulate intracellular pH and redox potential (Konings *et al.*, 1989; Molenaar *et al.*, 1993). Transport occurs via a histidine–histamine antiport system, while histidine itself is decarboxylated to produce histamine, which is related to food poisoning (Doeglas *et al.*, 1967a, b; Joosten and Nunez, 1996). Alternate pathways of histidine metabolism are yet to be discovered in LAB. The role of histidine was attributed to ATP generation by membrane potential by the F₁F₀ ATP synthase. However, the ATP synthase in LAB acts only as a proton efflux pump coupled with ATP hydrolysis (Poolman, 1993). A metabolic comparison to *B. subtilis* shows that lactococci and lactobacilli possess similar classes of less studied enzymes for histidine degradation to glutamate (<http://biocyc.org/>

META/server.html), which is metabolized via other pathways. These observations suggest that histidine metabolism in LAB needs further examination to fully explore the molecular details.

Catabolism of threonine

Threonine is less studied as a precursor of cheese-related flavor compounds. This amino acid is deaminated to produce α -ketobutyrate and propionic acid (<http://biocyc.org/server.html>). The physiological role for threonine metabolism is potentially ATP production and pH regulation. Threonine aldolase action is also known in LAB to generate glycine and acetaldehyde (Lees and Jago, 1976), which can then act as flavor compounds or be further metabolized to other compounds. However, threonine was catabolized to acetic, propionic, isobutyric and caproic acids by LAB (Ganesan *et al.*, 2004a). Threonine may also serve as a precursor for BCFA production in cheese.

Catabolism of proline and lysine

Lysine and proline are biosynthetic products from glutamate and arginine metabolism. These amino acids are important components of cell wall structures in their D-form. The L-forms are still catabolized to produce energy. Flavor adjunct bacteria, such as *brevibacteria*, produce lysine during ripening (Su and Jane, 1995). Lysine and proline are catabolized to fatty acids in cheese-like conditions (Ganesan *et al.*, 2004a). Other metabolic products and the definitive pathways warrant further study and characterization.

Catabolism of glycine and serine

Glycine is synthesized via 3-phosphoglycerate, and is used as a precursor for serine biosynthesis (<http://biocyc.org/server.html>). Glycine is physiologically important as it is the only amino acid with a single-carbon side chain, and hence is essential for reactions that involve single-carbon transfer, as it provides the terminal methio-group for methionine. It plays a crucial role in S-adenosyl methionine biosynthesis as it provides the methylating carbon for tetrahydro-folate (<http://biocyc.org/server.html>). Glycine is hence indirectly connected to sulfur amino acid metabolism and flavor compound generation. However, the involvement of glycine metabolism needs further characterization in sulfur metabolism.

Serine is produced from 3-phosphoglycerate via glycine by bacteria. It is catabolized to hydroxy-pyruvate and pyruvate, and joins central metabolism and the partial Krebs cycle (<http://biocyc.org/server.html>). Serine is also a precursor for cysteine and methionine biosynthesis in LAB (Fernandez *et al.*, 2000; Golic *et al.*, 2005). During starvation and cheese ripening, the serine concentration increases due to lactococcal metabolism (Stuart *et al.*, 1999). It is known as the hallmark of sugar starvation in bacteria, but the role of serine metabolism is poorly defined for LAB cell physiology. Recently, Liu *et al.* (2003a, b) studied serine metabolism in *Lactobacillus plantarum* to show that serine is metabolized to acetate. This agrees with Ganesan *et al.* (2004a), who also found only acetate

production by lactobacilli; but lactococci produced propionic, isobutyric and caproic acids, all of which are involved in cheese flavor.

Catabolism of aromatic amino acids

Aromatic amino acid catabolism is important in LAB as it leads to compounds that cause off-flavor in cheese (Gao *et al.*, 1997). The rosy, floral, fruity and related flavor and aroma descriptors for defective cheese arise from products of tryptophan, tyrosine and phenylalanine catabolism (Gao *et al.*, 1997; Rijnen *et al.*, 1999b). Schematically, the pathways for the catabolism of these aromatic amino acids are similar (Fig. 4.6). The ring portion is typically intact; while the amino-carbon is typically transaminated to produce an aromatic pyruvate, which is further reduced to an aromatic acid, aldehyde or alcohol (Gao *et al.*, 1997; Rijnen *et al.*, 1999a). Lactate derivatives of the aromatic side chain are also noted in laboratory media and cheese. Additionally, tryptophan and tyrosine are also decarboxylated to form tryptamine and tyramine, respectively. Other end products with pathways yet to be defined but involved in off-flavor are *p*-cresol, indole, and related phenols (Christensen and Reineccius, 1995; Vandeweghe and Reineccius, 1990).

Aromatic amino acid metabolism has been extensively characterized in lactococci by Gao *et al.* (1997). The initiating reaction was determined to be an ATase, and no other side-chain activities were detected (Gao *et al.*, 1997). This was genetically verified by deletions of genes that encoded the ATase (Rijnen *et al.*, 1999a). Further work on lactobacilli for aromatic amino acid metabolism showed that a subsequent dehydrogenase activity was detected, but attributed the rest of the reactions leading to the end products to be spontaneous (Gummalla and

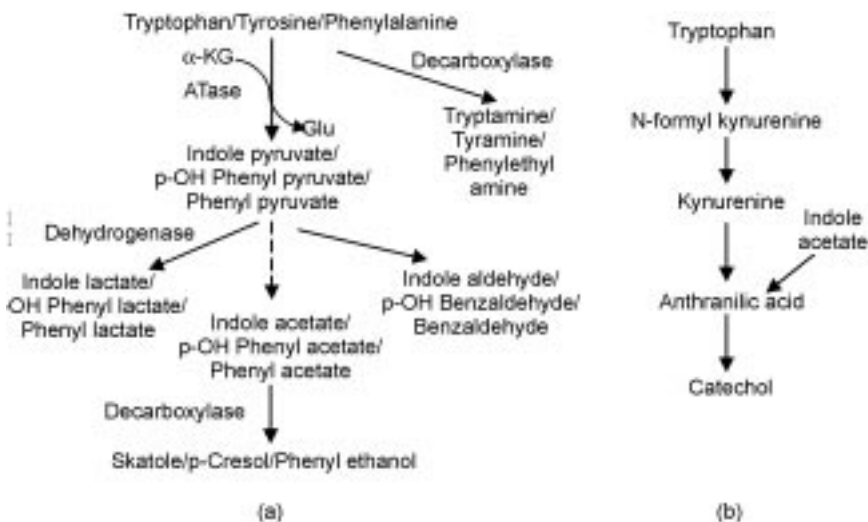


Fig. 4.6 Aromatic amino acid degradation pathways to off-flavor end products. Dotted lines represent multiple catabolic steps involved. (a) Pathways in LAB; (b) Pathway in *Brevibacterium linens*.

Broadbent, 1999). While this was extended to stabilize the products by forming the corresponding hydroxy acids, overall flavor development was retarded (Broadbent *et al.*, 2004). This also suggests that these compounds may contribute to overall flavor at lower levels, as observed for VSCs (Weimer and Dias, 2005; Weimer *et al.*, 1999). In *Brevibacterium linens*, tryptophan is degraded via the anthranilic acid pathway to produce kynurenine and anthranilic acid (Fig. 4.6), but not the products of tryptophan ATase (Ummadi and Weimer, 2001). Hence, this adjunct bacterium does not produce these off-flavor compounds while LAB do, which lead to off-flavors such as barny or utensil. This interaction between the metabolism of starter cultures and flavor adjunct bacteria is noted in culture pairs used to make low fat cheese (Weimer *et al.*, 1997).

Catabolism of glutamate and glutamine

Glutamate is the most recent addition to studies of cheese flavor production and metabolism (Rijnen *et al.*, 2000; Tanous *et al.*, 2002). Notably, metabolic analysis shows that glutamate participates in over 150 pathways in LAB (<http://biocyc.org/META/server.html>). Glutamine is inter-convertible with glutamate by glutamine synthase and an ATase (Lapujade *et al.*, 1998), which acts as a nitrogen fixing mechanism. In LAB glutamate metabolic studies have focused on regeneration of its source keto acids (i.e. α -ketoglutarate), which provides transamination precursors for catabolism of other amino acids, including BCAAs, aromatic amino acids, and methionine (Rijnen *et al.*, 2000; Tanous *et al.*, 2002). However, most studies have focused on aroma and have not conclusively demonstrated an increase in specific metabolites for flavor production, such as VSCs. The role of glutamate dehydrogenase, as a recent discovery in LAB, is not clearly understood, but is likely involved in redox regulation (Tanous *et al.*, 2005; Williams *et al.*, 2002). Recent work by Weimer's group found sulfur-containing compounds change substantially in the presence of the glutamate dehydrogenase gene in lactococci.

Alternate pathways also exist for glutamate degradation beyond its action as an amino-group donor/acceptor (Fig. 4.2). Genes related to the enzyme classes for catabolism of glutamate to succinate are found in LAB (<http://biocyc.org/META/server.html>), as well as for its metabolism to form arginine (Fig. 2.5). These may act as two different mechanisms for glutamate assimilation, either via the partial Krebs cycle from succinate, or via arginine metabolism to produce ATP. Glutamate is also catabolized to fatty acids by both lactococci and lactobacilli in cheese-like conditions (Ganesan *et al.*, 2004a). However, subverting the initial ATase reaction by providing α -ketoglutarate as the initial substrate resulted in increased isobutyric acid concentration and production of *n*-butyric acid by lactococci, while lactobacilli produced isovaleric acid but not *n*-butyric acid. While this study verified that glutamate is also catabolized to fatty acids, it also pointed out differences in the pathways through which it is potentially metabolized to produce different end products. Further investigations into the role of glutamate catabolism in LAB are needed apart from amino-group exchange, as it is associated with cheese flavor.

Catabolism of α -keto acids

The metabolism of amino acids in LAB typically requires an amino-acceptor keto acid to form another amino acid (Yvon *et al.*, 1997). α -Ketoglutarate has been the choice to promote ATase action by providing it as the primary acceptor (Yvon *et al.*, 1999). While this work ignored autodegradation of ketoglutarate to flavor-related fatty acids (Ganesan *et al.*, 2004a), metabolic engineering was done to convert back the glutamate produced from this reaction to ketoglutarate by glutamate dehydrogenase (Rijnen *et al.*, 2000; Tanous *et al.*, 2002). However, this body of work ignores other keto acids in cells and their ability to also act as amino-acceptors and flavor precursors.

Ganesan *et al.* (2004a) showed that keto acids are also converted to fatty acids, though not the same as products from their precursor amino acids. Several keto acids are present in intracellular fractions of long-term-starved lactococci (Ganesan, 2005), suggesting that amino-acceptors are not limiting for fatty acid production during starvation and nonculturability. The lack of an ATase caused lactococci to produce different end products from precursor amino acids and product keto acids (Ganesan and Weimer, 2004), suggesting that different ATases are involved in regulation of keto acid metabolism. The ability of long-term-starved lactococci to produce fatty acids without any external supplementation with keto acids (Ganesan, 2005; Ganesan *et al.*, 2006) also questions the importance of a single keto acid to be the primary amino-acceptor for amino acid metabolism.

Catabolism of sulfur amino acids

The production of VSCs was one of the earliest known mechanisms that directly related to positive cheese flavor development, especially in hard cheeses (Manning, 1974) where methanethiol is positively correlated with cheese flavor. The flavor profiles of VSCs have been discussed in this book and elsewhere in greater detail (Weimer *et al.*, 1999), and all known methionine pathways are summarized and illustrated in Fig. 4.6. The sources of the sulfur atom remain the sulfur-containing amino acids. Since cysteine is absent in caseins, the exclusive source of organic sulfur is methionine for LAB from casein in the ripening curd. However, the low number of methionine residues in caseins (1–2 residues per casein molecule) does not account for its increase beyond availability during cheese ripening. Recent studies demonstrate that both the starter culture and flavor adjunct cultures are capable of fixing inorganic sulfate into amino acids and VSCs (Ghosh, 2004). The increase in methionine during ripening is accompanied by an increase in its precursor amino acid serine (Stuart *et al.*, 1999). Sulfur fixation may account for the increase in methionine and VSCs during cheese ripening.

The products of methionine metabolism have hence been the focus of three decades of flavor research. Several studies using gas chromatography identified different VSCs (Weimer *et al.*, 1999). These were products of methionine catabolism to methanethiol (Fig. 4.7), which is then further chemically or enzymatically oxidized to form the polymeric organic sulfides such as di- and

Methionine is catabolized to methanethiol in both culture and cheese-like conditions (Dias and Weimer, 1998a, 1999). Addition of cultures to slurries increased VSC levels, which were also augmented by addition of adjunct bacteria to slurries made with the starter culture. LAB utilized both cystathionine and methionine to produce VSCs, but only low levels of cystathionine lyase activities were detected (Seefeldt and Weimer, 2000), suggesting that other enzymes and mechanisms were involved in VSC production in LAB. Notably, lactobacilli produced lower amounts of VSCs than lactococci, providing strength for a greater role of starters in flavor generation. Other organisms that produce VSCs include yeasts and molds associated with cheese (Bonnarme *et al.*, 2001a; Kadota and Ishida, 1972) and the adjunct organism, *B. linens* (Dias and Weimer, 1998b).

The majority of cheese-related organisms initiate methionine via an ATase. This reaction is found to occur commonly in LAB and yeasts and molds (Bonnarme *et al.*, 2001a, b; Dias and Weimer, 1998a). Lactococci are postulated to have only two functional ATases with specificity for methionine (Alting *et al.*, 1995; Yvon *et al.*, 2000). In spite of the lack of a specific sulfur amino acid ATase (SAT) being found to date in *L. lactis* (Rijnen *et al.*, 1999a, 2000; Yvon *et al.*, 1999, 2000), it is possible since the lactococcal and lactobacillic genomes contain ~12 ATases, many of which are not biochemically characterized, allowing options for additional enzymes to be found.

The sulfur-containing keto acid, α -keto- γ -methiobutyrate, is subsequently metabolized to yield methanethiol via formation of 3-methylthiopropionic acid (Weimer *et al.*, 1999), but the intermediates are yet to be identified (Gao *et al.*, 1998). In contrast, brevibacteria contain a unique enzyme, methionine- γ -lyase, that catalyzes a single-step reaction to make methanethiol and α -ketobutyrate (Dias and Weimer, 1998b). The keto acids and related products may then be converted to other flavor compounds such as fatty acids. LAB has produced fatty acids from methionine under cheese-like conditions (Ganesan *et al.*, 2004a), and may also use the carbon from methionine as a source during starvation. While methanethiol production and its subsequent oxidation to other VSCs are widely accepted, further delineation of the involved mechanisms for VSC production and their physiological role is necessary.

Catabolism of branched chain amino acids

Multiple genera produce fatty acids in cheese environmental conditions. Acetic, propionic, isobutyric, *n*-butyric, isovaleric and *n*-caproic acids are produced by lactococci and lactobacilli, the product depending on the strain (Nakae and Elliott, 1965b). The pH optima for fatty acid production vary for each organism. Lactobacilli produce valeric acid and its isomers from leucine and isoleucine at a lower pH (Nakae and Elliott, 1965a). *Lactobacillus delbrueckii* ssp. *bulgaricus* produces propionic and *n*-butyric acids, while *L. casei* ssp. *casei*, *L. delbrueckii* ssp. *lactis* and *Streptococcus salivarius* ssp. *thermophilus* produce acetic acid (Thornhill and Cogan, 1984). In similar experimental conditions, *L. lactis* ssp. *cremoris* produces more BCFAs than *L. lactis* ssp. *lactis* (Crow *et al.*, 1993). *Brevibacterium linens* produces acetic acid from glycine, alanine and leucine,

isovaleric acid from leucine, and caproic acid from cystine, alanine and serine (Hosono, 1968a,b). *Propionibacterium freudenreichii* produces isovaleric acid from leucine (Thierry *et al.*, 2002). Isovaleric and 3-methyl butyric acids found in Livarot and Pont l'Évêque cheeses are produced from leucine and isoleucine, respectively (Stark and Adda, 1971). Transamination and Strecker degradation yield branched chain aldehydes (Fox and Wallace, 1997).

Eleven genomes of LAB and related bacteria were recently sequenced by the LABGC and JGI (Walnut Creek, CA). Comparative metabolic analysis of the draft sequenced genomes (http://www.jgi.doe.gov/JGI_microbial/html/index.html) and the publicly available *Lactococcus lactis* subsp. *lactis* IL1403 genome (Bolotin *et al.*, 1999, 2001) revealed the presence of more than 100 genes that are either directly or indirectly involved in production of fatty acids or in production of substrates for catabolism to fatty acids via substrate-level phosphorylation to produce ATP. The presence of these genes indicates the feasibility of BCAA catabolism to fatty acids and survival during substrate exhaustion. Genomic information, along with metabolomics and bioinformatics, was recently used to characterize the catabolic pathways of BCAAs in lactococci (Ganesan *et al.*, 2006).

Lactococci produce 2-methyl butyric, isobutyric and isovaleric acids via a 12-step mechanism (Fig. 4.8) (Ganesan *et al.*, 2006). ATases are the enzymes involved in the first step of the conversion of amino acids to flavor compounds (Ganesan *et al.*, 2004a,b; Ganesan and Weimer, 2004; Yvon *et al.*, 1997). The pathway further uses general classes of enzymes such as dehydrogenases, acyl transferases, carboxylases, dehydratases, and acyl kinases to generate BCFAs from leucine, isoleucine and valine. Lactococci possess more than one branched chain ATase (BAT) or other ATases (Chambellon and Yvon, 2003) with overlapping substrate specificities. Mutants of BAT are capable of growth on α -ketoisocaproate (Atiles *et al.*, 2000). BAT catabolizes the BCAAs leucine, isoleucine and valine to yield their corresponding α -keto acids (Yvon *et al.*, 2000). Since the genes related to these pathways are present in LAB (Bolotin *et al.*, 1999, 2001) (http://www.jgi.doe.gov/JGI_microbial/html/index.html), they may be expressed during cheese ripening and allow cell survival. In cheese, BCAAs may be transported faster at a low pH of 5.2 than at pH 7.2 (Konings *et al.*, 1989). BCAAs are then utilized by the cell to produce ATP for ATP-driven transport systems. This is one of the roles of BCAA catabolism by the starter culture in cheese. Amino acid catabolism to fatty acids, especially BCAA catabolism, provides energy as ATP and other molecules essential for cellular survival. Notably the BCAA catabolic pathway of lactococci shares motifs and purposes similar to glycolysis (Ganesan *et al.*, 2006).

Amino acid to fatty acid catabolic pathways are identified in both lactic and non-lactic genera (Bolotin *et al.*, 1999, 2001; Fraley *et al.*, 1998) (http://www.jgi.doe.gov/JGI_microbial/html/index.html), and are characterized in some genera both enzymatically and genetically. Though the purposes are different, the series of reactions in both cases above are initiated by an ATase (Choi *et al.*, 2000; Heath and Rock, 1996; Ward *et al.*, 1999, 2000). A global

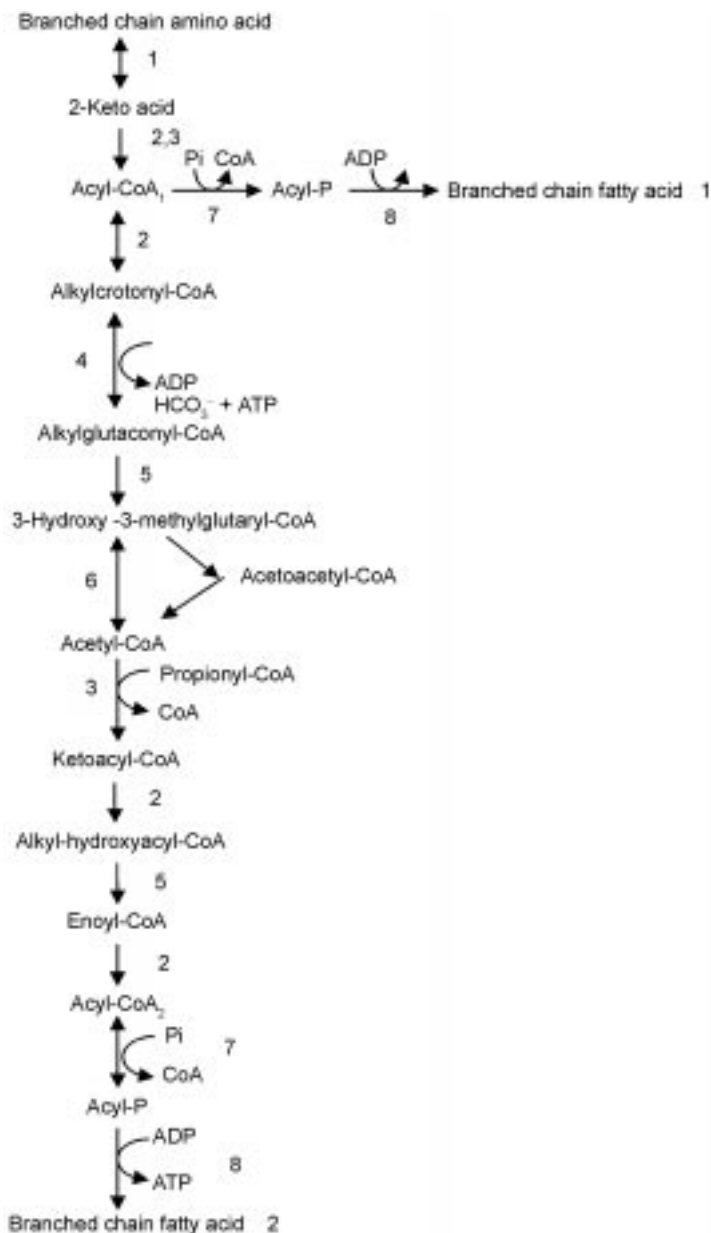


Fig. 4.8 Branched chain amino acid catabolism pathways of *Brevibacterium linens* (producing branched chain fatty acid 1) and *Lactococcus lactis* (producing branched chain fatty acid 2), reproduced with modifications and combined from Ganesan *et al.* (2004a) and Ganesan *et al.* (2006), respectively. Enzymes involved in the various steps are: (1) amino transferase, (2) dehydrogenase, (3) acyl transferase, (4) carboxylase, (5) dehydratase, (6) 3-hydroxy-3-methylglutaryl-CoA synthase, (7) phosphotransacylase/phosphatase, and (8) acyl kinase.

Table 4.3 Bioenergetics of amino acid catabolism to fatty acids

Amino acid	Pathway	Fatty acid product	$\Delta G^{0'}$ (kJ/mol)
Glycine		Acetic acid	−98.60
Isoleucine		Isovaleric acid	−56.29
Leucine		2-Methyl-valeric acid	−46.38
Threonine		Propionic + formic acid	+53.69
Valine		Isobutyric acid	−52.74
Glutamic acid	Hydroxypropionate	Butyric + acetic acid	−376.60
Glutamic acid	Methylaspartate	Butyric + acetic acid	−234.63

transcriptional regulator, *codY*, senses intracellular levels of BCAAs and aids in catabolism of amino acids via AAT and BAT (Chambellon and Yvon, 2003; Guedon *et al.*, 2001; Petranovic *et al.*, 2004). The physiological rationale for BCAA catabolism to BCFAs seems to be sugar starvation (Ganesan *et al.*, 2004a, b, 2006).

Fatty acid production pathways are active in conditions analogous to cheese ripening (Heath and Rock, 1996; Ward *et al.*, 2000). LAB generate fatty acids, whose purpose is yet unknown, except for cell wall fatty acid biosynthesis, but a common theme in all known amino acid to fatty acid pathways is coupling reactions with ATP generation by substrate level phosphorylation and/or regeneration of redox compounds, like NADH, under anaerobic conditions. These pathways need to be active in the absence of fermentable carbohydrates for the bacteria to survive.

Table 4.3 shows Gibbs free energy change values ($\Delta G^{0'}$) at pH 7 for the catabolism of amino acids to fatty acids. A negative $\Delta G^{0'}$ value means that the pathway produces energy (i.e. it is exergonic) and is energetically favorable. From the values of $\Delta G^{0'}$ in the table, the catabolic mechanisms of most amino acids to fatty acids, except threonine, are favorable. Especially, the catabolic mechanisms of BCAAs to BCFAs are favorable. The energy needed to generate one ATP is ~ -30.5 kJ/mol. Hence, from the $\Delta G^{0'}$ values, we see that multiple ATP molecules can be generated from these pathways. One such physiological condition in which these pathways will be relevant is the onset of carbohydrate exhaustion during cheese ripening and growth in culture, commonly referred as carbohydrate starvation.

4.5 Carbohydrate starvation in LAB

The catabolism of amino acids to fatty acids takes place only in the absence of carbohydrates (Nakae and Elliott, 1965a, b). Carbohydrates are the primary energy and carbon source for LAB that grow in laboratory media and natural products such as milk. During fermentation processes LAB are subject to vagaries of stress like water activity, pH, redox potential and substrate availability (Kim *et al.*, 1999; O'Sullivan and Condon, 1997; Rallu *et al.*, 1996;

Sanders *et al.*, 1999). Lactococci survive stress conditions and remain metabolically active (Kunji *et al.*, 1993; Stuart *et al.*, 1999; Thomas and Batt, 1968). They survive with intracellular glycolytic reserves for moderate periods of starvation (Thompson and Thomas, 1977). During this period, available energy within the cells is utilized towards protein and nucleic acid synthesis. Available proteins can then be degraded by lactococci over time to generate peptides and amino acids that aid survival (Thomas and Batt, 1968, 1969a).

Starvation and the stress response in LAB lead towards cheese flavor generation mechanisms. During the course of ripening, lactose is depleted to near zero levels by the first week in hard cheeses (Crow *et al.*, 1993). The pH of cheese drops to ~5.0. Salt in moisture of cheese is around 4% (Crow *et al.*, 1993). Due to lack of other energy-yielding substrates, lactococci revert to nitrogen compound metabolism and utilize amino acids from protein breakdown. The metabolic activities may cause the redox potential within the cheese matrix to fall due to low oxygen levels. These conditions cause stimulation of stress responses, activating or inactivating enzymes towards metabolism for bacterial survival (Rallu *et al.*, 1996).

One such specific physiological condition is the nonculturable physiological state in LAB that is induced by carbohydrate starvation in culture (Stuart *et al.*, 1999). This condition may be induced in cheese also due to depletion of available carbohydrates in the cheese matrix. The induction of VBNC and carbohydrate starvation may be linked with catabolism of amino acids in the later stages of cheese ripening.

4.6 The nonculturable state

The lactococcal population, when grown on selective media during ripening, appears to decrease, while lactobacilli populations increase concurrently. The total plate count assay measures cells capable of replication. However, this is not the only population of cells in cheese. While controversial, the nonculturable (NC) state in many organisms is a real phase, especially in micrococci where soluble peptides can induce regrowth (REF). Lactococci are capable of entering the nonculturable state, wherein they do not replicate and hence do not grow on plates – but they do remain intact and metabolically active. The cells are capable of survival in even minimal media for at least two weeks, without the external addition of an energy source (Stuart *et al.*, 1999), and are also capable of extended ‘survival’ for as long as four years (Ganesan, 2005). The ability of lactococci to survive under stress conditions (Rallu *et al.*, 1996) and continue protein turnover, RNA synthesis (Thomas and Batt, 1969a) and degradation also indicates their ability to actively metabolize proteins and amino acids. Hence, they shift towards a non-lactic, nitrogenous metabolism in their NC state due to lack of a sugar source and the presence of amino acids.

Previous studies focused on the growth and proliferation of starter bacteria. Overnight fermented products result from glycolysis and subsequent metabolism

of pyruvate. This led to extensive characterization of the regulation of glycolysis and pyruvate catabolism (Broome *et al.*, 1980; Cancilla *et al.*, 1995; Cocaign-Bousquet *et al.*, 1996; Neves *et al.*, 1999; Wouters *et al.*, 2000), and acid stress conditions and related genes in LAB (Huisman and Kolter, 1994; Kim *et al.*, 1999; Kunji *et al.*, 1993; O'Sullivan and Condon, 1997; Rallu *et al.*, 1996; Sanders *et al.*, 1999; Stuart *et al.*, 1999). Due to their nutritional fastidiousness (Ayad *et al.*, 1999; Jensen and Hammer, 1993; van Niel and Hahn-Hagerdal, 1999), genes related to amino acid synthesis are also well characterized (Delorme *et al.*, 1999; Godon *et al.*, 1992; Lapujade *et al.*, 1998; Nomura *et al.*, 1999). Amino acid catabolism of LAB is of considerable interest now from the perspectives of Cheddar cheese flavor (Puchades *et al.*, 1989; Yvon *et al.*, 1997, 1999) and bacterial survival (Stuart *et al.*, 1999).

During Cheddar cheese ripening, the lactococcal starter population decreases over time whereas non-starter bacterial population, primarily that of lactobacilli, increases. Lactococci are added at 10^5 cfu ml⁻¹ of milk to initiate cheese manufacture and increase to 10^8 to 10^9 cfu g⁻¹ of cheese curd at salting. Depending on the strain, lactococci decline by ~99.9% at the end of four weeks to $< 10^5$ to 10^6 cfu g⁻¹ cheese (Desmazeaud and Cogan, 1996). The lactobacilli in cheese begin at cell densities of 10 to 10^4 cfu g⁻¹ of cheese during initial storage, rise to around 10^8 cfu g⁻¹, and plateau at 10^6 to 10^8 cfu g⁻¹ (Desmazeaud and Cogan, 1996). The reduction in lactococcal population and the subsequent increase in non-starter population lead to contradictory views of possible theories of cheese ripening and the associated mechanisms to produce flavors during ripening.

One hypothesis attributes the reduction in lactococcal numbers in cheese during enumeration by cultivation on non-selective media to cell lysis and death (Crow *et al.*, 1995; Ostlie *et al.*, 1995; Wilkinson *et al.*, 1994). Autolysis releases intracellular enzymes of lactococci that aid non-starter growth and cheese ripening (Crow *et al.*, 1993). But this theory neglects other possible physiological states of bacteria in cheese and ignores the problem of enzyme diffusion in a semi-solid matrix, the need for co-factors with most enzymes involved in flavor generation, the salt concentration in the moisture phase, and the matrix pH on the enzyme activity. It is likely that a mixed population of actively growing, lysed, and NC cells is responsible for cheese flavor development.

An alternate hypothesis attributes starter bacterial metabolic activity in the state of nonculturability, also described as the viable-but-nonculturable state of bacteria, to cheese flavor generation. Lactococci become NC on laboratory media and remain metabolically active (Stuart *et al.*, 1999). At the onset of lactose starvation, the cells have a sufficient ATP pool (Thomas and Batt, 1969a), and sufficient reserves of glycolytic intermediates for active intake of substrates (Thompson and Thomas, 1977). Since the starter bacteria may also exist in the NC state in cheese, they are still metabolically capable of generating ATP (Stuart *et al.*, 1999) and other substrates essential for viability (Thomas and Batt, 1968, 1969a).

Based on plate count data available in the literature (Desmazeaud and Cogan, 1996; Lynch *et al.*, 1999), culturable lactococci are present in Cheddar cheese at

higher numbers than lactobacilli initially, yet appear to reduce (i.e. die and lyse) to a constant level of $\sim 10^4$ cfu g⁻¹ during cheese ripening, while lactobacilli begin at non-detectable amounts and grow to $\sim 10^9$ cfu g⁻¹ during the same time. Overlaying the likelihood that lactococci remain NC during cheese ripening, rather than lyse, as demonstrated previously (Ganesan, 2005), it is possible to make a theoretical estimate to determine the relative proportion of the possible metabolic states – actively growing, NC, and lysed. This analysis found that over 10^9 cfu ml⁻¹ cells remained metabolically active as NC after reaching a plateau of cell numbers during growth. In other words, lysis was observed during active growth, while after sugar exhaustion no further lysis occurred. If this is true, the proportion of the population that provides intracellular enzymes is static after ~ 30 days of ripening. Extrapolating this calculation to cheese, where lactococcal counts decrease from 10^9 cfu g⁻¹ to 10^4 cfu g⁻¹, there is still a population of $\sim 10^9$ cells g⁻¹ cheese ($10^9 - 10^4$) that are NC, meaning that only 0.001% of the total lactococcal population is estimated to be culturable. Therefore, almost the entire population of lactococci remains NC in cheese, despite the appearance that the cells die and lyse. The limited amount of lysis that occurs during cell growth in cheese would explain the limited amount of intracellular components that are found in cheese during ripening (Weimer *et al.*, 1997). It may also be due to the growth of lactobacilli (Peterson and Marshall, 1990).

Based on fatty acid production data by log phase cells under identical assay conditions and equal cell numbers (Ganesan *et al.*, 2004a) lactococci produce fatty acids in quantities ranging from equal amounts to five times higher than lactobacilli. While even the same number of log phase cells ensures that lactococci produce more fatty acids, there is also the possible presence of 10^9 cfu g⁻¹ lactococcal cells that are NC and culturable, compared to $\sim 10^7$ cfu g⁻¹ lactobacilli. The cumulative effect of NC and log phase lactococci in cheese would lead to fatty acid production that is around 100–500 times higher than lactobacilli. Considering the higher population of lactococci and of the increased production of fatty acids compared to lactobacilli, it is likely that lactococci are the primary source of flavor compounds found in cheese that are only produced during the NC phase.

The pathways active under such stressful conditions could be multipurpose pathways that help the starter cells survive. Bacteria may synthesize flavor precursors by these pathways and hence contribute to cheese flavors. In the presence of metabolically active starter cells, the contribution of non-starter bacteria is of questionable importance with respect to ideal flavor production, and hence, ultimately, the quality of cheese (Laleye *et al.*, 1990; Lee *et al.*, 1990).

4.7 Future trends

In past decades of flavor research, extensive efforts were made using multi-step purification and analytical tools. Genetic characterization and development of gene control systems were directed towards understanding basic metabolic

mechanisms and their control. The advent of high-throughput technologies such as genomics and metabolomics provides capabilities of studying multiple compounds and genes simultaneously in one experiment. As demonstrated by Ganesan *et al.* (2006), the use of bioinformatics with genomics and metabolomics is the forerunner for novel pathway characterization and extension of gene characterization for incomplete pathways. Such efforts will eventually lead to better control of off-flavors and production of compounds with preferable flavor attributes in a given cheese type.

4.8 Sources of further information and advice

A definitive body of work on defining entire pathways for beneficial flavor production from amino acid metabolism is limited. Studies by Steele, Yvon, and Broadbent for selective flavor compounds are recommended as starting points. A review by Weimer *et al.* (1999) is highly recommended for a better in-depth understanding of sulfur metabolism and flavor production. Public resources for metabolic reconstruction from genomic sequence (<http://biocyc.org/server.html>; <http://www.genome.jp/kegg/pathway.html>) are recommended as a useful tool to gain novel perspectives on flavor compound production mechanisms and alternate metabolic pathways for different substrates.

4.9 References

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5

Lipolysis and cheese flavour development

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5.1 Introduction

Cheese ripening is a complex set of biochemical events that involve at least three flavour generating pathways – glycolysis, lipolysis and proteolysis. Lipolysis refers to the breakdown of the milk fat content of cheese during ripening and occurs in most natural cheese varieties to varying extents. Generally, Italian, Blue and certain mould- and smear-ripened types undergo extensive lipolysis, Swiss-type cheeses undergo intermediate levels of lipolysis, while Cheddar and Dutch-types undergo relatively low levels of lipolysis. Despite the occurrence of lipolysis in many cheese varieties, it is only in recent years that the importance of lipolysis to cheese flavours is well recognised. One of the main reasons for this is that the study of lipolysis in cheese has been hampered by a lack of clear analytical methodology with which to identify lipolytic end products and elucidate their flavouring properties. However, this obstacle appears to have been overcome with the development of reliable methods principally based on gas chromatography and mass spectroscopy.

Typically, bovine milk has a fat content ranging from 3.5% to 5% depending on stage of lactation, breed and diet of the cow. The fat content in milk is present as emulsified globules surrounded by a thin membrane layer called the milk fat globule membrane (MFGM). The major free fatty acids (FFA) present in milk are butanoic (C_{4:0}), hexanoic (C_{6:0}), octanoic (C_{8:0}), decanoic (C_{10:0}), dodecanoic (C_{12:0}), tetradecanoic (C_{14:0}), hexadecanoic (C_{16:0}), octadecanoic (C_{18:0}), *cis*-9-octadecenoic (C_{18:1}), *cis,cis*-9,12-octadecadienoic (C_{18:2}), and 9,12,15-octadecatrienoic acids (C_{18:3}) (Banks, 1991a; Jensen *et al.*, 1962, 1991). Hexadecanoic and octadecanoic are the most abundant FFA (Banks, 1991b; Gunstone *et al.*, 1994), comprising ~25% and ~27% of total lipids, respectively

(Jensen *et al.*, 1962). Some notable features of the fatty acid profiles of bovine milk lipids include the high level of butanoic acid and other short-chain fatty acids, the low levels of polyunsaturated fatty acids and the fact that these lipids are rich in medium-chain fatty acids (Oba and Wiltholt, 1994).

The principal lipids in milk are triacylglycerides, which consist of a glycerol backbone with three fatty acids esterified at particular *sn*-positions on the triacylglyceride molecule, i.e. position 1, 2 or 3. Triacylglycerides comprise up to 98% of the total lipids. C_{4:0} and C_{6:0} are predominately located at the *sn*-3 position and the *sn*-1 and *sn*-3 positions, respectively. As chain length increases up to C_{16:0} an increasing proportion is esterified at the *sn*-2 position. C_{18:0} is generally located at the *sn*-1 position, while unsaturated fatty acids are esterified mainly at the *sn*-1 and *sn*-3 positions (Balcao and Malcata, 1998).

Phospholipids represent less than 1% of total lipids but play an important role in the MFGM. Phospholipids are amphipolar in nature and are strongly surface-active, enabling them to stabilise both oil-in-water and water-in-oil emulsions (Banks, 1991a). On average, phospholipids contain longer and more unsaturated fatty acids than triacylglycerides (Banks, 1991a; Jensen *et al.*, 1991). The principal phospholipids found in milk fat are phosphatidyl choline, phosphatidyl ethanolamine and sphingomyelin (Christie, 1983; Grummer, 1991; Gunstone *et al.*, 1994). Trace amounts of other polar lipids have also been reported in milk fat, including ceramides, cerobrosides and gangliosides. Cholesterol is the dominant sterol of milk (>95% of total sterols) (Anderson and Cheesman, 1971; Christie, 1983; Jensen *et al.*, 1991) and accounts for ~0.3% of the total lipids. MFGM consists of a complex mixture of proteins, phospholipids, glycoproteins, triacylglycerides, cholesterol, enzymes, and other minor components, and acts as a natural emulsifying agent enabling the fat to remain dispersed in the aqueous phase of milk (Anderson *et al.*, 1972; Kanno, 1980; Keenan *et al.*, 1983; Kinsella, 1970; Mather, 1978; Mather and Keenan, 1975; McPherson and Kitchen, 1983).

5.2 Lipolysis and cheese flavour

The necessity of the milkfat content of natural cheese for development of typical flavour and texture is well known (Foda *et al.*, 1974; Wijesundera *et al.*, 1998). However, the biochemical event of lipolysis, involving enzymatic hydrolysis of triacylglycerides to FFA, is now seen as contributing significantly to the flavour profile in many cheese varieties (McSweeney and Sousa, 2000).

Lipolysis in cheese is due to the action of two classes of enzymes, esterases and lipases. Esterases hydrolyse acyl ester chains from two to eight carbon atoms in length, while lipases hydrolyse acyl ester chains from 10 or more carbon atoms. Esterases act on soluble substrates in aqueous solution, while lipases act on emulsified milkfat substrates. In cheese, lipolytic enzymes originate from the following agents: milk, rennet preparation (rennet paste), the starter culture, adjunct cultures, non-starter bacteria (NSLAB), and exogenous

enzyme preparations (Deeth and Fitz-Gerald, 1995; Fox and Wallace, 1997; McSweeney and Sousa, 2000). Short- and intermediate-chain FFA released during lipolysis contribute directly to the flavour profile of cheese (Bills and Day, 1964; Woo and Lindsay, 1984; Woo *et al.*, 1984).

Lipolysis in Cheddar cheese generates free C_{6:0} to C_{18:3} in proportions similar to those in milk fat, while free butanoic acid (C_{4:0}) occurs at a greater relative concentration in cheese than in milk fat through either lipase selectivity or its synthesis by the microflora in cheese (Bills and Day, 1964).

The indigenous milk lipase, lipoprotein lipase (LPL), is quite active in milk, but ~97% of the activity is inactivated by HTST pasteurisation; consequently, this enzyme never reaches full activity during cheese ripening (Fox and Stepaniak, 1993; Fox *et al.*, 1993). LPL exhibits a preference for hydrolysis of medium-chain triacylglycerides (MCT) including C_{6:0}, C_{8:0}, C_{10:0} or C_{12:0}, and preferentially releases short- and medium-chain fatty acids. Most of the LPL activity (~90%) in milk is enclosed by MFGM or associated with the casein micelle. However, damage to the MFGM may allow excessive lipolysis by LPL, resulting in off-flavours in cheese and other dairy products (Darling and Butcher, 1978; Deeth and Fitz-Gerald, 1978; Fox *et al.*, 2000). O'Mahony *et al.* (2005) manufactured Cheddar cheese with milk containing normal (3.58 µm), small (3.45 µm) or large (4.68 µm) milkfat globules and noted that FFA release during ripening increased with globule size. This work suggests that decreased stability/integrity in MFGM of the larger milkfat globules may alter the compartmentalisation of lipolytic enzymes and their triacylglyceride substrates and result in increased lipolysis. The lipolytic action of LPL plays a significant role in flavour development in cheeses made using raw milk, while in cheeses made from pasteurised milk residual LPL activity has a minor role in the lipolytic activity and cheese flavour.

5.2.1 Enzyme addition

Rennet pastes, containing the lipolytic enzyme pregastric esterase (PGE), are used in the manufacture of some hard Italian varieties (e.g., Provolone, Romano) (Nelson *et al.*, 1977). PGE is highly specific for short-chain fatty acids esterified at the *sn*-3 position (Fox and Stepaniak, 1993; Nelson *et al.*, 1977). However, differences in the specificity of calf, kid and lamb PGEs appear responsible for differing flavour characteristics in some cheese varieties. Calvo and Fontecha (2004) compared substrate specificities, pH and temperature optima of hygienised rennet paste (HRP) and purified kid pregastric esterase (KPGE). The pH optimum of both preparations were similar, ~7.0–7.5. Interestingly, HRP was stable over the temperature range 40–45°C with 56% residual activity at 50°C, while KPGE had an optimum of 37°C with 90% loss in activity at 50°C. Marked differences in substrate specificity were also noted for both enzymes: KPGE had maximum activity towards short-chain fatty acid substrates, especially butanoic acid, when present as an ester in solution, with a major reduction in activity when present as a tributyrin emulsion, thus exhibiting the

characteristics of an esterase; while HRP had a low activity towards butanoic acid substrates but a higher activity towards longer-chain fatty acid substrates.

Recent work on four rennet pastes prepared from lambs fed different diets before slaughter found that diet significantly affected both the level and specificities of lipolytic activity in the resulting enzyme preparations. Addition of these enzyme preparations to Fiore Sardo, an uncooked Italian sheep milk cheese, resulted in differences in the levels of lipolysis and the FFA profiles developed in cheeses during ripening (Addis *et al.*, 2005).

5.2.2 Microbial enzymes

LAB possess lipolytic enzymes that hydrolyse FFA esters, tri-, di- and mono-acylglycerides. In general, LAB lipolytic activities appear to be quite weak in comparison to other bacteria and moulds. In many LAB strains esterase, rather than lipase activity, appears dominant (Piatkiewicz, 1987). However, as LAB persist in natural cheese their contribution to lipolysis over an extended ripening period may be substantial.

Based on genetic evidence, the esterase gene in LAB does not code for an *N*-terminal signal sequence, which is required for extracellular secretion, indicating that lipases and esterases of LAB are located intracellularly (Fernandez *et al.*, 2000). Generally, lipolytic activities in LAB appear to have a pH optimum of 7.0–8.5 and a temperature optimum of 37°C (Khalid and Marth, 1990). Because of their intracellular location, LAB esterases and lipases appear to require cell autolysis or permeabilisation that allows access to the substrate in cheese. However, surprisingly few studies have reported on this particular relationship. Meyers *et al.* (1996) compared the effect of incubation of whole, and presumably intact, LAB strains with intracellular extracts prepared by sonication of the cells on the release of FFA from triacylglyceride substrates. These studies found that the highest FFA levels were released on incubation with whole cells of various LAB strains, suggesting that the microenvironment within the whole cells may be more conducive to lipase activity. This observation may reflect amino acid metabolism, rather than lipolysis (Ganesan *et al.*, 2004a,b). Conversely, Collins *et al.* (2003a) concluded that some evidence existed for a relationship between the extent of autolysis of starter culture and the resulting lipolysis in Cheddar cheese. In this study, cheese made using the highly autolytic starter culture *Lactococcus lactis* subsp. *cremoris* AM2 developed significantly higher levels of a number of FFAs including octanoic acid (C_{8:0}), tetradecanoic acid (C_{14:0}), hexadecanoic acid (C_{16:0}) and octadecanoic acid (C_{18:0}) during ripening compared with cheese made with the poorly autolytic strain *L. lactis* subsp. *cremoris* HP.

Holland *et al.* (2005) studied the characteristics of LAB esterases and noted that these enzymes mainly displayed activity towards monoacylglycerides up to C₁₈, with C₈ being the preferred substrate, while little activity was found towards diacylglycerides of FA > C₆. LAB esterases did not hydrolyse intact milk fat but could act on hydrolysed milk fat containing mono-, di- and

triacylglycerides, with monoacylglycerides being the preferred substrate. In Cheddar cheese made using various ratios of milk treated with or without PGE, lipolysis was higher in cheeses made from lipolysed milks over ripening, providing further evidence for the esterase nature of LAB activity during cheese ripening (Holland *et al.*, 2005). Another aspect to LAB esterase activity in cheese concerns the synthetic reaction performed by these enzymes whereby esters are synthesised via alcoholysis from alcohols and glycerides other than ethanol and tributyrin. Liu *et al.* (2003) first demonstrated the formation of ethyl butanoate by whole LAB cells in a transferase (alcoholysis) reaction with the transfer of butyryl groups from tributyrin to ethanol.

Propionic acid bacteria (PAB) deserve particular mention for their lipolytic activity, which has been estimated as being 10 to 100 times more active than LAB, and most reports suggest a predominantly intracellular location for these activities (Kakariari *et al.*, 2000). PAB play a significant role in the development of characteristic flavour in Swiss-type cheese varieties through conversion of lactate to acetate, propionate and CO₂. However, FFAs generated during ripening are key components of the flavour profile in these varieties. The effects of variation in the ratio of the LAB strains *Streptococcus thermophilus* and *Lactobacillus helveticus* to PAB, and of the duration of warm room treatment on the formation of water-soluble FFAs (C_{2:0} to C_{12:0}) during Swiss-type cheese ripening were examined by Ji *et al.* (2004). Increasing the ratio of LAB to PAB (0.33:1, 0.66:1 or 1:1) affected the formation of C_{2:0}, C_{3:0} and C_{10:0}. Increasing the duration of warm room treatment at 25°C from two to three weeks resulted in higher concentrations of C_{2:0} and C_{3:0} with little difference for the formation of C_{4:0} or C_{6:0}. Similar concentrations of 3-methylbutanoic acid *i*-C_{5:0} developed in the two-week warm room treatments irrespective of the ratio of LAB to PAB used in cheese manufacture.

Lipolytic enzymes from bacteria, yeasts and moulds used in the manufacture of varieties such as Limburger, Brie, Roquefort and Blue make an important contribution to the flavour profile of these cheeses (Collins *et al.*, 2003b; McSweeney and Sousa, 2000). In surface smear-ripened cheese varieties the lipolytic activity of *B. linens* makes an important contribution to lipolysis during ripening. Rattray and Fox (1997) purified an intracellular esterase from *Brevibacterium linens* with pH and temperature optima of 7.5 and 35°C, respectively. This enzyme showed a preference for the hydrolysis of short-chain fatty acid ester substrates and was capable of releasing ethanoic, butanoic, hexanoic, octanoic and decanoic acids from β -naphthyl substrates. Most reports on the lipolytic activity of *Brevibacterium* spp. assign an intracellular location for these enzymes which appear to be predominantly esterases. *Penicillium* spp. used in the ripening of mould-ripened cheeses such as Brie, Camembert Roquefort, Danablu and Gorgonzola have been shown to possess a number of lipases (Gripon, 1993; McSweeney and Sousa, 2000). *Penicillium roqueforti* has two lipases, one with a pH optimum from 7.5 to 8.0, the other with a more alkaline pH optimum (9.0 to 9.5) (Niki *et al.*, 1966); while *P. camemberti* produces an extracellular lipase optimally active at pH 9.0 and 35°C (Lamberet and Lenoir,

1976; Menassa and Lamberet, 1982). *Geotrichum candidum*, a component of the surface flora of cheeses such as Camembert, possesses a lipase highly specific for unsaturated fatty acids with a double bond at position *cis*-9 or *cis*, *cis*-9,12 with a preference for *cis*-9-octadecanoic acid, C_{18:1}. The lipase from *G. candidum* is optimally active over the pH range 5.5 to 7.5 and hence may play an important role in the ripening of certain surface-ripened varieties (Boutrou and Gueguen, 2005).

Yeast species such as *Debaryomyces hansenii*, *Yarrowia lipolytica* and *Cryptococcus laurentii* play an important role in the lipolysis during ripening of cheeses such as Picante, a traditional Portuguese variety manufactured using mixtures of ovine and caprine milk (Freitas *et al.*, 1999). Of the above strains examined by Freitas *et al.* (1999) only *Yarrowia lipolytica* and *Cryptococcus laurentii* released butanoic acid from tributyrin. The importance of yeast lipolytic activity in the ripening of Raclette cheese was demonstrated by Wyder *et al.* (1999). Cheeses were ripened with or without foil wrapping to exclude the surface flora, which included the yeast species *Galactomyces geotrichum*, *Debaryomyces hansenii*, *Pichia jadinii* and *Yarrowia lipolytica*. Examination of the lipolytic patterns of cheeses inoculated by yeast strains individually, or in combination, indicated an interactive effect between *Pichia jadinii* and *Yarrowia lipolytica* as these strains released highest levels of short-chain FFA when used in combination for cheese manufacture.

5.3 Identification of fat-related aroma compounds important for cheese flavour

Levels of lipolysis vary considerably between cheese varieties. Highest levels of lipolysis are found in traditional mould-ripened cheese types with 5–10% of total triacylglycerides hydrolysed in Camembert and up to 25% hydrolysed in blue-vein cheeses (Gripon *et al.*, 1991; Gripon, 1993). In the case of Danish Blue cheese, 18–25% of total fatty acids may be released as FFA (Anderson and Day, 1966). Indeed, conditions conducive to extensive lipolysis in Danish Blue cheese are deliberately created by homogenisation, which damages the MFGM, reduces fat globule size and increases the total fat globule surface area, thereby providing a greater lipid–serum interface for lipase activity to occur (Nielsen, 1993).

Extensive lipolysis also occurs in Italian varieties such as Grana Padano, Parmigiano-Reggiano, Romano and Provolone where the use of raw milk allows the lipolytic action of LPL to occur to a significant extent (Bosset and Gauch, 1993; Contarini and Toppio, 1995; Woo and Lindsay, 1984). However, in the manufacture of Parmigiano-Reggiano and Grana Padano, high curd cooking temperatures reduce the contribution by LPL activity during ripening. The manufacture of Provolone and Romano cheeses may involve the addition of PGE originating from kid or lamb rennet pastes, resulting in extensive lipolysis, release of short-chain FFAs, C_{4:0} to C_{10:0}, and the generation of the ‘piccante’

flavour characteristic of these varieties (Battistotti and Corradini, 1993; Fox *et al.*, 2000; McSweeney and Sousa, 2000). In Romano cheese, a direct relationship between flavour intensity and butanoic acid content exists (Arnold *et al.*, 1975). Levels of lipolysis in Ragusano, a brine-salted *pasta-filata* cheese in which rennet paste is used as coagulant, reach $\sim 6400 \text{ mg FFA kg}^{-1}$ cheese. FFA release in this variety also appears to be positively related to salt-in-moisture levels in the cheese and brine temperature. Increasing proportions of short-chain FFA were released in cheese with increasing brine temperature, especially at higher salt-in-moisture contents, 6–8% (Melilli *et al.*, 2004).

According to Gripon (1993), levels of lipolysis in Gouda, Gruyère and Cheddar cheeses should not exceed 2% of the triacylglycerides. In Emmental cheese, ~ 2 to 7 g FFA kg^{-1} , are released during ripening, in cheese made from raw or pasteurised milk (Chamba and Perreard, 2002; Steffen *et al.*, 1993). Significant lipolysis occurs in traditional Feta cheese made using thermised milk, starter cultures and either artisanal rennets, derived from lamb or kid abomasa, or a blend of artisanal and commercial rennets. At the end of a 120-day ripening period, Feta made using artisanal rennet or a commercial and artisanal enzyme blend released $\sim 12,000$ or $\sim 7000 \text{ mg FFA kg}^{-1}$, respectively. Short-chain FFAs ($\text{C}_{2:0}$ to $\text{C}_{8:0}$) comprised from 33% or 44% respectively of the total FFA content in these cheeses. Artisanal rennet increased the butanoic acid ($\text{C}_{4:0}$) content significantly compared to the rennet blend, and after 120 days of ripening this FFA comprised $\sim 20\%$ of the total FFA released in Feta cheese (Georgala *et al.*, 2005).

In Manchego-type cheese, after a 70-day ripening period, total FFA values reached 7 mg kg^{-1} milk fat comprising $\sim 10\%$, 20% or 70% short-, medium- or long-chain FFAs, respectively, as a result of residual LPL activity after pasteurisation and microbial lipases/esterases. Short-chain FFAs, including butanoic acid, make an important contribution to the final cheese flavour profile of this variety (Pavia *et al.*, 2000).

Lipolysis in Cheddar and Gouda cheeses is quite moderate, and is increasingly recognised as being important to their overall flavour profiles. Lipolysis in Cheddar cheese manufactured over a four-month period was studied by Collins *et al.* (2003a) to find the total FFA levels in cheeses ripened for 238 days at 8°C ; it ranged from ~ 1200 to 3200 mg kg^{-1} cheese. Hexadecanoic acid and *cis*-9-octadecanoic acid doubled in concentration over ripening with an overall steady increase in all the FFAs ($\text{C}_{4:0}$ to $\text{C}_{18:2}$).

The impact of a seasonal milk supply on lipolysis and flavour in Cheddar cheese was examined by Hickey *et al.* (2006) who manufactured Cheddar cheese over nine months on three separate occasions corresponding to three stages of lactation: ~ 90 days (early), ~ 180 days (mid) and ~ 250 days (late). These authors found that lipolysis in all cheeses was primarily influenced by initial levels of free fatty acids in the milk, which were highest in late lactation and lowest in early lactation. Lipolysis increased in cheeses over ripening but was not influenced by stage of lactation (SOL). In all cheeses, volatile short-chain FFAs were preferentially hydrolysed from the triacylglycerides. SOL appeared to

significantly ($p < 0.05$) influence mean levels of $C_{12:0}$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$, but had no significant effect on mean levels of $C_{4:0}$, $C_{6:0}$, $C_{8:0}$ and $C_{10:0}$. The most abundant FFAs in all cheeses in decreasing order were $C_{18:1}$, $C_{16:0}$, $C_{18:0}$, $C_{14:0}$ and $C_{4:0}$. Except for $C_{18:1}$ and $C_{16:0}$, the individual FFA composition of each cheese over ripening did not appear to be affected by SOL (Hickey *et al.*, 2006).

Alewijn *et al.* (2005) monitored lipolysis in Gouda cheeses made from raw or pasteurised milk over a 96-week ripening period and found that FFA levels reached 700–1200 mg kg⁻¹ dry matter, corresponding to 0.3% of the total fatty acid glycerides in the fat fraction in the cheese. Short-chain FFA levels released in the cheeses were lower than their mole percentage in milk or in milk triacylglycerides, which the authors suggest may arise from their loss in the whey during manufacture. However, there was a preferential release of short- or medium-chain FFAs in the cheese over ripening. In cheese made from raw milk, FFA release was highest and was attributed to elevated LPL activity producing mono- or diacylglycerides which may have been more suitable substrates for subsequent LAB esterase activity.

Production of enzyme-modified cheese (EMC), concentrated cheese flavours produced enzymatically from immature cheese or other dairy substrates, typically involves the generation of a high level of lipolysis (Kilcawley *et al.*, 1998). Kilcawley *et al.* (2001) compared the levels of FFAs ($C_{4:0}$ to $C_{18:3}$) in natural Cheddar cheese and in a range of commercial Cheddar-type EMCs. While the overall profiles of Cheddar EMCs were similar to that of natural Cheddar cheese, most EMCs had significantly higher levels of FFA as a result of exogenous lipases added during their production. EMC contained ~24,000–32,000 mg FFA kg⁻¹ with $C_{16:0}$, $C_{18:1}$, $C_{14:0}$ and $C_{18:0}$ representing ~30, ~19, ~13 and ~11% of total FFA, respectively. Based on FFA profiles of Cheddar EMCs, some evidence was provided for the use of short- or medium-chain specific esterases/lipases in their manufacture. In particular, enzymes specific for the release of butanoic acid may have been used in the manufacture of a number of the Cheddar EMC samples analysed.

FFAs released as a result of lipolysis, especially short- and medium-chain fatty acids, contribute directly to cheese flavour. They also act as precursor molecules for a series of catabolic reactions leading to the production of flavour and aroma compounds, such as methyl ketones, lactones, esters, alkanes and secondary alcohols (Curioni and Bosset, 2002; Fox *et al.*, 1993; Gripon *et al.*, 1991; McSweeney and Sousa, 2000).

Methyl ketones, derived from FFA by mould metabolism, are particularly important for the unique flavour of Blue cheese, especially heptan-2-one and nonan-2-one (Arnold *et al.*, 1975; Jolly and Kosikowski, 1975a,b; King and Clegg, 1979; Molimard and Spinnler, 1996). Methyl ketones are present in Camembert cheese at 250–600 $\mu\text{mol kg}^{-1}$ of fat (Molimard and Spinnler, 1996). The two major methyl ketones in Blue and Camembert cheeses are nonan-2-one and heptan-2-one (Anderson and Day, 1966; Gripon, 1993). Odd-chain methyl ketones, from $C_{3:0}$ to $C_{15:0}$, constitute some of the most important components in

the aroma of surface-mould ripened cheese, (e.g., St Paulin, Tilsiter and Limburger) (Dartley and Kinsella, 1971). During a 40-week ripening period for Gouda cheese, Alewijn *et al.* (2005) found that the methyl ketone concentration doubled to reach levels of 2 mg kg^{-1} .

Lactones are formed by the intramolecular esterification of hydroxy fatty acids with the loss of water and formation of a ring structure (Molimard and Spinnler, 1996). Lactones may contribute to overall cheese flavour by giving a buttery-type character to cheese (Dirinck and De Winne, 1999; Fox and Wallace, 1997; Fox *et al.*, 1993, 2000; McSweeney and Sousa, 2000). δ -Lactones have generally higher detection thresholds than those of γ -lactones and give rise to pronounced fruity notes such as 'peach', 'apricot' and 'coconut' (Dufossé *et al.*, 1994; O'Keefe *et al.*, 1969). Dirinck and De Winne (1999) found that δ -dodecalactones were more abundant in Gouda cheeses than in Emmental cheeses and attributed the buttery notes in Gouda cheeses to the higher concentrations of lactones. Alewijn *et al.* (2005) reported that in Gouda cheese the concentrations of δ - and γ -lactones increased during ripening from 3 to 50 mg kg^{-1} and from 0.2 to 5 mg kg^{-1} , respectively. Production of γ -lactones continued for a number of weeks after δ -lactone formation ceased, which the authors suggest may arise from the depletion of a precursor molecule rather than a loss of a particular enzyme activity.

Esters are highly flavoured and are formed when FFAs react with alcohols. Esterification reactions resulting in the production of esters occur between short- to medium-chain fatty acids and the alcohols derived from lactose fermentation or they may arise from amino acid catabolism (Alewijn *et al.*, 2005; Collins *et al.*, 2003b; Molimard and Spinnler, 1996). Engels *et al.* (1997) found high concentrations of the ester ethyl butanoate in cheeses with a 'fruity' note such as Gruyère, Parmesan and Proosdij. However, this fruity flavour is considered to be a defect when present in the flavour profile of Cheddar cheese (McSweeney and Sousa, 2000; Urbach, 1997). Generally, esters contribute positively to the flavour profile of Parmigiano-Reggiano cheese, with ethyl ethanoate, ethyl octanoate, ethyl decanoate and methyl hexanoate the most abundant esters in this variety (Meinhart and Schreier, 1986).

In Gouda cheese, the majority of the esters present are ethyl esters of long-chain fatty acids (Alewijn *et al.*, 2003). Gouda cheese made from raw milk showed a greater and constant increase in ester formation compared to cheese made from pasteurised milk (Alewijn *et al.*, 2005). Thierry *et al.* (2004) found that *Propionibacterium freudenreichii* significantly influenced the formation of a range of esters, alcohols and, to a lesser extent, ketones in cheese juice and in mini-Emmental cheeses. The mechanism for their formation by PAB in Emmental cheese remains to be elucidated.

Ethyl esters are generated from esterification of ethanol with acetyl-coenzyme A (Yoshioka and Hashimoto, 1983). *Geotrichum candidum* and *Pseudomonas fragi* can generate esters, some of which have very pronounced fruity notes (Jollivet *et al.*, 1994; Molimard and Spinnler, 1996). 2-Phenylethyl acetate and 2-phenylethyl propanoate are important flavour compounds in

Camembert cheeses, and ethyl and methyl esters are found in high levels in Blue-type cheese (de Llano *et al.*, 1992; Roger *et al.*, 1988). Thioesters are formed by the reaction of FFA with free sulphhydryl groups and are capable of being formed by a range of cheese microflora including *Lactococcus* spp., *Leuconostoc* spp. and coryneform bacteria (Lamberet *et al.*, 1997; Molimard and Spinnler, 1996). More recently Arfi *et al.* (2005) showed that in a slurried Camembert cheese system, S-methyl thioacetate was synthesised by *Kluyveromyces lactis* and *Debaryomyces hansenii* only when associated with the growth of *Brevibacterium linens*.

Secondary alcohols, arising from lipolysis, may contribute to cheese flavour (Arora *et al.*, 1995). Propan-2-ol, butan-2-ol, octan-2-ol and nonan-2-ol are found in most soft cheese varieties (Engels *et al.*, 1997). Moinas *et al.* (1975) found that heptan-2-ol and nonan-2-ol represented 100–200 and 50–100 g kg⁻¹, respectively, of all aromatic compounds present in Camembert cheese. Oct-1-en-3-ol has a ‘mushroom’ type odour with a perception threshold of 0.01 mg kg⁻¹ and may be a key aromatic compound of Camembert cheese (Molimard and Spinnler, 1996).

Straight-chain aldehydes such as butanal, heptanal and nonanal may be formed through β -oxidation of unsaturated fatty acids and can give rise to ‘green grass-like’ aromas (Collins *et al.*, 2003b; Keeney and Day, 1957; Moio *et al.*, 1993). Qian and Reineccius (2003) calculated, based on their odour activity values, that 3-methylbutanal, 2-methylpropanal, 2-methylbutanal, methional and phenylacetaldehyde were likely to be important contributors to the aroma of Parmigiano-Reggiano cheese; other contributors included esters and short-chain FFAs.

5.4 Improving the flavour of cheese by manipulating lipolysis

Control and/or acceleration of cheese flavour has been a long-standing research objective aimed at shortening ripening times or ensuring consistently high quality flavour profiles (Wilkinson, 1993). Generally, attempts have focused on enhancing proteolysis through LAB and enzyme technology. However, a number of attempts have been made to accelerate cheese ripening through increasing lipolysis by addition of exogenous lipase preparations (other than PGEs), with variable success (Wilkinson, 1993).

Addition of lipases to Blue-type cheeses, Egyptian Ras and Domiati cheeses and Italian Romano and Fontina-types was reported to increase lipolysis along with some enhancement of sensory attributes (El-Neshawy *et al.*, 1982; El Salam *et al.*, 1978; Jolly and Kosikowski, 1975a, b, 1978; Peppler *et al.*, 1976; Rabie, 1989). Barron *et al.* (2004) compared the effect of addition of lamb PGE with that of fungal lipase on the lipolysis and sensory properties of Idiazabal cheese. The concentration of short-chain FFA was higher in cheese with added PGE than in cheese with added fungal lipase and comprised 70% or 30% of total FFA, respectively. Levels of 1,2-, 2,3- and 1,3-diacylglycerides were higher in cheese

made using PGE, reflecting differences in the specificities of the enzymes. After 90 days of ripening, total monoacylglyceride levels were significantly higher for cheese with added fungal lipase; however, by 180 days of ripening, total monoacylglyceride content was similar for both enzyme treatments. In terms of sensory impact, cheeses made with PGE had highest scores for odour and flavour intensity and, despite the clear differences at various stages of ripening in mono- and diacylglyceride profiles, textural attributes were not significantly affected by enzyme treatment. Overall, lamb PGE was considered more appropriate for generation of an authentic Idiazabal cheese flavour.

In the case of Cheddar cheese, addition of lipase preparations derived from animal or microbial sources to accelerate flavour development have produced conflicting results. Sood and Kosikowski (1979) reported acceleration in flavour development on addition of lipases, while Law and Wigmor (1985) found that rancidity had developed after two months of ripening in Cheddar cheese with added animal or microbial lipase preparations. An acceleration of Cheddar cheese flavour through enhanced lipolysis was reported by Kheadr *et al.* (2002) who added two encapsulated fungal lipases, derived from *Mucor miehei* or *Aspergillus niger*, to cheesemilk. Cheeses with an intermediate level of lipase addition were reported to have slightly better flavour intensities than untreated controls. However, at the highest level of lipase addition a soapy off-flavour developed after two and three months of ripening.

Blends of encapsulated enzymes consisting of a fungal lipase derived from *M. miehei* with either bacterial proteinase (BP), fungal proteinase (FP) or fungal proteinase/peptidase (ZP) were evaluated for their effects on Cheddar cheese ripening by Kheadr *et al.* (2003). Increases in volatile and non-volatile FFA were noted for all enzyme treatments. After two months of ripening, cheeses with BP and FP developed a fully mature Cheddar flavour. On ripening to three months, FP cheeses developed bitterness and a soft texture, while BP did not develop any flavour or textural defects.

The use of fungal lipase to accelerate lipolysis and flavour development in Tulum, a Turkish semi-hard variety, was reported by Yilmaz *et al.* (2005). FFA release in cheese increased with the level of lipase addition; however, sensory scores did not follow this trend. Indeed, while sensory scores indicated some degree of flavour improvement at 60 days of ripening with lipase addition, beyond this period a decrease in sensory scores was noted.

The potential for the use of microbial adjunct cultures to accelerate lipolysis and flavour development in cheese has been reported by Ferreira and Viljoen (2003) who added *Debaryomyces hansenii* and *Yarrowia lipolytica* individually, or in combination, as adjuncts to Cheddar cheese made using LAB starter culture. The effects on lipolysis in experimental cheeses was not reported; however, sensory analysis indicated that when both yeast strains were added in combination as adjuncts, improvements in flavour and an acceleration of ripening were noted over control cheeses.

Das *et al.* (2005) report the use of yeast adjunct cultures *Geotrichum candidum* and *Y. lipolytica* and three strains of *Propionibacterium freudenreichii*

ssp. *shermanii* in a washed-curd dry-salted cheese. *Lactobacillus fermentum* was added to generate ethanol from lactose, which subsequently acts as a substrate for ethyl ester synthesis. Yeast strains were added for their ability to liberate high levels of *cis,cis*,-9,12-octadecadienoic acid (linoleic acid), propionibacteria adjuncts were added for conversion of *cis,cis*,-9,12-octadecadienoic to conjugated linoleic acid (CLA), and *L. fermentum* was added to promote ester formation and mask the potential off-flavours produced by liberation of long-chain FFAs. A seven-fold increase in FFA levels over control cheeses was noted on inclusion of yeast adjuncts. However, propionibacteria strains in combination with yeast adjuncts did not further increase levels of lipolysis. Elevated CLA levels were noted on inclusion of yeast adjuncts but appeared to have resulted from the general lipolytic activity of the yeast strains. Ethyl ester concentrations increased in experimental cheeses and appeared to be due to the inclusion of yeast adjuncts, with a fruity flavour note also detected in these cheeses.

Inclusion of various mesophilic lactobacilli as adjuncts in a model Caciotta cheese system caused strain-related increases in FFA; evidence for the predominance of esterase activity in certain strains was indicated in the release of high levels of short-chain FFAs (Di Cagno *et al.*, 2005).

In general, enzyme-accelerated lipolysis has not always resulted in enhancement in cheese flavour, especially in varieties where lipolysis is normally quite moderate, e.g. Cheddar and Gouda. Apart from the addition of PGE, the best results appear to have occurred when using fungal lipases for acceleration of lipolysis and flavour development. Recently, Hernandez *et al.* (2005) concluded that PGE when added to Idiazabal cheese enhanced short-chain FFA release and gave better sensory qualities compared to cheeses with added fungal lipase or control cheeses.

A particular process where extensive lipolysis appears important is in the production of EMC. This process involves creation of an emulsified substrate followed by enzymatic hydrolysis by lipases and/or proteinases to generate high levels of FFA and proteolytic end products. Little published information is available on the lipases used industrially; however, some reports indicate the use of PGE or fungal lipases (Kilcawley *et al.*, 1998, 2001).

5.5 Future trends

The influence of the end products of lipolysis on the development of cheese flavour is well recognised in a range of varieties including soft surface and internal ripened types, Spanish, Italian, and Swiss varieties. In many of these cheese types, the contribution of LPL and PGE is considerable, with a lesser contribution from microbial esterases/lipases. In the ripening of Cheddar and Dutch types, the contribution of moderate levels of lipolysis to flavour appears more important than previously thought. Despite the advances in flavour chemistry, our knowledge of the contribution of LPL and microbial enzymes to lipolysis and secondary flavour compounds in these varieties is still not well understood.

In order to maximise the potential of LPL and microbial enzymes in these cheese varieties, a thorough understanding of their mechanism of activity in the cheese matrix is required.

A key point for elucidation in LAB research is the quantification of the influence of strain-related autolysis and cell permeabilisation on the release of intracellular esterases/lipases into cheese and the stability of these enzymes within the cheese physico-chemical environment. As discussed previously, the recently discovered dual hydrolytic–synthetic nature of esterases requires further study regarding its impact on flavour chemistry and sensory properties of cheese. However, manipulation by enzyme engineering of esterase characteristics under various physico-chemical and/or ripening conditions may offer the possibility for creation of specific flavour notes/profiles in a more controlled and directed fashion.

For enhancement of flavour through culture or enzyme technology, the selection or biotechnological creation of LAB with esterases/lipases which mimic the action of PGE in cheese appears warranted. When allied to liposome entrapment technology, enzyme engineering of esterases/lipases offers the exciting possibility for both a targeted and controlled release mechanism of flavour-enhancing esterases/lipases into cheese.

In varieties where LPL action is minimised through heat treatment, the pre-hydrolysis of triacylglycerides by specific lipases may provide increased levels of substrates for the action of LAB esterases during ripening. Initial trials by Holland *et al.* (2005) indicate that this approach may be a potential industrial-scale route to enhance lipolysis in a range of cheese varieties; however, extensive validation and a correlation with sensory attributes of the resulting cheeses is imperative before widespread acceptance of this technology.

The role of amino acid metabolism and its interaction with lipolysis needs to be examined. The starter cultures and adjunct bacteria are adept at production of FFA from amino acids; however, the exact amount from each source is not understood. Enhancing lipolysis in a controlled fashion must also include examination of the bacterial metabolism of amino acids during ripening.

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The relative contributions of starter cultures and non-starter bacteria to the flavour of cheese

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6.1 Introduction

Cheese is the most diverse group of dairy products. The most common and simple criterion to classify cheese varieties into meaningful groups is texture (very-hard, semi-hard, semi-soft, soft) which is related mainly to the moisture content of the cheese (Burkhalter, 1981). This classification could be improved by considering milk-producing species, moisture to protein ratio, method of coagulation, cooking temperature and microorganisms (Fox and McSweeney, 2004). This latter characteristic is fundamental. There is a general agreement in that cheese cannot be made without the presence of certain microbial species, in most cases lactic acid bacteria. The contribution of microorganisms to cheese flavour is influenced by the protocol of cheese-making. Microorganisms gain entry into the cheese either by deliberate addition as starter cultures or by being naturally associated with the environment and ingredients used in cheese-making. Therefore, technology is central to defining the biodiversity of cheese microorganisms.

The production of the majority of rennet-coagulated cheese varieties can be subdivided into two well-defined phases, manufacture and ripening, both of which involve a number of processes. The manufacturing phase, defined as those operations performed during the first *ca.* 24 h, includes steps which are in some part common or specific for cheese varieties. Milk pasteurization, coagulation, dehydration (cutting the coagulum, cooking, stirring, pressing and other operations that promote gel syneresis), shaping and salting either have a direct

effect on the cheese microorganisms or influence the environment in which they proliferate. As the cheese manufacture is essentially a dehydration process in which fat and casein in milk are concentrated between 6- and 12-fold, depending on the variety, it predetermines moisture, NaCl in moisture and pH of the curd during ripening. These environmental factors, together with temperature and time of ripening, influence, in turn, the biochemical changes that occur during ripening and contribute to the unique characteristics of the cheese flavour (Fox *et al.*, 2000). Notwithstanding the role of coagulants, indigenous milk enzymes (e.g., plasmin and lipoprotein lipase) and cathepsins, the microbial contribution to the biochemical events that characterize cheese flavour could be considered as indispensable.

6.2 Cheese-related microorganisms

The microorganisms associated with cheese are extremely diverse. They may be conveniently divided into two main groups: primary and secondary starter cultures. Besides, adventitious (autochthon, indigenous) microorganisms, represented mainly by non-starter lactic acid bacteria (NSLAB), are a significant proportion of the microbial population of, probably, all ripened cheese varieties.

6.2.1 Primary starter cultures

These microorganisms are so called since they 'start' the production of lactic acid from lactose, which occurs early in the manufacturing phase of cheese. A useful rule is to decrease the value of pH to <5.3 in milk in ~ 6 h at $30\text{--}37^\circ\text{C}$, depending on the cheese variety. Generally, the primary starter cultures are lactic acid bacteria carefully selected and deliberately added to milk during cheese manufacture. Starter cultures provide the most significant contribution to the microbial biomass in young curd, typically attaining densities of $\geq 10^8$ cfu g $^{-1}$ within one day.

Primary starter cultures are usually classified as mesophilic or thermophilic (Table 6.1). The latter (e.g., *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*) are mainly used in Italian (e.g., Grana, Pecorino, Mozzarella) and Swiss (e.g., Emmentaler, Sbrinz, Gruyère) cheese varieties, where a high temperature ($37\text{--}52^\circ\text{C}$) prevails during manufacture. Mesophilic starter cultures (e.g., *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*) are used in all cheese varieties in which the temperature of the curd during the early stage of acid production does not exceed *ca.* 40°C .

Based on the complexity of the culture and the way it is reproduced, natural (traditional) and commercial mixed-strain (defined-strain) starters are distinguished (Limsowtin *et al.*, 1996). Natural starter cultures are propagated daily at the cheese plant by some form of back-slopping and/or by application of selective pressure (e.g., heat treatment, incubation temperature, low pH). Natural

Table 6.1 Examples of cheeses and related primary starters

Cheese	Type of starter	Starter microorganisms ¹								
		Lc	Lc.Cit ⁺	Lh	Ldb	Ldl	Strt	Strm	Lm	Ec
Asiago	Natural whey and milk culture, thermophilic			+	+		+			
Brie	Commercial culture, mesophilic	+	+						+	
Camembert	Commercial culture, mesophilic	+	+						+	
Canestrato Pugliese	Natural whey culture, thermophilic			+		+	+			
Castelmagno	Natural whey and milk culture, thermophilic					+	+			
Cheddar	Commercial culture, mesophilic	+								
Cottage	Commercial culture, mesophilic	+	+						+	
Edam	Commercial culture, mesophilic	+	+						+	
Emmental	Commercial culture, thermophilic			+		+	+			
Fiore Sardo	Natural whey starter, thermophilic				+		+			
Fossa	Natural whey and milk culture, thermophilic				+		+			
Gorgonzola	Commercial culture, thermophilic				+		+			
Gouda	Commercial culture, mesophilic	+							+	
Grana Padano	Natural whey culture, thermophilic			+	+	+	+			
Gruyère	Commercial culture, thermophilic			+		+	+			
Manchego	Natural milk and commercial culture, mesophilic	+								
Montasio	Natural whey culture, mesophilic and thermophilic	+		+			+			
Mozzarella	Natural culture, mesophilic and thermophilic,	+		+		+	+		+	+
	Commercial culture, thermophilic									
Parmigiano Reggiano	Natural culture, thermophilic			+	+	+	+			

Table 6.1 Continued

Cheese	Type of starter	Starter microorganisms ¹								
		Lc	Lc.Cit ⁺	Lh	Ldb	Ldl	Strt	Strm	Lm	Ec
Pecorino Romano	Natural culture in scotta, thermophilic			+		+	+			
Pecorino Sardo	Natural whey and milk culture, thermophilic			+	+		+			
Pecorino Siciliano	Natural whey culture, thermophilic			+	+		+			
Provolone Italiano	Natural whey culture, thermophilic			+			+			
Quarg	Commercial culture, mesophilic	+	+						+	
Roncal	Natural milk culture, mesophilic	+								
Roquefort	Commercial culture, mesophilic	+	+						+	
Sbrinz	Commercial culture, thermophilic			+		+	+	+		
Stilton	Commercial culture, mesophilic	+							+	
Taleggio	Commercial culture, thermophilic			+			+			
Tilsit	Natural milk culture, thermophilic						+			+

¹ Lc, *Lactococcus lactis* subsp. *lactis* and/or *cremoris*; Lc.Cit⁺, *Lactococcus lactis* subsp. *lactis* citrate positive ; Lh, *Lactobacillus helveticus* ; Ldb, *Lactobacillus delbruechii* subsp. *bulgaricus*; Ldl, *Lactobacillus delbruechii* subsp. *lactis*; Strt, *Streptococcus thermophilus*; Strm, *Streptococcus macedonicus*; Lm, *Leuconostoc mesenteroides*; Ec, *Enterococcus faecium* and/or *faecalis*.

Source: Adapted from Parente and Cogan (2004).

whey- and milk-starter cultures are distinguished depending on the medium and techniques used for their propagation. Natural whey-starter cultures are prepared by incubating some of the whey drained from the cheese vat overnight under more or less selective conditions (e.g., 45°C under temperature gradient to a final pH of *ca.* 3.3 for Grana cheeses) (Limsowtin *et al.*, 1996). The resulting whey-starter is dominated by aciduric and/or thermophilic strains: mainly *Lb. helveticus* (>85%) and *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus fermentum* and *Str. thermophilus*. Other types of whey-starters are made from deproteinized whey and used for Pecorino and Swiss-type cheeses (Limsowtin *et al.*, 1996; Mannu *et al.*, 2002) by selecting almost the same thermophilic strains in addition to enterococci. Natural milk-starters are used in small cheese-making plants of southern and northern Italy for the manufacture of traditional cheeses. The selective pressure includes thermization/pasteurization of raw milk (62–65°C for 10–15 min) followed by incubation at 37–45°C until the desired titratable acidity is reached. These starter cultures are usually dominated by *Str. thermophilus* with *Streptococcus macedonicus*, enterococci and mesophilic lactobacilli (Limsowtin *et al.*, 1996; Andrighetto *et al.*, 2002).

Commercial mixed-strain (defined-strain) starters are derived from the best natural starter cultures, reproduced under controlled conditions by specialized institutions (Dairy Research Centres or commercial starter companies) and distributed to cheese plants which use them to build up bulk starter or for direct-vat inoculation (Table 6.1). Mesophilic mixed-strain starter cultures are usually classified based on citrate fermentation and composition: citrate-negative ‘O’ starters which contain Cit[−] *Lc. lactis* subsp. *lactis* and subsp. *cremoris*, and citrate-positive ‘L, D and DL’ starters containing *Leuconostoc mesenteroides*, Cit⁺ *Lc. lactis* subsp. *lactis* or both, in addition to acid-producing strains (Limsowtin *et al.*, 1996). Thermophilic mixed-strain starters usually contain *Str. thermophilus* alone or in mixture with thermophilic lactobacilli (*Lb. delbrueckii* subsp. *lactis* and *Lb. helveticus*) (Glattli, 1990).

6.2.2 Secondary starter cultures

In many cheese varieties, secondary starter cultures are added intentionally and/or encouraged to grow by favourable environmental conditions (secondary microorganisms). The terms secondary starters or secondary microorganisms are used interchangeably depending on the deliberated addition to cheese milk. These microorganisms are so called to distinguish them from the primary acid-producing starters since they are involved in cheese ripening only (Table 6.2). Secondary starter cultures include yeasts (e.g., *Geotrichum candidum*, *Debaryomyces hansenii*) in mould and bacterial surface-ripened cheeses (e.g., Brie, Camembert), moulds (e.g., *Penicillium camemberti*, *Penicillium roqueforti*) in mould-surface-ripened soft cheeses (e.g., Brie, Camembert) and blue-veined cheeses (e.g., Roquefort, Gorgonzola), coryneform bacteria (e.g., *Arthrobacter*, *Brevibacterium*), *Staphylococcus* and *Micrococcus* in smeared soft and semi-hard cheeses (e.g., Limburger, Taleggio) and propionibacteria (e.g.,

Table 6.2 Examples of cheeses and related secondary starters and/or secondary microorganisms

Cheese	Secondary starters and/or secondary microorganisms
Brie	<i>Geotrichum candidum</i> , <i>Debaryomyces hansenii</i> , <i>Penicillium camemberti</i> , <i>Penicillium roqueforti</i> , <i>Staphylococcus</i> sp.
Camembert	<i>G. candidum</i> , <i>D. hansenii</i> , <i>P. camemberti</i> , <i>P. roqueforti</i> , <i>Corynebacterium ammoniagenes</i> , <i>Corynebacterium variabilis</i> , <i>Arthrobacter nicotianae</i> , <i>Brevibacterium linens</i> , <i>Rhodococcus</i> sp., <i>Brachybacterium</i> sp., <i>Staphylococcus</i> sp.
Castelmagno	<i>Penicillium</i> sp.
Emmental	<i>Propionibacterium acidopropionici</i> , <i>Propionibacterium freudenreichii</i> , <i>Propionibacterium shermanii</i>
Gorgonzola	<i>P. camemberti</i> , <i>P. roqueforti</i>
Gouda	<i>Propionibacterium</i> sp.
Gruyère	<i>P. shermanii</i> , <i>Brachybacterium</i> sp., <i>Staphylococcus</i> sp., <i>Corynebacterium</i> sp.
Gubbeen	<i>Staphylococcus</i> sp., <i>Corynebacterium casei</i> , <i>Corynebacterium flavesces</i> , <i>Microbacterium gubbeenense</i>
Limburger	<i>A. nicotianae</i> , <i>Arthrobacter</i> sp., <i>C. ammoniagenes</i> , <i>C. variabilis</i> , <i>Corynebacterium</i> sp., <i>B. linens</i> , <i>Staphylococcus</i> sp., <i>Micrococcus</i> sp., <i>Rhodococcus</i> sp.
Romadour	<i>A. nicotianae</i> , <i>Arthrobacter</i> sp., <i>C. ammoniagenes</i> , <i>C. variabilis</i> , <i>Corynebacterium</i> sp., <i>B. linens</i> , <i>Staphylococcus</i> sp., <i>Micrococcus</i> sp., <i>Rhodococcus</i> sp.
Roquefort	<i>P. camemberti</i> , <i>P. roqueforti</i>
Sbrinz	<i>P. shermanii</i>
Stilton	<i>P. roqueforti</i> , <i>Staphylococcus</i> sp.
Taleggio	<i>Arthrobacter</i> sp., <i>Brevibacterium</i> sp., <i>Staphylococcus</i> sp., <i>Micrococcus</i> sp., <i>Corynebacterium</i> sp.
Tilsit	<i>B. linens</i> , <i>Arthrobacter globiformis</i> , <i>A. citreus</i> , <i>A. nicotianae</i> , <i>C. ammoniagenes</i> , <i>C. variabilis</i> , <i>Curtobacterium poinsettiae</i> , <i>Cu. betae</i> , <i>Cu. oxidans</i> , <i>Cu. helvolum</i> , <i>Clavibacterium insidiosum</i> , <i>Microbacterium liquefaciens</i> , <i>M. lacticum</i> , <i>M. gubbeenense</i> , <i>Staphylococcus</i> sp., <i>Rhodococcus</i> sp.

Propionibacterium acidopropionici, *Propionibacterium freudenreichii*) in Swiss-type cheeses. Except for propionibacteria and *P. roqueforti*, the secondary starter cultures grow mainly on the cheese surface. Currently, secondary starter cultures are manufactured in the form of commercial preparations or, especially, as natural cultures. Traditionally, the secondary microflora originates from the milk, the cheese-making utensils and/or the cheese factory environment. Like the manufacture of traditional smear-ripened cheeses, mature curds are smeared (e.g., washed with diluted solutions of NaCl, which may also contain some of the surface microflora) and the cheese surface microorganisms are transferred from the old to the young curds.

6.2.3 Non-starter lactic acid bacteria (NSLAB)

NSLAB are not deliberately added as a part of primary and secondary starter cultures but are adventitious contaminants, which grow during ripening. They do not contribute to acid production during cheese manufacture, but impact on flavour development of most of the ripened cheese varieties. Since this contribution, some authors include the NSLAB as a part of the secondary microflora also. The principal bacterial groups are non-starter lactobacilli (e.g., *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus plantarum*, *Lactobacillus casei*), leuconostocs (e.g., *Leuconostoc lactis*, *Leuc. mesenteroides*), pediococci (e.g., *Pediococcus acidilactici*, *Pediococcus pentosaceus*) and enterococci (e.g., *Enterococcus faecalis*, *Enterococcus faecium*). Some of them also correspond to strains used as primary starter cultures in certain cheese varieties (e.g., leuconostocs, enterococci). Non-starter facultatively heterofermentative lactobacilli constitute the majority of the NSLAB population in most cheese varieties during ripening (Table 6.3) (Beresford *et al.*, 2001). They grow at 2–53°C, are acid-tolerant and tolerate the lack of fermentable carbohydrates, low pH and a_w (mainly due to NaCl), and the presence of bacteriocins which make the environmental conditions very hostile during cheese ripening. Overall, lactobacilli exhibit fastidious nutritional requirements, but NSLAB find ample nutritional opportunities for growth in ripening cheese (e.g., lactate, citrate, glycerol, amino sugars, amino acids and other metabolites) (Peterson and Marshall, 1990; Wouters *et al.*, 2002).

The use of non-starter lactobacilli (mainly *Lb. casei*, *Lb. paracasei* subsp. *paracasei*, *Lb. plantarum*, *Lactobacillus curvatus*, *Lactobacillus rhamnosus*) as adjunct starter cultures in the manufacture of semi-hard cheeses has been considered as a tempting strategy to overcome the loss of sensory quality in cheese subsequent to milk pasteurization. Although the role of these bacteria in overall cheese quality is still debated due to the unpredictable and dynamic nature of non-starter lactobacilli influenced by compositional and environmental factors (Lane *et al.*, 1997; Lynch *et al.*, 1996), the positive contributions to flavour development by adjunct starter cultures have been reported in several cheese varieties (Requena *et al.*, 1992; Corsetti *et al.*, 1998; Weimer *et al.*, 1997). Overall, when selecting strains for use as adjuncts, two factors have to be usually considered: the ability to outgrow adventitious lactic acid bacteria and the ability to produce good and balanced flavour (Crow *et al.*, 2001).

6.2.4 Changes in microflora during cheese ripening

Cheese during ripening is frequently characterized by successions of microbial communities. These successions are determined by manufacture and ripening conditions, and by the interactive associations among microorganisms. Overall, the large part ($\geq 10^8$ cfu g⁻¹) of primary starter biomass (e.g., thermophilic starters) declines throughout ripening. Depending on the strain and environmental conditions, primary starter cultures release intracellular enzymes due to autolysis which favour immediate access to the cheese matrix (Feirtag and

Table 6.3 Examples of cheeses and related non-starter lactic acid bacteria

Cheese	Dominant non-starter lactic acid bacteria ¹																
	aci	bre	cas	cur	cyp	fer	hil	pbu	pca	ppl	pen	pla	rha	Ped	Ef	Ln	del
Arzua									+			+					
Batzos									+	+	+						
Caciocavallo Pugliese			+						+			+					
Caciocavallo Silano								+	+								
Camembert	+		+						+			+					+
Canestrato Pugliese		+		+					+		+	+				+	
Cheddar		+	+	+					+			+	+				
Comté						+			+				+				
Emmental									+								
Fiore Sardo				+					+		+						
Fontina									+			+					
Fossa				+					+		+	+					
Gouda																+	
Grana Padano			+						+				+	+			
Gruyère			+										+				
Idiazabal			+	+	+							+	+			+	

Kefalotyri									+			+		
Mahòn												+		
Manchego			+	+								+		+
Montasio									+					
Mozzarella				+			+					+		
Parmigiano-Reggiano			+						+				+	+
Pecorino Romano				+										
Pecorino Sardo				+										
Pecorino Toscano					+				+			+		
Ragusano							+							
Ricotta Forte	+	+	+						+					
Roncal			+	+	+							+	+	+
Roquefort														
Serra de Estrela			+						+		+	+		
Toma									+				+	

¹ The abbreviations used for the non-starter lactic acid bacteria are as follows: aci, *Lactobacillus acidophilus*; bre, *Lactobacillus brevis*; cas, *Lactobacillus casei*; cur, *Lactobacillus curvatus*; cyp, *Lactobacillus cypricasei*; fer, *Lactobacillus fermentum*; hil, *Lactobacillus hilgardii*; pbu, *Lactobacillus parabuckneri*; pca, *Lactobacillus paracasei*; ppl, *Lactobacillus paraplantarum*; pen, *Lactobacillus pentosus*; pla, *Lactobacillus plantarum*; rha, *Lactobacillus rhamnosus*; Ped, *Pediococcus* sp.; Ef, *Enterococcus faecium*; Ln, *Leuconostoc* sp.; del, *Lactobacillus delbrueckii*.

Source: Adapted from Beresford and Williams (2004)

McKay, 1987; O'Sullivan *et al.*, 2002; Bottazzi *et al.*, 1992) and, in general, their proteolytic activity is beneficial to the growth of NSLAB (Martley and Crow, 1993) and propionibacteria (Piveteau *et al.*, 2002). The growth rate of *Lb. plantarum* under conditions which mimicked cheese ripening seemed to depend on the type of starter proteinase which was associated (Di Cagno *et al.*, 2003b).

NSLAB are present in cheeses made from both raw and heat-treated milk. Hygienically produced raw milk may contain $\sim 10^2$ cfu lactobacilli ml^{-1} and it is likely to be the main source of NSLAB in cheeses made from raw milk (Berthier *et al.*, 2001). NSLAB were isolated from 12 Italian ewes' cheeses, and the cheeses manufactured from raw ewes' milk contained a larger number of more diverse strains than cheeses produced from pasteurized milk (De Angelis *et al.*, 2001). Some lactobacilli are inactivated by pasteurization (Turner *et al.*, 1986), but some strains may survive the heat treatment and proliferate in cheese during ripening (Jordan and Cogan, 1999). Nevertheless, it has been suggested that manufacturing equipments are the more probable source of NSLAB in cheeses made from pasteurized milk (Martley and Crow, 1993). NSLAB have been isolated from the floor and drains in the dairy environment and from the surfaces of equipment used in cheese manufacture and vacuum packaging. Lactobacilli are able to form and persist in biofilms on cheese-making equipment and could be re-isolated from batches of cheese produced after the plant had been cleaned, implying that they survive cleaning and sanitizing treatments (Somers *et al.*, 2001).

Independent of the way of entry, NSLAB (e.g., *Lactobacillus parabuchneri*, *Lb. paracasei* subsp. *paracasei*, *Lb. plantarum*, *Lb. casei* and *Lb. curvatus*) reach $\sim 10^8$ cfu g^{-1} and dominate the viable microflora of Cheddar, extra-mature Dutch, and semi-hard and hard Italian cheese varieties (Bosset *et al.*, 1997; Braun and Olson, 1986; Broome *et al.*, 1990; Fox and McSweeney, 1996a). A generation time of ~ 8.5 days in cheese ripened at 6°C has been estimated for non-starter lactobacilli which are recovered as viable cells from cheeses stored at 10°C for 3 years (Jordan and Cogan, 1993). While showing a marked decrease with respect to the concentration found after 4 months of ripening, $\sim 10^4$ NSLAB g^{-1} were still determined after 24 months of Parmigiano-Reggiano cheese ripening (Coppola *et al.*, 1997).

One NSLAB strain can affect the flavour development potential of another. The outgrowth of a *Lb. rhamnosus* strain, added on purpose as an adjunct in a Cheddar cheese trial, could be retarded by a simultaneously added *Lb. casei* strain (Martley and Crow, 1993). The presence of *Leuconostoc* species in the primary starter cultures affected the development of adventitious NSLAB (Martley and Crow, 1993). This may explain why the reports on the occurrence of NSLAB in Gouda cheese, manufactured with starter cultures containing *Leuconostoc*, are rather limited (Wouters *et al.*, 2002). A succession of microorganisms is usual in smear-ripened cheeses where the lactate deacidification activity by yeasts favours the increase of pH at the surface and the subsequent growth of moulds and corynebacteria.

Depending on the cheese varieties, primary and secondary starter cultures, and NSLAB may play a different role in lactose, lactate and citrate metabolisms,

lipolysis and proteolysis which are considered the primary events for characterizing cheese flavour.

6.3 Lactose, lactate and citrate metabolisms

6.3.1 Metabolism of lactose

Although *ca.* 98% of the lactose is removed in the whey as lactose or lactate (Huffman and Kristoffersen, 1984), the complete and rapid metabolism of the lactose in cheese curd is essential for the production of good quality cheese since the presence of fermentable carbohydrate may lead to the development of an undesirable contaminant microflora (Fox *et al.*, 2000). In Cheddar cheese, the residual lactose is fermented at a rate dependent on the salt-in-moisture content of the curd (Turner and Thomas, 1980). At low salt-in-moisture concentrations and low populations of NSLAB, residual lactose is converted mainly to L-lactate by the primary starters. At high populations of NSLAB (e.g., high ripening temperature), considerable D-lactate is formed, partly by fermentation of residual lactose and partly by isomerization of L-lactate (Turner and Thomas, 1980).

The fermentation of residual lactose has been studied in Parmigiano Reggiano cheese during the first 48 h after manufacture (Mora *et al.*, 1984). It depends mainly on the rate at which the curd cools after removal from the cheese vat. The temperature at the centre of the curd remains relatively high (e.g., > 50°C for 12–16 h), while the exterior of the cheese cools rather suddenly (e.g., *ca.* 2 h at 42°C). Consequently, the fermentation of residual lactose starts earlier and is more intense in the external zone.

The fermentation of lactose in Swiss-type cheeses is quite complicated (Fox *et al.*, 1990). Lactose is metabolized by *Str. thermophilus* within 12 h but only the glucose moiety is used. Subsequently, lactobacilli metabolize residual lactose and galactose to a mixture of D- and L-lactate during a further 14 days of ripening. Thereafter, the concentration of lactic acid changes little until the curd is transferred to the hot room, when the propionibacteria begin to grow (Turner *et al.*, 1983).

The pathway for the metabolism of lactose depends on the starter culture or NSLAB species. Glycolysis is the pathway used by the major part of the lactic acid bacteria, while *Leuconostoc* spp. use the phosphoketolase pathway, producing lactate, ethanol and CO₂ as end-products. Lactate contributes to the flavour of cheese, particularly early during maturation, but the major effect of acidification on flavour development is indirect since, together with the buffering capacity of the curd, it influences pH and thus the growth of secondary starter cultures and NSLAB, and the activity of ripening enzymes.

6.3.2 Metabolism of lactate

Lactate is an important substrate for a range of reactions which contribute positively or negatively to cheese ripening (Fig. 6.1). Cheddar cheese contains a

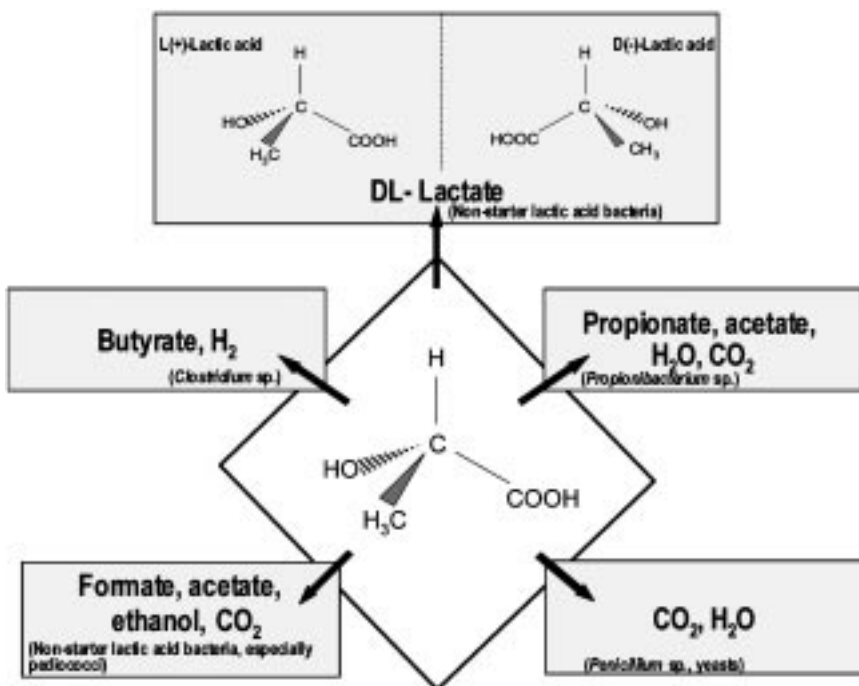


Fig. 6.1 Metabolism of lactate during cheese ripening.

considerable concentration of D-lactate which could be formed by racemization of L-lactate produced by primary starter cultures such as *Lactococcus* (Tinson *et al.*, 1982). Racemization of L-lactate is likely to occur more rapidly in cheese made from raw milk due to high numbers of NSLAB. Racemization presumably involves oxidation of L-lactate by L-lactate dehydrogenase (LDH) to pyruvate which is, in turn, reduced to D-lactate by D-LDH. It seemed that pediococci racemize L-lactate more actively than lactobacilli (Thomas and Crow, 1983). Nevertheless, pediococci constitute only a small proportion of the NSLAB microflora compared to lactobacilli (Crow *et al.*, 2001). The racemization of L-lactate is probably not significant from the flavour viewpoint. However, Ca-lactate may crystallize in cheese, causing undesirable white specks, especially on cut surface (Dybing *et al.*, 1988). The solubility of Ca-DL-lactate is lower than that of pure Ca-L-lactate; hence, racemization of lactate favours the development of crystals in cheese.

Lactate is metabolized by lactic acid bacteria, depending on strain, to acetate, ethanol, formate and CO₂ (Fox *et al.*, 2000). The oxidation of lactate to acetate in cheese depends on the NSLAB population and on the availability of O₂, which is determined by the size of the block and oxygen permeability of the packaging material (Thomas, 1987). Pediococci, if present in cheese together with high concentrations of O₂, produce 1 mol of acetate and 1 mol of CO₂ and consume 1 mol of O₂ per mole of lactate utilized (Thomas *et al.*, 1985).

The catabolism of lactate is extensive in surface mould-ripened and smear-ripened cheese varieties (e.g., Camembert, Brie, Limburger, Taleggio). During ripening of Camembert cheese, secondary microorganisms and/or secondary starter cultures (e.g., *G. candidum* and *D. hansenii*) quickly colonize the surface, followed by a dense growth of *P. camemberti* and by low numbers of Gram-positive bacteria similar to those found on the surface of smear-ripened cheeses, which do not colonize the cheese surface until the pH has increased to >5.8 (Addis *et al.*, 2001). *G. candidum* and *P. camemberti* rapidly metabolize lactate to CO₂ and H₂O, causing an increase in pH. De-acidification occurs initially at the surface, resulting in a pH gradient from the surface to the centre and causing lactate to diffuse outwards. When the lactate is exhausted, *P. camemberti* metabolizes proteins, producing NH₃ which diffuse inwards, further increasing the pH. The concentration of calcium phosphate at the surface exceeds its solubility at the high pH and precipitate as a layer of Ca₃(PO₄)₂ on the surface, thereby causing a calcium phosphate gradient within the cheese, resulting in its outward diffusion; reduction of the concentration of calcium phosphate in the interior helps to soften the body of the cheese. De-acidification at the surface is also indispensable for the growth of *B. linens*, *Corynebacterium* spp. and *Arthrobacter* spp. that contribute to the colour and flavour properties of the smear-ripened cheeses.

Catabolism of lactate is particularly important in Swiss-type cheeses. Although the presence of other minor pathways has been supposed (Deborde and Boyaval, 2000), the propionic acid fermentation by *Propionibacterium freudenreichii* subsp. *shermanii* (secondary starter) in the Emmental cheese produces 2 propionate + 1 acetate + 1 CO₂ + H₂O from 3 mol of lactate. Propionate and acetate contribute to the flavour of Swiss cheese and CO₂ migrates through the curd to points of weakness where it collects to form the large characteristic eyes. The proportion of lactobacilli used as primary starter cultures influences the production of CO₂ and volatile acids. Increasing the number of starter lactobacilli accelerates sugar metabolism and causes higher concentrations of both D- and L-lactate but suppresses the growth of propionibacteria, due to a lower pH, and thus delays the production of propionic and acetic acids.

6.3.3 Metabolism of citrate

Although citrate is present at low levels in milk (ca. 8 mmol l⁻¹), its concentration in the aqueous phase of the curd is approximately 3 times higher than that in the whey, presumably reflecting the concentration of colloidal citrate (Fryer *et al.*, 1970). Cheddar cheese contains 0.2–0.5% citrate. Citrate is not metabolized by most primary starter Cit⁻ *Lc. lactis* subsp. *lactis* and subsp. *cremoris* strains, but is metabolized, with the production of diacetyl, acetate, acetoin, 2,3-butyleneglycol and CO₂, by Cit⁺ strains of lactococci (formerly referred to as *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* or *Streptococcus diacetylactis*), and *Leuc. mesenteroides* subsp. *cremoris* and *Lc. lactis* or by thermophilic lactobacilli

(Fox *et al.*, 2000). The CO₂ produced is responsible for the characteristic eyes of Dutch-type cheeses and for the undesirable openness in Cheddar cheese. Due to mainly the formation of diacetyl, citrate metabolism is significant in flavour development in Cottage (Antinone *et al.*, 1994), Quarg (Mohr *et al.*, 1997) and Dutch-type cheeses (Milo and Reineccius, 1997). Citrate may be metabolized by some strains of facultatively heterofermentative lactobacilli to acetoin, acetate and probably diacetyl (Palles *et al.*, 1998). In Cheddar cheese citrate decreases slowly, to almost zero at 6 months, presumably as a result of the metabolism of lactobacilli that are the major components of the NSLAB at this stage of ripening.

6.4 Proteolysis

Proteolysis is the most complex and, in most varieties, the most important biochemical event that occurs in cheese during ripening because it provides substrates for flavour formation. The extent of proteolysis in cheese varies from minor (e.g., Mozzarella) to extensive (e.g., Blue, Parmigiano Reggiano). Proteolysis contributes to development of cheese texture, flavour and off-flavours. Proteolysis of casein is the initial reaction in the production of flavour compounds, which is followed by the liberation of amino acids as precursors for a complex series of catabolic reactions that produce many important volatile flavour compounds. Proteolysis in cheese during ripening is potentially catalysed by proteinases and peptidases from several sources: coagulant, milk indigenous proteinases (e.g., plasmin), somatic cells (e.g., cathepsins D and B), primary and secondary starter cultures, NSLAB, and exogenous proteinases and peptidases. Manufacturing practices, particularly cooking temperature, and development of a highly proteolytic secondary microflora and ripening time, influence the pattern and extent of proteolysis. Residual coagulant activity retained in the curd is the major source of proteinase activity in most cheeses, except *pasta-filata* varieties and those with a high cooking temperature, in which enzymes such as chymosin are denaturated extensively. The proteinase activity of plasmin is of particular importance for the above cheeses, since it is a heat-stable enzyme, and also in cheeses whose pH increases during ripening (the pH optimum of plasmin is *ca.* 7.5). With a few exceptions (e.g., moulds), the major role of cheese-related microorganisms is concerning peptidase activities and catabolism of amino acids.

6.4.1 The proteolytic system of lactic acid bacteria

The concentrations of amino acids in milk are below the nutritional requirements for the growth of auxotrophic lactic acid bacteria and, therefore, their complex proteolytic system degrades mainly caseins into small peptides and amino acids, which fulfil their nutrition and inadvertently contribute to the cheese flavour (Law and Mulholland, 1995). The best-studied proteolytic system

among lactic acid bacteria is that of *Lactococcus* (Kunji *et al.*, 1996; Siezen, 1999). The main components are cell-envelope-associated proteinase (CEP or lactocepin), although intra-cellular proteinases have been reported (Stepaniak *et al.*, 1996) and are found in the genome of these organisms, amino acid and peptide transport systems, and a range of intracellular peptidases. During growth in milk, the initial step in casein degradation is performed by lactocepin and the short peptides produced are taken up by the cell via peptide transport systems (Juillard *et al.*, 1995). Further degradation to amino acids is catalysed by a number of intra-cellular peptidases (Kunji *et al.*, 1996).

Lactocepins of *Lactococcus* are homologous with the subtilisin family of serine-proteinases and were initially classified into two broad groups, P_I-type and P_{III}-type proteinases (Tan *et al.*, 1993). P_I-type enzymes (e.g., produced by *Lc. lactis* subsp. *cremoris* HP and Wg2) degrade β -casein rapidly but act only slowly on α_{s1} -casein, whereas P_{III}-type proteinases (e.g., strain SK11) hydrolyse β -casein differently from P_I-type strains and hydrolyse α_{s1} - and κ -caseins (Law and Haandrikman, 1997). Although this broad classification scheme remains useful, it soon became apparent that the lactocepin of certain strains of *Lactococcus* had a specificity intermediate between P_I- and P_{III}-types with additional groupings being defined as more protease characterization is done (Broadbent *et al.*, 1998).

Peptides isolated from Cheddar cheese, the N- or C-terminus of which corresponds to the specificity of lactocepin, do not contain a major chymosin or plasmin cleavage sites (Fox and McSweeney, 1996a), suggesting that chymosin and/or plasmin act first and that lactocepin then hydrolyses the resulting intermediate-sized peptides. Cell-envelope-associated proteinases with properties similar to the lactococcal lactocepins have also been isolated from a number of strains of thermophilic *Lactobacillus* used as primary starter cultures (Kunji *et al.*, 1996; Law and Haandrikman, 1997).

Many different peptidases have been characterized from lactic acid bacteria (Fig. 6.2). They include endopeptidases that degrade oligopeptides to shorter peptides and exopeptidases which release one or two amino acids at a time from short peptides (Kunji *et al.*, 1996; Law and Haandrikman, 1997; Siezen *et al.*, 2002). On the basis of the substrate specificity, lactic acid bacteria possess three types of endopeptidases: PepO which is capable of hydrolysing several casein fragments but not di-, tri- and tetrapeptides; PepF which specifically cleaves Phe-Ser bond; and PepE, the general properties of which indicated a different substrate specificity from the other two metallo-endopeptidases. Tripeptidases (PepT) and general dipeptidase (PepV) of lactic acid bacteria have a broad specificity and are capable of hydrolyse specifically tri- and dipeptides, respectively. Carboxypeptidases are exopeptidases that catalyse the hydrolysis of peptides from the C-terminal. No carboxypeptidase activity has been detected in lactococci but some activity towards N-terminal-blocked peptides has been reported in strains of lactobacilli (El-Soda *et al.*, 1987).

The most thoroughly studied exopeptidase from lactic acid bacteria is the general aminopeptidase (PepN). It has a broad specificity, being capable of

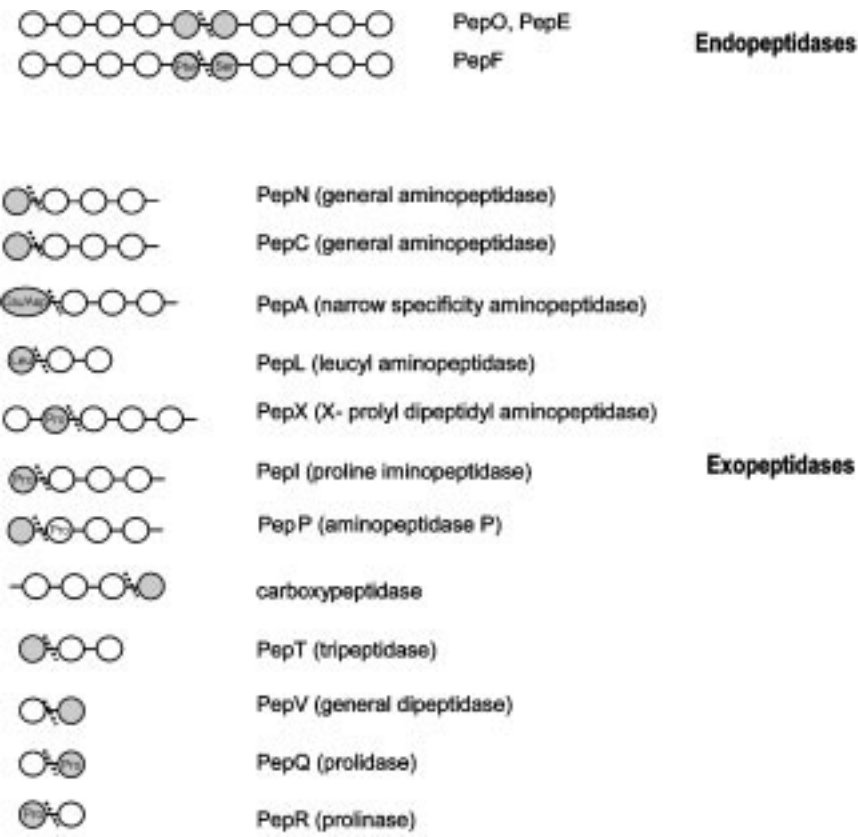


Fig. 6.2 Mechanism of hydrolysis of peptidases in lactic acid bacteria.

hydrolysing a wide range of peptides differing in both size and amino acid composition. Substrates with a hydrophobic or basic amino acid residue at the *N*-terminal are hydrolysed preferentially (Tan *et al.*, 1992). PepC in lactic acid bacteria is a metal-independent general aminopeptidase (Kunji *et al.*, 1996). It shows broad specificity including little activity on peptides with positively charged amino acid residues (Mistou and Gripon, 1998). Lactococcal PepA is a narrow-specificity peptidase which releases only an *N*-terminal Glu or Asp from di-, tri- and oligopeptides with up to ten amino acid residues (Bacon *et al.*, 1994). Glutamate is a well-recognized flavour enhancer and, therefore, the role of PepA in the development of flavour in cheese may be of great importance. The presence of more than one leucyl-aminopeptidase (PepL) has been reported in lactic acid bacteria (Banks *et al.*, 1998). PepL preferentially hydrolyses dipeptides and some tripeptides with an *N*-terminal leucyl residue.

Caseins are rich in the imino acid proline. Because of its cyclic structure, specialized peptidases are required to hydrolyse peptide bonds involving proline, thus making peptides accessible to the action of other peptidases (Cunningham and O'Connor, 1997). Several proline-specific peptidases with distinct substrate

specificities have been found in lactic acid bacteria. X-prolyl dipeptidyl aminopeptidase (PepX) is a peptide hydrolase capable of releasing X-Pro and sometimes X-Ala dipeptides from the *N*-terminal of oligopeptides. This enzyme influenced proteolysis and sensorial characteristics of Gruyère cheese (Meyer and Spahni, 1998). Proline iminopeptidase (PepI) catalyses the release of an *N*-terminal proline residue from di-, tri- and oligopeptides. Prolinase (PepR) and prolidase (PepQ) are specific dipeptidases which hydrolyse dipeptides with the sequence Pro-X and X-Pro, respectively. Aminopeptidase P (PepP) is a specific aminopeptidase that catalyses the removal of the *N*-terminal amino acid from oligopeptides having the sequence X-Pro-Pro-(X)_n or X-Pro-(X)_n.

6.4.2 Proteolytic enzymes from secondary starter cultures

In mould-ripened, smear-ripened and Swiss-type cheeses, microorganisms other than lactic acid bacteria play a pivotal role in the development of characteristic flavour. While the proteolytic enzymes of lactic acid bacteria have been well characterized, there have been fewer studies on organisms associated with such cheese varieties. Proteolytic systems of *P. camemberti* and *P. roqueforti* are somewhat similar; both synthesize an aspartyl-proteinase, a metalloproteinase, an acid carboxypeptidase and an alkaline aminopeptidase. The aspartyl-proteinase of *P. camemberti* hydrolyses α_{s1} -casein faster than β - and κ -caseins (Gripon, 1993). *Geotrichum candidum* also synthesizes extracellular and intracellular proteinases but the contribution of these enzymes to cheese ripening is less than that of enzymes from *Penicillium* spp. (Gripon, 1993). Growth of *B. linens* on the cheese surface is thought to play an important role in the development of the characteristic colour and flavour of smear surface-ripened cheese varieties (Ratray and Fox, 1997). Extracellular enzymes of *B. linens* include proteinases, aminopeptidases and esterases. Species of the genus *Arthrobacter* are the major components of the microflora of surface mould-ripened cheeses such as Brie and Camembert, and read-smear cheeses. Two extracellular serine proteinases have been purified from *A. nicotianae* that preferentially hydrolysed β -casein over α_{s1} -casein (Smacchi *et al.*, 1999a). An extracellular PepI from the same strain was also purified and characterized (Smacchi *et al.*, 1999b). Some *Micrococcus* spp. are very proteolytic and produce extracellular proteinases, and intra-cellular proteinases and peptidases (Fox *et al.*, 1993). The extracellular proteinases from certain micrococci preferentially hydrolysed α_{s1} -casein (Nath and Ledford, 1972). *Propionibacterium* spp. are weakly proteolytic; their caesinolitic potential was estimated to be 5–15 times less than that of lactococci (Dupuis *et al.*, 1995). Nevertheless, propionic acid bacteria are highly peptidolytic, especially on proline-containing peptide bonds, thus contributing to the characteristic flavour of Swiss-type cheeses. Endopeptidases and PepX have been isolated from various strains of *P. freudenreichii* subsp. *shermanii* (Fernández-Espla and Fox, 1997; Stepaniak *et al.*, 1998). Nevertheless, autolysis of propionic acid bacteria in cheese is limited and lower than autolysis of lactic acid bacteria (Ostlie *et al.*, 1999).

6.4.3 Primary and secondary proteolysis

The general pattern of proteolysis of many cheese varieties may be summarized as follows. Initially, caseins are hydrolysed by residual coagulant activity and/or by plasmin, and perhaps other indigenous proteolytic enzymes to a range of large and intermediate-sized peptides (primary proteolysis) that are hydrolysed by proteinases and peptidases of primary starters, NSLAB and secondary starters/microorganisms to shorter peptides and amino acids (secondary proteolysis).

Primary proteolysis is similar during ripening of most cheeses: chymosin hydrolyses the Phe₂₃–Phe₂₄ bond of α_{s1} -casein, except in cheeses that are cooked at a high temperature (*ca.* 55°C, e.g., Swiss, *pasta-filata* and hard Italian cheeses), in which plasmin is the principal proteolytic agent. In Blue-veined cheeses, after sporulation, enzymes from *P. roqueforti* hydrolyse α_{s1} -casein (f24-199) and other peptides, changing the peptide profile (Gripone, 1993). In many cheeses (e.g., Italian ewe's milk and Manchego cheeses), α_{s1} -casein is hydrolysed faster than β -casein (Sousa and McSweeney, 2001). In Blue-veined cheeses, both α_{s1} - and β -caseins are completely hydrolysed at the end of ripening. In Swiss-type cheeses, β -casein is hydrolysed faster than α_{s1} -casein, with concomitant increases in γ -caseins, indicating a role of plasmin. Plasmin and *Lactobacillus* proteinases are responsible for the extensive proteolysis in Parmigiano-Reggiano cheese that is ripened for a long period (*ca.* 24 months) at an elevated temperature (*ca.* 18–20°C) (Battistotti and Corradini, 1993).

Several water-soluble peptides from Cheddar cheese have been isolated and characterized; quantitatively, they correspond mainly to the *N*-terminal half of α_{s1} -casein and are related to the specificity of the starter and non-starter proteinases and peptidases (Fox and McSweeney, 1996a). After chymosin hydrolysis, the peptide α_{s1} -casein (f1-23) is hydrolysed at the bonds Gln₉–Gly₁₀, Gln₁₃–Glu₁₄, Glu₁₄–Val₁₅ and Leu₁₆–Asn₁₇ by lactocepin (Fox and McSweeney, 1996a). The proteinase activity of NSLAB seems to be less than that of primary starter cultures and their contribution to casein hydrolysis during ripening of Cheddar cheese appears to be relatively small (Lynch *et al.*, 1997).

The contribution of the peptidases from lactic acid bacteria is well recognized. Although these enzymes are intracellular, they are liberated in cheese following autolysis of the cells. Indeed, the rate of secondary proteolysis is higher in cheese made with fast-lysing than that with slow-lysing starter strains (Martínez-Cuesta *et al.*, 2001; Hannon *et al.*, 2003). NSLAB affect cheese quality and almost certainly contribute to the intensity of flavour, although sometimes they may cause off-flavours in cheese. Comparison of raw and pasteurized milk cheese generally showed that raw milk cheese ripens more quickly and develops a stronger flavour than pasteurized milk cheese due to an increase of water-soluble peptides and free amino acids (FAA) (McSweeney *et al.*, 1993; Ur-Rehman *et al.*, 1999). Cheeses containing elevated numbers of *Lb. casei* and *Lb. plantarum* developed higher levels of FAA and received higher flavour intensity scores than control cheeses (Lee *et al.*, 1990a,b). Since the proteolytic systems of NSLAB are generally similar to those of other lactic acid

bacteria, they appear to contribute to secondary proteolysis in a similar way to the starter, but probably to a lesser extent since maximum NSLAB numbers in cheese ($\sim 10^7$ – 10^8 cfu g⁻¹) are usually lower than maximum numbers of starter ($\geq 10^8$ cfu g⁻¹). Nevertheless, peptidases of NSLAB strains seemed to retain a higher level of activity than lactococcal enzymes under cheese ripening conditions (Gobbetti *et al.*, 1999a,b). The activity of the NSLAB appears to supplement the proteolytic action of the starter. To show this the proteolytic system of *Lc. lactis* subsp. *lactis* was enriched with lactobacilli peptidases; the level of FAA during ripening approximately tripled (Courtin *et al.*, 2002). The pattern of secondary proteolysis by NSLAB seems to depend on the type of primary starter proteinase that is associated during ripening (Di Cagno *et al.*, 2003b).

Significant concentrations of FAA, the final products of proteolysis, occur in most of the cheeses. Cheddar contains low concentrations of FAA; the principal amino acids are Glu, Leu, Arg, Lys, Phe and Ser. Studies on mature Cheddar cheese have shown that Glu is important for Cheddar cheese flavour (Fox *et al.*, 1994). FAA accumulate in Parmigiano-Reggiano until 15 months of ripening, after which their concentration remains relatively constant (*ca.* 230 mg g⁻¹) (Resmini *et al.*, 1988). This elevated concentration of amino acids contributes to the characteristic flavour of Parmigiano-Reggiano. Glu, Pro, Val, Leu and Lys are the amino acids present at high concentrations in several hard varieties such as Parmigiano-Reggiano, Pecorino Romano, Canestrato Pugliese, Fossa, Mahón and Manchego cheeses (Ordóñez *et al.*, 1980; Frau *et al.*, 1997; Di Cagno *et al.*, 2003a). Many amino acids have characteristic flavours (McSweeney *et al.*, 1997); although none has a cheese-like flavour, it is believed that they contribute to the savoury taste of mature cheese. However, the principal role of amino acids in flavour development is as precursors of volatile compounds produced through their catabolism.

6.4.4 Catabolism of amino acids

Catabolism of amino acids plays a major role in flavour development during cheese ripening (McSweeney and Sousa, 2000). Although much more work has to be done to elucidate the biochemistry of the catabolism of amino acids, two major pathways are well recognized. The first series of reactions (Fig. 6.3) is initiated by the activity of an aminotransferase that transfers the amino group from an amino acid to an α -keto acid (usually α -ketoglutaric acid) and results in the production of new α -keto acid and amino acid (usually Glu). α -Keto acids produced by the transamination of aromatic and branched-chain amino acids and Met are further degraded to other compounds (e.g., aldehydes, alcohols) by enzyme-catalysed or chemical reactions. The second major series of reactions (Fig. 6.4) by which amino acids are catabolized is initiated by the activity of amino acid lyases that cleave the side chains of amino acids. These pathways are particularly important for the catabolism of aromatic amino acids and Met. Other pathways by which amino acids may be catabolized include the

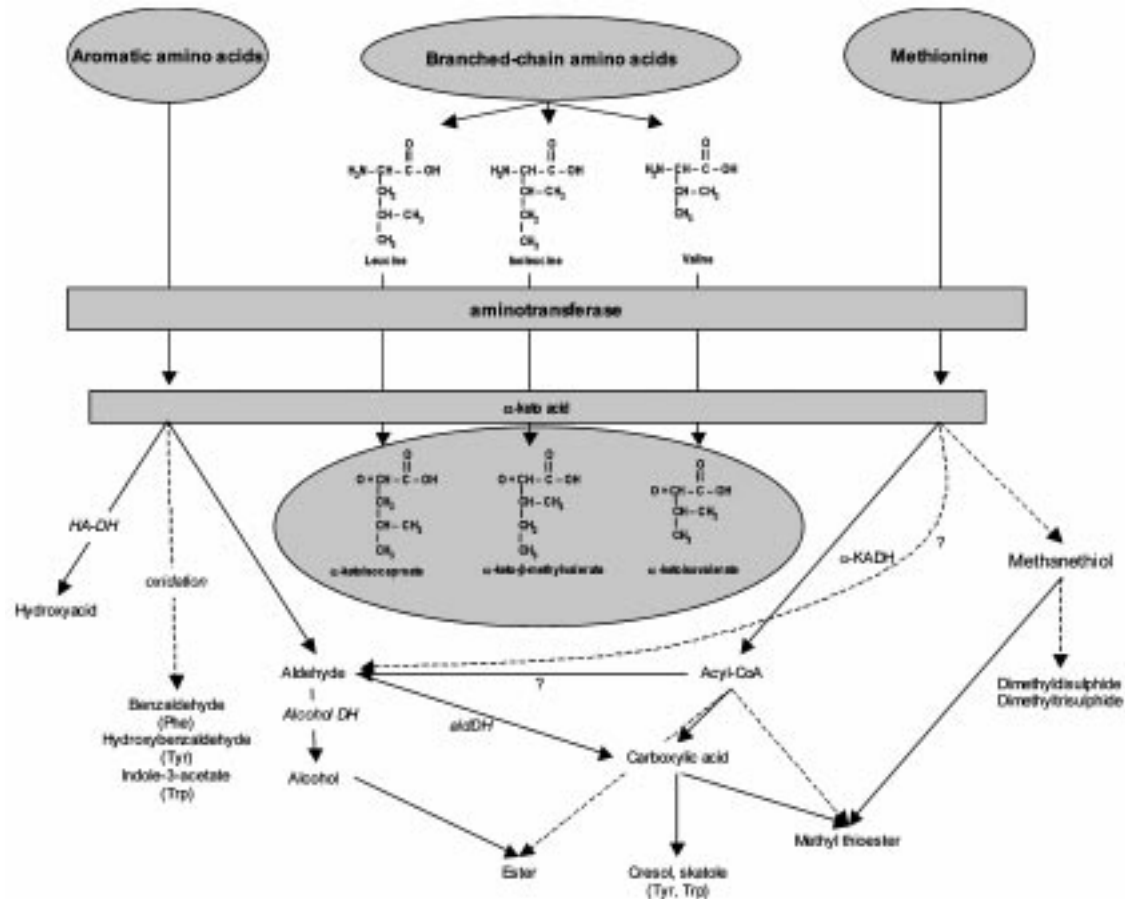


Fig. 6.3 Pathway for amino acid catabolism as started by aminotransferase reaction. HA-DH: hydroxyacid dehydrogenase; α -KADH: α -keto acid dehydrogenase; aldDH: aldehyde dehydrogenase; alcohol DH: alcohol dehydrogenase.

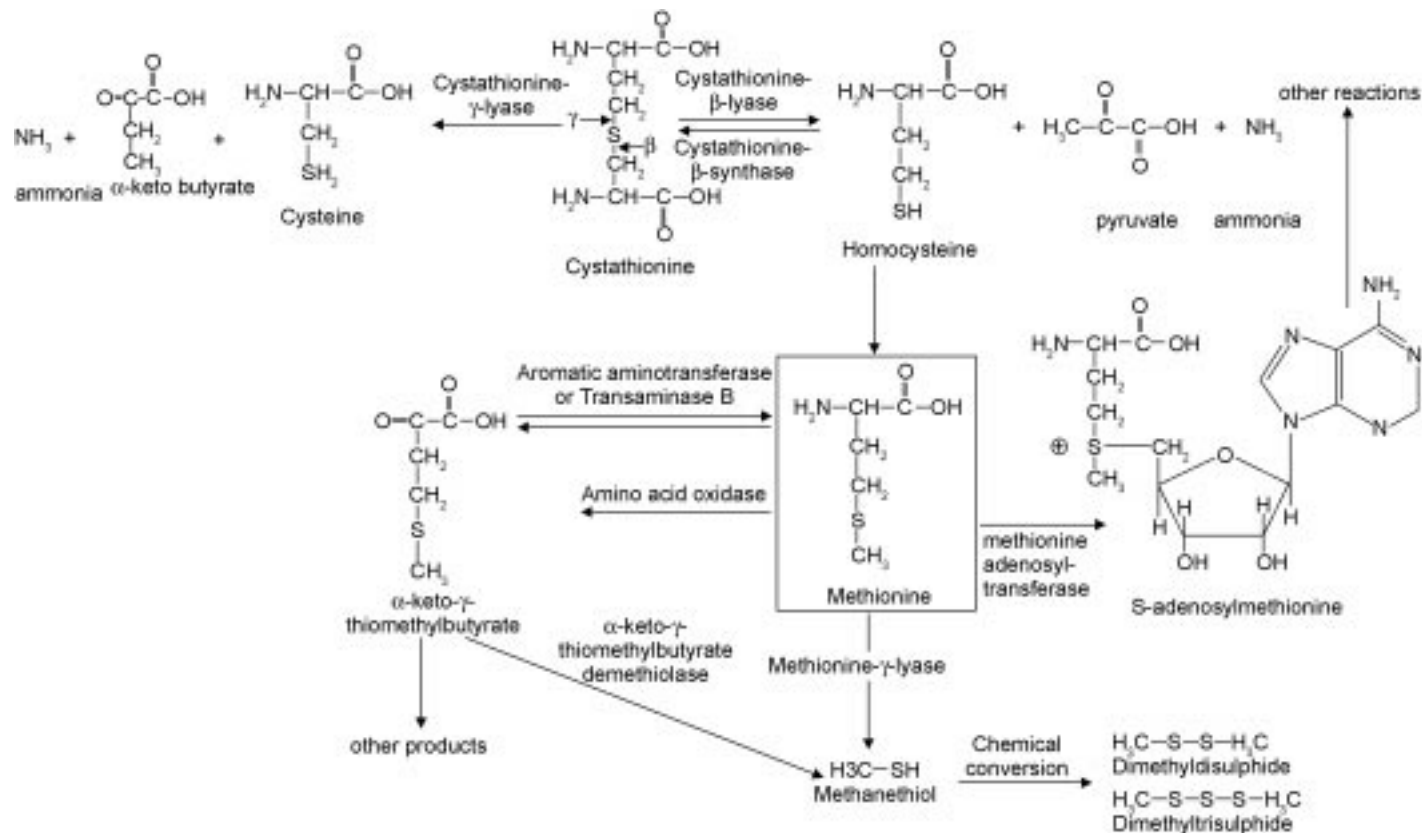


Fig. 6.4 Pathway for amino acid catabolism as started by elimination reaction.

production of amines by decarboxylases and the production of NH_3 by deaminases. There are also specific pathways for the metabolism of Thr, Asp, Glu and Arg. In the context of cheese-related microorganisms, most of the studies have concerned the incubation of resting cells or cellular extracts in cheese models or in synthetic media containing casein or free amino acids. Few reports have shown the *in situ* catabolism of amino acids. Lactic acid bacteria, coryneform bacteria, propionic bacteria, moulds, yeasts and *G. candidum* seem to be capable of producing flavour compounds from amino acids, but this capacity is highly strain dependent (Ganesan *et al.*, 2004a; Seefeldt and Weimer, 2000; Yvon and Rijnen, 2001).

Aminotransferases of lactic acid bacteria (Ganesan and Weimer, 2004; Yvon and Rijnen, 2001), *B. linens* (Ganesan *et al.*, 2004b) and *P. freudenreichii* (Thierry *et al.*, 2002) have been studied. Two branched-chain aminotransferases, one from *Lc. lactis* subsp. *lactis* NCDO763 (Yvon *et al.*, 2000), with activities on Ile, Val and Met, and one from *Lc. lactis* subsp. *lactis* LM0230 (Atilas *et al.*, 2000) and *Lc. lactis* subsp. *cremoris* B78 (Engels, 1997) with activities on Ile, Leu, Val, Met and Phe have been characterized. These enzymes tolerate cheese-ripening conditions. A third aminotransferase with activities on Leu has been described in *Lc. lactis* (Rijnen *et al.*, 2000). Transamination has been found in *Lb. helveticus* also (Klein *et al.*, 2001). It seems that transamination is the first and the main step in the conversion of FAA by *Lb. helveticus*, with Phe and Tyr being converted most efficiently. Aminotransferase activity has been shown in NSLAB strains (Williams *et al.*, 2002). *Lb. paracasei* subsp. *paracasei* strains generate aldehydes, alcohols and acids from branched-chain amino acids, Phe and Met when grown in media containing casamino acids or lactalbumin hydrolysate (Tammam *et al.*, 2000). It is clear that many bacteria found in cheese are capable of amino acid transamination but it is uncertain what role these reactions play in cheese flavour development. Some FFA found in ripening cheese cannot be found in milkfat, but Ganesan *et al.* (2004a) found these FFA to be produced during the metabolism of starter and adjunct cultures on amino acids. Some authors (Yvon *et al.*, 1998) concluded that aminotransferase activity does not play a major role, but that subsequent steps are limiting in the formation of flavour compounds. On the contrary, other reports (Banks *et al.*, 2001; Ur-Rehman and Fox, 2002) have shown that supplementation of Cheddar cheese with α -ketoglutaric acid caused statistically significant effects on the production of certain volatile flavour compounds.

Volatile sulphur compounds are found in most cheeses and are important components of flavour (Fox and McSweeney, 1996b). Since Met is present in the caseins at a higher concentration than Cys, sulphur compounds in cheese presumably originate mainly from Met. The major aroma compounds produced from Met are methional, methanethiol and its oxidation products, dimethylsulphide and dimethyltrisulphide. Methionine- γ -lyase catalyses the conversion of methionine to α -ketobutyrate, methanethiol and ammonia. Cystathionine- β -lyase and cystathionine- γ -lyase catalyse the conversion of cystathionine to homocysteine, pyruvate and ammonia, and to cysteine, ammonia and

α -ketobutyrate, respectively. Homocysteine is, in turn, converted to Met by the activity of homocysteine methyltransferase. Several primary starters such as *Lc. lactis* subsp. *lactis* and subsp. *cremoris*, *Lb. helveticus* and *Lb. delbrueckii* subsp. *bulgaricus* are capable of degrading Met to methanethiol, dimethylsulphide and dimethyltrisulphide (Dias and Weimer, 1998a; Imhof *et al.*, 1995; Law and Sharpe, 1978), but this ability is strain dependent (Dias and Weimer, 1998a; Seefeldt and Weimer, 2000; Weimer *et al.*, 1999). In general, lactococci are auxotrophic for methionine while lactobacilli are auxotrophic for both cysteine and methionine. In addition, it seems that lactococci possess greater cystathionine lyase activity than lactobacilli. The production of total volatile sulphur compounds was found to be four times higher in slurries acidified by *Lc. lactis* subsp. *cremoris* S3 than in those chemically acidified with gluconic acid- δ -lactone. The cystathionine- β -lyase of *Lc. lactis* subsp. *cremoris* B78 is reported to be active under the conditions of pH and NaCl of Gouda cheese during ripening (Alting *et al.*, 1995) and lysis of cells is required for full activity. Nevertheless, some authors (Dias and Weimer, 1998a) suggest that the cystathionine lyases from lysed lactococci make an insignificant contribution to the production of volatile sulphur compounds from methionine in cheese during ripening.

The ability of NSLAB strains of *Lb. casei* and *Lb. plantarum* to produce flavour compounds has been investigated; some strains are capable of transaminating methionine to α -keto- γ -methylthiobutyrate but methionine lyase or amino acid decarboxylase activities have not been detected (Amarita *et al.*, 2001). Moreover, *Lb. fermentum* and *Lb. reuteri* produce aroma compounds from sulphur amino acids but *Lb. brevis*, *Lb. paracasei* and *Lb. curvatus* do not (De Angelis *et al.*, 2002). *Lb. fermentum* DT41 was isolated from the natural starter for Parmigiano-Reggiano cheese (Smacchi and Gobetti, 1998). It contains a cystathionine- γ -lyase which retains activity under cheese-ripening conditions. The same enzyme has been purified from *Lb. reuteri* DSM20016 (De Angelis *et al.*, 2002). This microorganism, together with other lactobacilli, has been used as an adjunct in the manufacture of Canestrato Pugliese-type cheese, and cheeses containing an adjunct composed of *Lb. fermentum* DT41 and *Lb. reuteri* DSM20016 had the highest levels of methanethiol, dimethylsulphide, dimethyldisulphide and dimethyltrisulphide.

Coryneform bacteria, and especially *B. linens*, are much better producers of methanethiol and dimethylsulphide than lactic acid bacteria (Dias and Weimer, 1998a). The methionine- γ -lyase of *B. linens* BL2 is active under cheese ripening conditions (Dias and Weimer, 1998b). Methanethiol is a precursor of other volatile sulphur compounds which contribute to the garlic flavour of smear-ripened cheese (Hemme *et al.*, 1982). Moreover, these bacteria are capable of producing S-methylthioesters from methanethiol and different carboxylic acids such as acetic, propionic, isobutyric or isovaleric acids (Bloes-Breton and Bergère, 1997). *B. linens* GC71 is capable of esterifying acetic, propionic and methyl branched-chain acids with methanethiol to produce thioesters (Lambert *et al.*, 1997). S-methylthioesters are important flavour compounds in surface-

ripened cheese. Their specific flavours depend on their chain length and configuration (Weimer *et al.*, 1999). *G. candidum* may produce sulphur-containing flavour compounds; dimethyldisulphide is the main compound but also methanethiol and dimethyltrisulphide are produced (Berger *et al.*, 1999). Also *G. candidum* may play an important role in development of flavour in smear-ripened cheeses. Yeasts, micrococci and *B. linens* are capable of cleaving the side chain of tyrosine and tryptophan, releasing phenol and indole, respectively (Jollivet *et al.*, 1992; Parliament *et al.*, 1982). Such activities have never been detected in any lactic acid bacteria (Gummalla and Broadbent, 1999) and *B. linens* (Ummadi and Weimer, 2001). The enzymes involved in this β -elimination of tyrosine and tryptophan are tyrosine-phenol lyase and tryptophan-indole lyase, respectively. They produce, in addition to phenol and indole, ammonia and pyruvate.

6.5 Lipolysis

Ripening of most cheese varieties (e.g., Edam, Swiss and Cheddar) is accompanied by a low level of lipolysis (*ca.* 200–1000 mg of free fatty acids kg^{-1}) but extensive lipolysis occurs in Blue and some hard Italian cheese varieties. Lipids play an important role in cheese flavour by acting as a source of free fatty acids (FFA) which in turn may be catabolized to other flavour compounds (e.g., methylketones) and by acting as a solvent for sapid compounds produced from lipids or other precursors.

Lipolytic enzymes may be classified as esterases or lipases, which are distinguished according to three main characteristics: length of the hydrolysed acyl ester chain, physico-chemical nature of the substrate (whether emulsified or not) and enzymatic kinetics. Lipases/esterases in cheese originate from six possible sources: milk, rennet paste, primary starter bacteria, secondary starter microorganisms, NSLAB and exogenous lipase preparations. Milk contains an indigenous lipoprotein lipase that is more important in cheeses made from raw milk than in that made from pasteurized milk since the enzyme is inactivated by pasteurization. Rennet paste contains a potent lipase, pregastric esterase, which is responsible for lipolysis in Italian Provolone and Pecorino cheeses varieties. With the exception of moulds, the lipolytic activities of cheese-related microorganisms are markedly lower compared with the other potential enzyme sources.

Lactic acid bacteria possess intracellular esterolytic/lipolytic enzymes capable of hydrolysing a range of derivatives of FFA. Lipases and esterases of lactic acid bacteria seems to be the principal lipolytic agents in Cheddar and Dutch-type cheeses made from pasteurized milk (Fox *et al.*, 2000). Evidence for this comes from studies on aseptic starter-free cheeses acidified with gluconic acid- δ -lactone, where very low levels of FFA are released during ripening (Reiter *et al.*, 1967), and from the relationship between autolysis of primary starter cells and FFA levels during ripening (Collins *et al.*, 2003). Esterases have been purified from primary starters such as *Lc. lactis* subsp. *lactis* (Chich *et al.*,

1997) and *Str. thermophilus* (Liu *et al.*, 2001), and intracellular esterolytic activities were found in *Lb. helveticus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (El-Soda *et al.*, 1986). The major tributyrin esterase of *Lc. lactis* subsp. *lactis* has been cloned, over-expressed and characterized (Fernandez *et al.*, 2000). The purified enzyme showed a preference for short-chain acyl esters and also phospholipids. The presence of lipase and esterase activities has been shown in NSLAB also (Khalid and Marth, 1990). In the majority of strains, activities increased as the carbon chain length of the fatty acid decreased. Intracellular lipases and esterases have been purified and characterized from *Lb. plantarum* (Gobbetti *et al.*, 1996, 1997a), *Lb. casei* (Castillo *et al.*, 1999) and *Lb. fermentum* (Gobbetti *et al.*, 1997b). The response of esterase activity to the effects of salt, temperature and pH is strain-dependent (Gobbetti *et al.*, 1999a). The major beneficial effect of enterococci in cheese-making has been attributed to the hydrolysis of milk fat by esterases (Tsakalidou *et al.*, 1993; Sarantinopoulos *et al.*, 2001). Multiple esterase activities were present and whereas *Enterococcus faecalis* strains were the most lipolytic, *E. faecium* strains were more esterolytic. Despite *Lactococcus* spp. and *Lactobacillus* spp. being weakly lipolytic in comparison to species such as *Pseudomonas* and *Flavobacterium*, because they are present at high numbers over an extended ripening period, lactic acid bacteria are responsible for the liberation of significant levels of FFA in many cheese varieties which do not have strongly lipolytic enzymes and/or a secondary microflora. However, FFA and volatile sulphur compounds also arise from amino acid metabolism by the starter and adjunct cultures (Dias and Weimer, 1998b, 1999; Ganesan *et al.*, 2004a, b; Ganesan and Weimer, 2004).

Propionibacteria used as secondary starters are well known for their lipolytic activity and have 10–100 times more activity than lactic acid bacteria (Dupuis *et al.*, 1993). *In vitro* studies as well as data from experimental cheeses have shown that propionic acid bacteria release FFA in cheese (Chamba and Perreard, 2002). The lipase activity of *P. freudenreichii* subsp. *shermanii* contributes to the low level of lipolysis in Swiss cheeses. Among secondary starters and/or adventitious microorganisms related to smear-ripened cheeses, *Y. lipolytica* (Freitas *et al.*, 1999), *B. linens* (Sørhaug and Ordal, 1974) and staphylococci (Curtin *et al.*, 2002) were found to possess a considerable esterase/lipase activity.

Microbial lipolysis is very extensive in mould-ripened cheeses, especially blue-veined cheeses (ca. 30,000 mg of FFA kg⁻¹). The main agents are *Penicillium* spp. used as secondary starters (Fig. 6.5). *P. roqueforti* and *P. camemberti* synthesize potent extracellular lipases which contribute to the release of FFA during ripening of Blue and white mould-ripened cheese varieties, respectively. In cheese, FFA are also precursors of many important flavour compounds such as methylketones, lactones, esters, alkanes and secondary alcohols. Methylketones (heptan-2-one and nonan-2-one) are the most important flavour components in Blue cheese generated by the activity of *P. roqueforti* (Urbach, 1997). *P. camemberti* and *G. candidum* may also produce methylketones (Cerning *et al.*, 1987; Molimard and Spinnler, 1996). Metabolism of fatty acids by *Penicillium* spp. involves four main steps corresponding to the early stages of

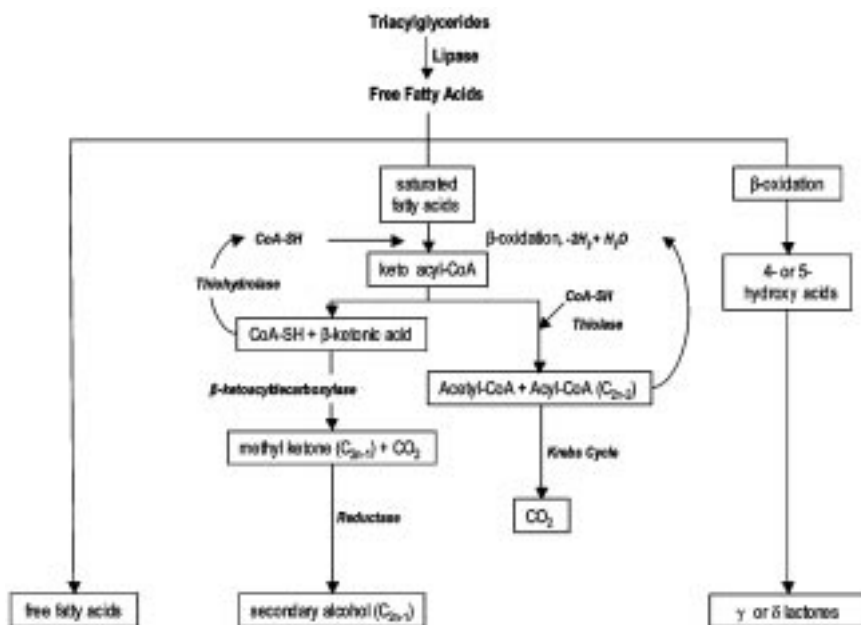


Fig. 6.5 Catabolisms of free fatty acids by *Penicillium* spp. used as secondary starter in blue-veined and white mould-ripened cheeses.

β -oxidation. Initially, FFA are released by lipases, followed by the oxidation of FFA to β -ketoacids, and decarboxylation to alkan-2-ones, of one less carbon atom than parent FFA; alkan-2-ones may be reduced to the corresponding secondary alcohol (alkan-2-ol). *P. roqueforti* is responsible for the reduction of methylketones to secondary alcohols (e.g., 2-pentanol, 2-heptanol, 2-nonanol) in Blue cheeses (Martelli 1989; Engels *et al.*, 1997).

6.6 Flavour improvement

Consumer demand for variations in the flavour of cheeses and the desire to improve the flavour of cheeses made of pasteurized milk is creating the interest for new strains and emerging technologies to drive the development of cultures with the appropriate characteristics. The easiest route is concerning the supplementation of conventional starters with bacteria found in high quality aged cheeses (Weimer *et al.*, 1999).

The use of attenuated or adjunct starter cultures seems to be promising. Attenuated starter cultures of the *Lactococcus* and *Lactobacillus* genera, in parallel with primary starter cultures increased proteolysis and lipolysis, decreased ripening time and improved flavour (Klein and Lortal, 1999). A comparison between wild lactococci and those of industrial cultures has shown that wild strains generally produce specific flavours quite distinct from those

produced by industrial strains (Ayad *et al.*, 1999). Wild-type strains produce high levels of primary alcohols and branched aldehydes as the result of the catabolism of amino acids. This correlates well with the low requirements of amino acids for growth and, probably, with the more active amino acid convertase enzymes in wild lactococci. If the high versatility of heterofermentative lactobacilli makes their contribution to cheese ripening uncertain (Fox *et al.*, 1993), careful metabolic selection may permit their use as adjunct starter cultures. Adjunct starter cultures may eliminate defects by the adventitious NSLAB, since they inhibit their outgrowth (Wouters *et al.*, 2002) and, in particular, strains isolated from raw milk have been shown to improve the cheese flavour (McSweeney *et al.*, 1994). Good descriptions of the screening process to select adjunct starter cultures as well as new systematic approaches for selecting the right combination of starter and adjunct are reported (Crow *et al.*, 2001; Beresford *et al.*, 2001), and Dairy Research Centres or commercial starter companies are increasing the portfolios of heterofermentative lactobacilli usable as cheese adjunct starter cultures.

Perhaps the most sophisticated technique for controlling and improving upon flavour development is the metabolic engineering of essential pathways leading to flavour generation. Increasing the release, rather than the activities, of the relevant enzymes for cheese flavour holds most promise (Hugenholtz and Kleerebezem, 1999). The nisin-controlled expression of a bacteriophage lysine and holing was manipulated in *Lc. lactis* (de Ruyter *et al.*, 1997). It results in complete cell lysis, a concomitant sharp increase in the release of intracellular peptidases and amino acid converting enzymes, and heightens production of flavour components in cheese.

6.7 Future trends

Proteolysis and subsequent amino acid catabolism appear to give the greatest intensity to cheese flavour and lactic acid bacteria or secondary microorganisms possess a highly intricate proteolytic machinery dedicated to the synthesis of key odorants. The continued study of the specificity and mode of action of the principal enzyme pathways will enable scientists to select strains for producing the most desirable and cost-effective cheeses with required flavour. This will be achieved only through the collaborative efforts of multidisciplinary groups comprised of microbiologists, food technologists, food chemists, flavour chemists and sensory specialists that precisely dissect and develop the critical formulation required for flavour optimization.

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Starter culture development for improved cheese flavour

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7.1 Introduction to starter cultures

Starter cultures are species of lactic acid bacteria (LAB) that are, in most cases, deliberately added to milk where their primary role is to initiate or start the production of lactic acid for the cheese manufacturing process. Historically, starter cultures consisted of undefined mixtures of LAB that either were allowed to grow naturally in milk or were added to milk from a previous product batch. However, with increasing industrial-scale cheese production there was a requirement for more reproducible starter performance and freedom from undesirable organisms. As a result starter cultures are now supplied by specialist starter production organisations or companies either as commercial undefined mixed strain cultures that have been selected from the original natural cultures or as defined mixtures of pure characterised strains. There are a number of lactic acid bacteria species that are used as starter cultures with the specific organism usually dependent on the cheese variety. The principal starter culture species used in cheese manufacture are *Lactococcus lactis* subsp. *cremoris*, *Lc. lactis* subsp. *lactis*, *Lactobacillus delbrueckii* subsp. *lactis*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. helveticus*, *Streptococcus thermophilus* and *Leuconostoc* species.

In this chapter the primary focus is to examine the role of starter cultures in the development of cheese flavour and aroma as well as an examination of factors that influence the production of flavour and aroma compounds by starter cultures. In many cases further details of metabolic pathways and of flavour compounds are found in other chapters.

7.1.1 Principal functions of starter cultures

The production of lactic acid at a controlled and consistent rate is the most important factor in the control of cheese quality, with the consequent decrease in pH influencing many aspects of the cheese manufacturing process (Lawrence *et al.*, 1984). Acid production determines the basic cheese structure through its effect on coagulant activity, the rate of whey expulsion and the extent to which the colloidal calcium phosphate present in the casein micelles dissolves. Lactic acid produced by starter cultures also contributes to the inhibition of acid-sensitive pathogenic organisms and spoilage organism growth in cheese, in addition to contributing to the depression of water activity and acting as a substrate for secondary cultures or non-starter lactic acid bacteria (NSLAB) in the formation of various flavour compounds and CO₂.

Starter cultures also have a number of secondary functions, with one of the more significant being their contribution, both directly and indirectly, to the production of flavour and aroma compounds during cheese maturation. Starter cultures can directly affect cheese flavour and aroma through the degradation of lactose, citrate, milk proteins and to a limited extent milk fat, as well as having an indirect effect in establishing the environmental conditions that influence the activity of other secondary cultures, adventitious NSLAB and adjunct organisms. However, it is important to appreciate that with cheese maturation, starter cultures are only one of a number of ripening agents that include the coagulant, natural milk enzymes, secondary cultures and adventitious NSLAB which contribute to overall flavour development.

7.1.2 Starter cultures used in cheese manufacture

In modern cheesemaking the majority of starter culture strains used commercially belong to the genera *Lactococcus*, *Streptococcus*, *Leuconostoc* and *Lactobacillus*, although *Enterococcus* strains normally found in raw milk cheese are also increasingly being used in defined starter cultures. Within each genus there are a number of species or subspecies that are used specifically for cheesemaking, while species and subspecies can in turn be grouped into specific strains traditionally based on the type and range of metabolic end products, enzyme activities and bacteriophage (phage) sensitivity. In more recent times, though, the differentiation of strains has been made easier by the introduction of molecular techniques such as Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE), Pulsed Field Gel Electrophoresis (PFGE) and Randomly Amplified Polymorphic DNA (RAPD) (Marilley and Casey, 2004).

In Europe many of the starter cultures for large-scale cheese manufacture are produced as undefined mixtures of different strains and/or species which have been carefully selected from the natural starter cultures that were present in milk. These cultures have been preserved and propagated under controlled laboratory conditions by commercial starter companies and are supplied to the cheese manufacturing plant in a frozen or freeze-dried form. These undefined mixed cultures can be classified into either a mesophilic group with an optimum

growth temperature of around 30°C or a thermophilic group with an optimum temperature of around 42°C. The mesophilic group are dominated by strains of *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*, while thermophilic cultures usually contain *Sc. thermophilus* alone or in combination with thermophilic lactobacilli such as *Lb. delbrueckii* subsp. *lactis*, *Lb. delbrueckii* subsp. *bulgaricus* or *Lb. helveticus*. The mesophilic group can also be divided into a further two groups on the basis of their ability to ferment citrate, with the citrate-positive cultures containing *Leuconostoc mesenteroides* subsp. *cremoris* and/or citrate-positive *Lc. lactis* subsp. *lactis* together with the traditional acid producing strains of *Lc. lactis* subsp. *lactis* or *Lc. lactis* subsp. *cremoris*.

Citrate-negative mesophilic starter cultures are used in cheeses such as Cheddar or Feta where their prime requirement is to produce acid, whereas in Dutch-type cheeses or some mould-ripened cheeses citrate-positive mesophilic starter cultures are used for their role in flavour and eye formation. Thermophilic starter cultures are typically used in the production of Italian and Swiss-type cheeses.

In many countries outside Europe such as Australia, New Zealand, the USA and Ireland, the tendency is to use a small number of defined-strain starter cultures in which the strains and species in the starter culture mix are known. This enables the manufacture of a very consistent product, an important characteristic in large-scale, continuously operated cheese plants. However, in this system there is usually an ongoing requirement for introduction of new phage-resistant starter culture strains, although in recent years strategies based on improved factory design, aseptic propagation of starter cultures and better selection of starter strains have reduced the impact of phage infections (Heap and Harnett, 2003).

7.1.3 Role of starter cultures in cheese flavour development

As one of the principal ripening agents in cheese together with the coagulant, natural milk enzymes and adventitious non-starter bacteria, starter cultures play a significant part in the biochemical degradation of lactose, citrate, milk fat and caseins (McSweeney and Sousa, 2000). Many of the resulting degradation products have been implicated in cheese flavour, although as yet it is still not possible to attribute cheese flavour and aroma to any specific compounds. Rather it appears that cheese flavour is dependent on a mix of many compounds present in the correct ratios and concentrations.

Carbohydrate metabolism

Details of lactose and citric acid metabolism can be found in reviews by Monnet *et al.* (1996), Coccagn-Bousquet *et al.* (1996) and McSweeney and Sousa (2000), as well as in Chapter 1. As outlined previously, the prime function of starter cultures is to ferment lactose to lactic acid. In the case of *Lactococcus* species lactose is transported into the cell via an energy-dependent transport system that phosphorylates the lactose as it is transported across the cell membrane (Fig. 7.1).

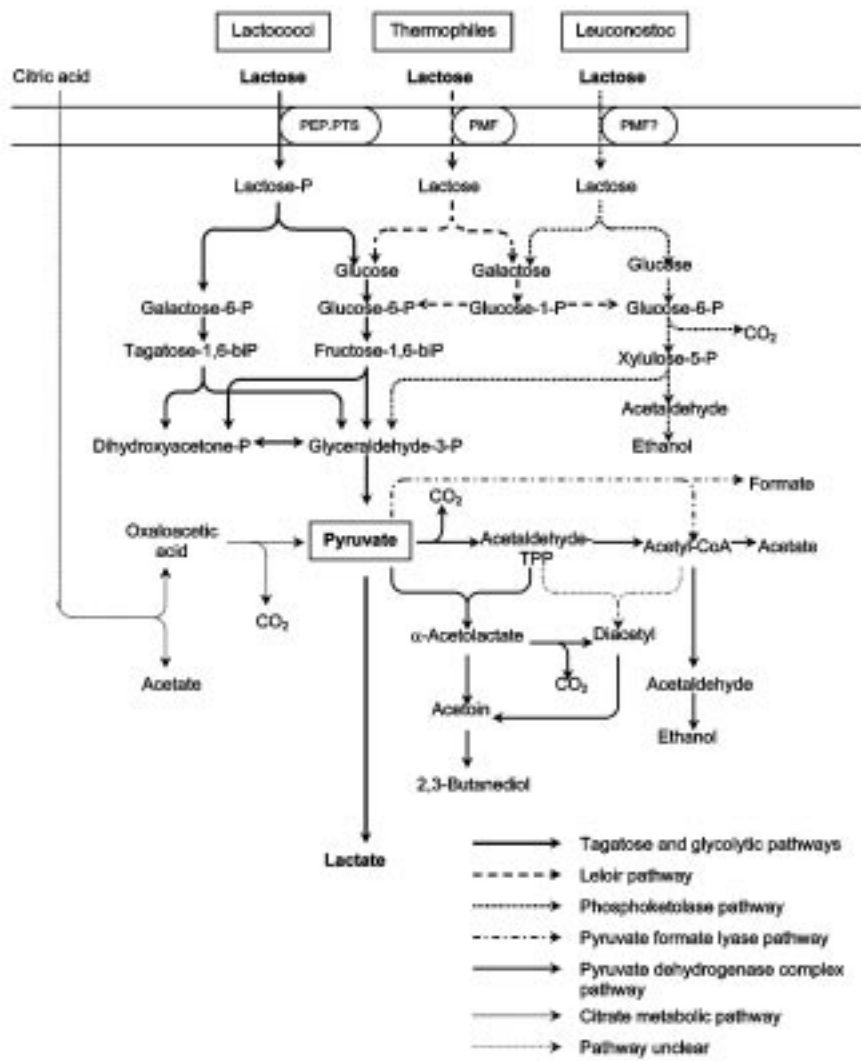


Fig. 7.1 Outline of pathways associated with carbohydrate catabolism in lactic acid bacteria. PEP:PTS, phosphoenol pyruvate phosphotransferase system; PMF, proton motive force. Adapted from Monnet *et al.* (1996) and Broome *et al.* (2003).

The phosphorylated lactose is first hydrolysed to glucose and galactose-6-phosphate by a phospho- β -galactosidase before they are subsequently metabolized via the glycolytic and tagatose pathways respectively to pyruvate, the vast majority of which is converted to lactic acid by lactate dehydrogenase. The other starter cultures, including thermophilic starters and *Leuconostoc*, transport lactose into the cell via a specific permease in which the lactose remains unmodified, whereupon it is hydrolysed by β -galactosidase into glucose and galactose. In thermophiles, glucose is metabolised by the glycolytic pathway

while the galactose is either excreted, such as in the case of strains of *Sc. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. delbrueckii* subsp. *lactis*, or metabolised through the Leloir pathway. In *Leuconostoc*, glucose is metabolised to carbon dioxide, ethanol and lactic acid via the phosphoketolase pathway while the galactose is converted to lactic acid via the Leloir pathway. Under certain conditions lactococci, rather than forming lactic acid from pyruvate, are also able to convert it to a number of other potential flavour compounds including formate, acetate, acetaldehyde, ethanol, acetoin, diacetyl and 2,3-butanediol.

Citric acid metabolism occurs in citrate-fermenting strains of *Lc. lactis* subsp. *lactis* where it is converted to acetate, carbon dioxide and pyruvate, with the pyruvate further metabolised to acetate, diacetyl, acetoin, 2,3-butanediol and carbon dioxide. In *Leuconostoc* the pyruvate formed from citrate is converted to lactate, although at low pH and in the absence of glucose or lactose *Leuconostoc* will also produce diacetyl and acetoin.

Lipolysis

The hydrolysis of milk fat triacylglycerides to free fatty acids and glycerol, monoacylglycerides or diacylglycerides is a critical process in the development of cheese flavour (McSweeney and Sousa, 2000). The free fatty acids can contribute directly to cheese flavour or react with alcohol or free sulphhydryl groups to form esters and thioesters. They can also act as precursors for a range of other flavour compounds via β -oxidation and decarboxylation to form flavour compounds such as lactones, methyl ketones and secondary alcohols. The lipase/esterase systems of a number of starter bacteria have been studied but generally they do not possess high lipolytic activity and their role in forming flavour compounds from milk fat is not clear. Free fatty acids can also be produced by an interconversion from amino acids to form various compounds including acetic, isobutyric, isovaleric and propionic acids (Ganesan and Weimer, 2004; Ganesan *et al.*, 2004, 2006).

Proteolysis and amino acid degradation

The major process associated with cheese flavour development is the degradation of milk proteins and as a result there have been numerous studies into the proteolytic system of starter cultures and the further catabolism of the resulting amino acids (Yvon and Rijnen, 2001; Sousa *et al.*, 2001). In order to grow in milk, starter cultures require a well-developed proteolytic system which in lactococci consists of a cell-envelope associated proteinase (CEP) that hydrolyses caseins to oligopeptides (4–10 amino acid residues), an oligopeptide transport system, various di/tripeptide transport systems for hydrophilic and hydrophobic substrates and at least 10 amino acid transport systems (Fig. 7.2). On reaching the cytoplasm the peptides are degraded by an array of peptidases with both broad and narrow specificities to amino acids, which are utilised for synthesis of the starter cultures' own protein. During cheese maturation much of the initial degradation of milk proteins is performed by the coagulant, and to a

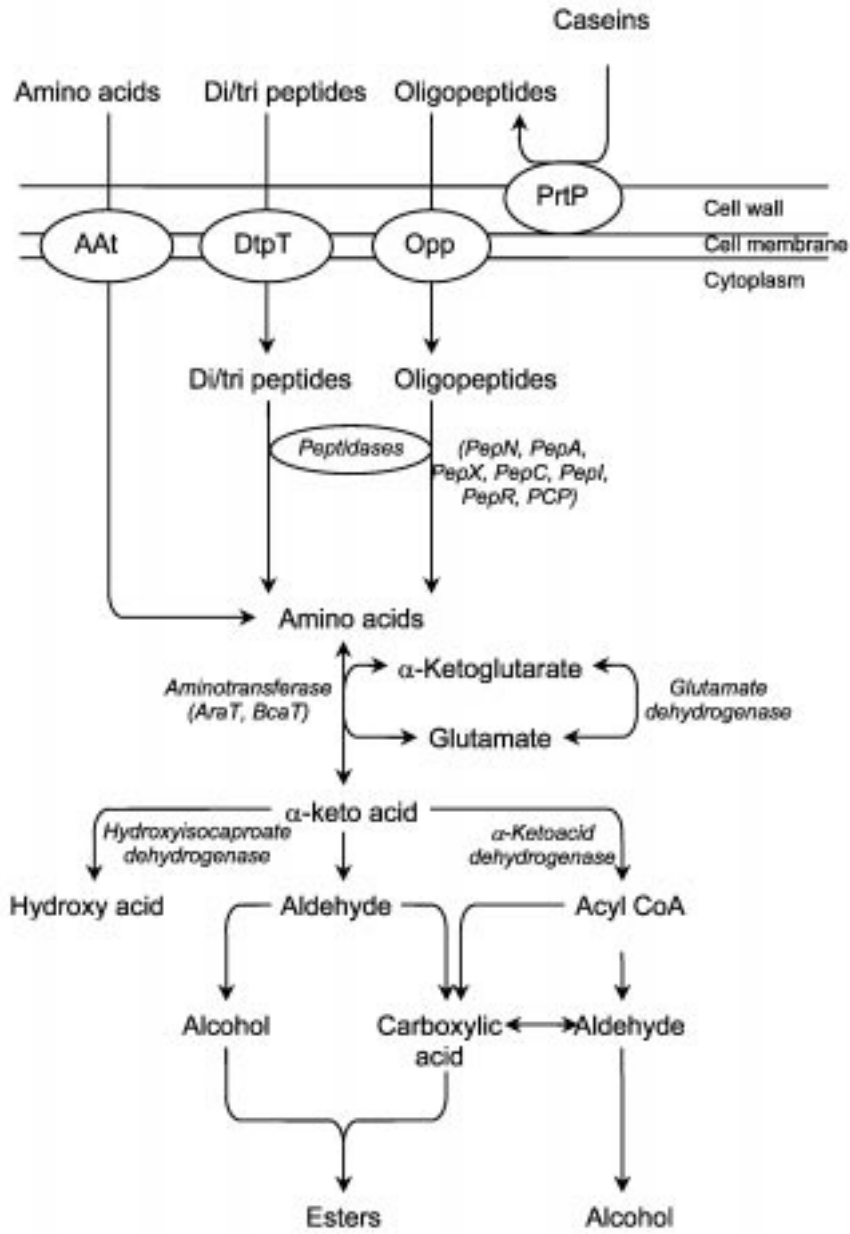


Fig. 7.2 Outline of pathways associated with casein catabolism and flavour compound formation in lactic acid bacteria. PrtP, cell wall-bound extracellular proteinase; Opp, oligopeptide transport system; DtpT, di/tripeptide transporter; Aat, amino acid transport system. Adapted from Monnet *et al.* (1996) and Smit *et al.* (2002).

lesser extent the plasmin, to form large and intermediate-sized peptides which are subsequently degraded by enzymes from starter cultures and non-starter lactic acid bacteria to small peptides and amino acids. In cheese, the non-growing starter culture cells then catabolise the small peptides and amino acids to a range of important aroma and flavour compounds, the majority of which are the result of the enzymatic breakdown of key aromatic (phenylalanine), branched chain (leucine) and sulphurous (methionine and cysteine) amino acids as well as serine and threonine.

The first step in amino acid catabolism is generally a transamination reaction catalysed by an aromatic or branched chain aminotransferase that transfers the α -amino group of the amino acid to a keto acid acceptor, generally α -ketoglutarate (Fig. 7.2). One of the limiting factors of the transamination reaction in cheese is the supply of α -ketoglutarate. This could be overcome by the addition of α -ketoglutarate to cheese or by the use of starter strains which have glutamate dehydrogenase activity and are able to form α -ketoglutarate from glutamate, an abundant amino acid present in cheese. The α -keto acids resulting from transamination can then be converted into hydroxy acids, carboxylic acids, aldehydes, alcohols and esters by a variety of enzymes. Interestingly, the deletion of a single aminotransferase gene in *Lc. lactis* subsp. *lactis* alters the type of fatty acids produced, but does not decrease the total concentration of fatty acid formed, thereby demonstrating that genetic redundancy in lactococci for amino acid metabolism will have an impact on cheese flavour compounds (Ganesan and Weimer, 2004).

7.2 Factors affecting flavour formation by starter cultures

7.2.1 Starter culture preparation

The production of starter cultures is generally undertaken by large specialist companies or institutions producing a diverse range of frozen or freeze-dried concentrated starter cultures that can be used to directly inoculate the cheese vat or for the preparation of fresh bulk starter culture on site at the cheese plant. In the bulk set system growth vessels of up to 10,000 litres are inoculated with starter cultures that are allowed to grow, usually with pH control of varying degrees of sophistication, before delivery to the cheese vat. Starter cultures prepared for the direct inoculation of cheese vats are typically grown using various media in large fermentation tanks of 5000–15,000 litres under pH and temperature control before being concentrated by centrifugation or membrane filtration. Cryoprotectants are added and the concentrated starter cultures are then frozen and/or freeze-dried before packaging and storage. In the case of starter cultures used for inoculation of bulk set growth, internal pH control systems utilising phosphate or citrate buffering agents can also be used and the concentration step is usually omitted. Further information on the procedures used to prepare starter cultures and on the advantages and disadvantages of direct vat inoculation and bulk set starter cultures can be found in a review by Sandine (1996).

The preparation of starter culture either for direct addition to the cheese vat or for addition to a bulk starter vessel at a cheese plant can vary considerably. There is a range of potential growth media which can be composed of milk (full fat or skimmed), reconstituted powdered milk, or a range of whey-based media supplemented with various nutrients such as yeast extract, vitamins and minerals. The growth medium can be subjected to a range of sterilisation procedures ranging from heating in the growth vessel itself to various external heat treatment regimes using an ultra high temperature (UHT) process. Following inoculation the temperature of the fermentation vessel is generally held at 18–30°C for mesophiles or 37–45°C for thermophiles, while the pH, which can vary between 5.5 and 6.0 depending on the starter culture, is controlled by the automatic addition of an alkali such as NaOH, NH₄OH, KOH, gaseous ammonia or Na₂CO₃. Factors including the rate of agitation, fermenter headspace gas composition, duration of incubation, and stage of starter culture growth at harvesting are further variables in the preparation of starter cultures.

Much of the work on the effects of the various processing variables has focused on maximising cell mass, starter activity and stability under storage, with less emphasis on factors such as proteolytic activity and production of aroma/flavour compounds. Although these variables are likely to affect potential flavour pathways of starter cultures, particularly proteolytic activity which is important to initiate the flavour forming pathways from protein, there is little published work covering the effect of growth media and conditions aimed at flavour enhancement. There has been some work on the regulation of the various components of the proteolytic pathway of starter cultures by growth media under laboratory conditions. Generally, the expression of components of the proteolytic pathway is highest in media containing amino acids only, while peptides generally down-regulate expression (Kunji *et al.*, 1996). Peptide transport is moderately affected by the composition of the growth media, with expression of the di/tri transporter and the oligopeptide transport system increasing when *Lc. lactis* is grown in chemically defined media containing only amino acids as compared to complex media containing both peptides and amino acids as sources of nitrogen. It has also been suggested that expression of peptidase activity is also regulated by media composition in a similar manner to the proteinase. However, when the cells are grown in milk the regulation of the components of the proteolytic system through changes in amino acid and/or peptide levels appear to be minimal (Kunji *et al.*, 1996).

7.2.2 Bulk starter culture media

The essential requirement for media used in the preparation of bulk starter is the minimisation of phage infection. This is more critical when bulk starters are prepared on site at a cheese plant where there is an environment conducive to the build-up and development of phage. A range of growth media, similar to the media used for the preparation of starter cultures for direct inoculation, can be used in addition to media that have been formulated to minimise opportunities

for phage infection. Generally, these media are whey-based and have added phosphate or citrate in order to sequester calcium, which is required by most phage to infect starter cultures.

The procedure used to control pH in the bulk starter culture growth vessel varies considerably between cheese plants. Continuous dosing with either NaOH or NH_4OH to maintain the pH at a constant value can be used; however, another common procedure is to deliver the neutralising agent in one or two shots after allowing the growth medium to reach a pH of about 5.0. Internal pH control can also be achieved by utilising a built-in soluble neutraliser (phosphates), insoluble neutralisers (calcium carbonate, trimagnesium phosphate) or encapsulated neutraliser (sodium carbonate encapsulated in ethyl or methyl cellulose). Following growth for typically 16–20 hours the culture is then chilled rapidly to below 10°C and held for up to 48 hours before addition to the cheese vat. Again, most of the work in this area has been on establishing conditions for maximum starter culture activity, and there is little published information on the effect of bulk starter culture media and growth conditions on the flavour-developing capacity of starter cultures. It is possible that factors such as media composition, neutralising procedure, incubation pH, incubation temperature, incubation duration and duration of holding may affect the expression of the various proteolytic enzymes.

In addition to having an influence on expression of starter culture flavour enzymes, in cases where a number of strains of starter bacteria are grown together in a bulk starter vessel as mixed cultures, variations in media composition, media treatment and incubation conditions can also affect starter composition. This in turn may have a significant impact on flavour development in maturing cheese should the starters in the mixed culture vary in their proteolytic activity or their potential to lyse and release their enzymes into the cheese matrix.

7.2.3 Starter culture preservation

Starter cultures produced for the inoculation of bulk starter culture vessels or that have been concentrated for the direct inoculation of cheese vats need to be preserved and stored in a state which does not restrict their viability or activity. This can be achieved by a number of means, including chilling of liquid starter cultures, air drying, vacuum drying or spray drying, although in most cases starter viability and activity are affected. The most effective method of preservation of the starter culture is by adding a cryoprotectant, such as reconstituted skim milk plus lactose, followed by rapidly freezing to below -60°C using liquid nitrogen before storing at between -20°C and -40°C . The disadvantage of this method is the necessity to keep the starter cultures frozen, which can be a problem during shipment to the cheese plant, particularly over long distances. An alternative procedure is freeze-drying where the water is removed from frozen starter cultures by sublimation under vacuum. The dried starter cultures are then stored under an inert atmosphere, which allows them to

be transported and stored at ambient temperature, although viability and activity are improved if the freeze-dried starter cultures are stored at lower temperatures.

The effect of the preservation treatment on starter culture proteolytic activity is not well documented. It is possible that the treatment has little effect and it could be that starter strain variation in viability during storage has the major impact on flavour development in maturing cheese.

7.2.4 Starter culture activity in the cheese matrix

The ripening cheese matrix is a complex and dynamic environment consisting of protein, milk fat, minerals and water, with casein (principally the α_s -caseins) acting as the main structural component in the form of a network. Dispersed within the network are fat globules, gas bubbles and pockets of water containing dissolved salts (principally of Na^+ , Cl^- , Ca^{2+} , PO_4^{3-} , H_2PO_4^- , HPO_4^{2-} and K^+), organic acids (lactate, citrate and acetate) and protein degradation products, all of which can vary considerably in concentration throughout the cheese.

In addition to salts, organic acids and protein degradation products, other factors in the moisture phase of cheese influence microbial and enzymic activities. The pH of the moisture phase, which can range between 4.7 and 5.6 depending on the cheese variety, is critical in determining the rate of flavour compound formation, while water activity and redox potential will also have an important bearing on enzymic and chemical reaction rates.

There have been few investigations into the influence of the cheese environment on flavour compound formation by starter cultures, with most studies of the proteolytic system of starter cultures carried out under optimal *in vitro* conditions. Studies that have examined cheese environment effects have made use of a range of model systems. The more basic of these range from buffered solutions adjusted to the cheese pH and containing salt at concentrations approximating that found in the moisture phase of cheese to systems that model the cheese aqueous phase composition (Broome and Limsowtin, 2002). In one such study using a buffered salt solution it was shown that while aminopeptidase activities of lactic acid bacteria are partially inhibited by the lower pH of Cheddar cheese, the presence of salts can either further inhibit activity or partially and even fully restore activity, depending on the organism (Laan *et al.*, 1998).

Various other model systems have made use of cheese slurries prepared from aseptic processed cheese curd, unsalted curd or directly from skim milk. Although they have been shown to replicate the ripening process of natural cheeses over a short time period, difficulties in maintaining aseptic conditions and uniform media composition were experienced. Model cheese systems have also been developed, including the miniature cheese system used by Ur-Rehman *et al.* (1999) to assess the ripening properties of *Lactococcus* species and the Cheddar model used at NIZO Food Research, The Netherlands, to study the effect of cheese ripening agents on aroma formation.

7.3 Starter culture selection criteria

7.3.1 Basic selection criteria

As the primary function of starter bacteria is the rapid and sustained production of lactic acid, the fundamental selection criteria of starter cultures are based on their ability to grow and produce lactic acid in milk under cheesemaking conditions. Starter culture activity, which is a measure of its rate of acid production, can vary for a variety of reasons. A number of genes encoding lactose metabolism are commonly located on plasmids, which are extrachromosomal circular pieces of DNA that are much smaller than the bacterial chromosome. These plasmids can be unstable compared to the chromosome, and during subculture one or more plasmids can be lost along with the properties encoded on the plasmid. If eventually the proportion of cells that have lost the plasmid encoding for lactose metabolism becomes high enough, acid production rates will be affected. Other starter culture properties such as proteinase production, citrate uptake and phage resistance can also be plasmid associated, although not necessarily on the same plasmid, and so properties such as the ability to grow in milk and phage resistance may also be affected should the proportion of cells missing these plasmids become significant.

Reduced starter culture activity can also result from inhibitors present in milk, although if the starters have been assessed in a standard milk medium such as reconstituted skim milk, this type of inhibition may not be evident until the starter is used in a cheese plant. Natural milk inhibitors responsible for reduced starter culture activity can be immunoglobulins or the lactoperoxidase system, while inhibition can also be due to chemical contaminants resulting from disinfectant/sanitiser misuse or animal health treatments (Desmazeaud, 1996).

The other key factor to be taken into consideration for the selection of starter bacteria is resistance to phage. Starter cultures are very susceptible to phage, which are viruses that multiply only within the bacterial cell and can normally be controlled in the cheese plant through good factory design, aseptic propagation of starter cultures and better selection of cultures. Where several strains of starter cultures are used for the preparation of bulk starter, they should differ in their phage sensitivity, while any starter strains that are affected by phage in a cheese plant should be replaced by a resistant strain.

7.3.2 Selection for flavour characteristics

Starter cultures are one of the key agents involved in the development of cheese flavour and consequently selection of starter cultures for their ability to influence flavour is an important consideration. In the past the emphasis has been on avoiding starter cultures that contribute to bitterness; however, with a better understanding of the mechanisms involved in flavour compound formation, particularly those involved in protein degradation, cultures can now be selected on their ability to form specific enzymes or compounds. Variations in the cleavage specificity towards caseins by the cell-envelope proteinase of starter cultures can be used to indicate the potential for forming bitter peptides,

while large variations in specific peptidases such as the aminopeptidase PepN suggest differences in the debittering potential of starter cultures. Starter cultures can also be characterised on their ability to degrade amino acids and the activity of specific enzymes involved in the process (Smit *et al.*, 2002).

7.4 Improving the flavour potential of starter cultures

Although starter cultures are only part of the complex microbial and enzymic ecosystem that contributes to cheese ripening, they can have a significant influence on the direction of cheese flavour development. Initially, starter cultures are the predominant organisms present in cheese reaching numbers of up to $\sim 10^8$ – 10^9 cfu g⁻¹ within a day of manufacture. They are mainly involved with the further metabolism of lactose to lactic acid, the rate of which is determined by the salt-in-moisture level. However, during maturation there is a decline in cell numbers as the cells either undergo autolysis or revert to a viable but non-culturable state (Stuart *et al.*, 1999; Ganesan *et al.*, 2006). The rate at which the starter culture cells lyse varies considerably, and although influenced by a number of factors it is highly strain dependent. During lysis intracellular enzymes, particularly peptidases, are released into the cheese matrix and continue to have an impact on protein degradation and flavour formation.

In order to increase the impact that the starter cultures have on flavour development, a number of strategies have been adopted, mainly based on increasing proteolytic activity, increasing the rates of lysis and improving their amino acid converting activities. Much of this work has relied on *in vitro* genetic manipulation carried out under laboratory conditions. However, while this has contributed significantly to our understanding of the mechanisms involved and identification of key pathways, there is still a question over the use of genetically modified organisms in industrial cheese manufacture. Incorporation of these organisms into industrial cheese manufacture depends on whether they can consistently perform the function they were designed for under industrial conditions, their cost-effectiveness, regulatory approval and market acceptance. Until the issue of genetic manipulation of starter cultures is fully resolved, natural selection of starter culture strains for specific attributes that contribute to the improvement of flavour in cheese is still active, although laborious and time consuming.

7.4.1 Autolysis

Starter culture lysis is caused by an *N*-acetylmuramidase, which is released into the growth medium where it hydrolyses the bacterial cell wall and allows the release of intracellular enzymes into the cheese matrix. It is still unclear how autolysis is controlled in the cell, although it has been shown that the autolysin of *Lc. lactis* is degraded by the CEP; however, the extent of degradation is influenced by the CEP specificity, its amount and the location of the enzyme

(Buist *et al.*, 1998). Starter cultures that have the CEP of the PI-type caseinolytic specificity (PI) quickly degraded the autolysin, so limiting the extent to which these cells could lyse, whereas starter cultures with a CEP of the PIII or PI/PIII type caseinolytic specificities degraded the autolysin much more slowly, making these cells more susceptible to autolysis. Some strains have a natural propensity to lyse during the first few weeks of maturation and this attribute has been exploited by starter technologists.

Lysis can also be induced in a number of starter culture strains through the induction of temperate phage at the cooking temperatures of Cheddar cheese (Feirtag and McKay, 1987). There is evidence to suggest that these thermo-inducible strains tend not to produce bitterness in cheese while non-inducible strains result in bitter cheese (Lepeuple *et al.*, 1998). Increasing salt-in-moisture levels, decreasing pH and higher maturation temperatures also appear to increase the rate at which starter cultures lyse during cheese maturation.

In general a positive relationship between the extent of starter culture autolysis, the release of free amino acids in cheese and enhanced ripening has been demonstrated and consequently a measure of cell lytic ability has become an important consideration when selecting starter cultures. Lysis within the cheese matrix can be assessed through enumeration of viable cells on a selective medium or by determining the activity of intracellular marker enzymes such as lactate dehydrogenase, glucose-6-phosphate dehydrogenase and lysyl-aminopeptidase. Direct measurement of intracellular enzymes by immunological and proteomic analysis of cheese aqueous extracts can also be utilised to determine lysis of starter cultures in cheese. However, since assessment of lysis in the cheese matrix can be time consuming and costly, simpler alternative predictive tests have been used based on cheese slurries or pseudo-curd in addition to buffers containing salt and adjusted to the pH of cheese (Boutrou *et al.*, 1998). In the buffer system, starter culture cells are suspended in pH 5.0 citrate buffer containing 15 g l^{-1} of NaCl, incubated at 13°C for 30 days, and the extent of lysis is assessed from the decrease in absorbance.

Several approaches have been used to increase the lytic capacity of starter cultures. Phage have been deliberately added to cheesemilk, resulting in varying degrees of starter culture lysis early in the maturation process and an associated increase in amino acids (Crow *et al.*, 1995). Crude bacteriocin has also been added to milk; however, it had a variable affect on starter culture lysis and appeared to be dependent on the type of starter culture and the growth conditions.

In spite of the evidence suggesting that accelerated lysis of starter cultures has a beneficial affect on cheese flavour development, excessive lysis can result in problems. Many reactions that require cofactors and energy rely on intact cells and are, therefore, restricted if the proportion of lysed cells becomes too high. For example, residual lactose has been shown to be higher in cheese where there are greater levels of starter culture autolysis, which may result in flavour variability as the lactose is utilised by various non-starter lactic acid bacteria. It is also possible that a number of amino acid metabolic pathways which are

cofactor-dependent and therefore reliant on intact cells may be affected, with excessive lysis resulting in modified flavour development (Crow *et al.*, 1995). In both cases this suggests that there may be an optimum balance or ratio of lysed to intact cells, the value of which may actually vary as maturation proceeds. However, the determination of optimum ratios and the factors affecting them in the cheese is an area that requires further investigation.

Evidence is mounting suggesting that carbohydrate depletion and starvation during cheese ripening induces a non-culturable but metabolically active state in lactococci, which results in lactococci producing alternative compounds that are not found during normal growth (Stuart *et al.*, 1999; Ganesan *et al.*, 2006). It is possible that cells previously thought to be lysed or dead may actually exist in this state and therefore the importance placed on the ability of starter cultures to lyse may need to be reassessed (Ganesan *et al.*, 2006).

7.4.2 Specific enzyme activities

Much of the work involved in the selection of starter cultures on the basis of specific enzyme activities is associated with proteolysis. Determination of specific peptidase activities has been used to assess strains for their potential to form bitter peptides while in an alternative procedure for determining the debittering potential of starter cultures, strains are examined for their ability to degrade the C-terminal part of β -casein (β -cn_{193–209}). This peptide, formed by the action of rennet and starter cultures, is a primary cause of bitterness in Gouda and Cheddar cheese. In reality though, the debittering activity of starter cultures cannot be determined by one specific measurable characteristic, as ultimately their ability to degrade bitter peptides in cheese will be determined by a combination of growth conditions, enzyme activity, ability to lyse and influence of cheese ripening conditions.

As the role of amino acid degradation in flavour formation becomes clearer, starter cultures can now be characterised in terms of specific amino acid degrading enzymes (Smit *et al.*, 2002). At present the key enzymes appear to be the aminotransferases, glutamate dehydrogenase, hydroxyisocaproate dehydrogenase and isocaproic acid decarboxylase, although this list may expand as our understanding of the amino acid degrading pathways of starter cultures increases (Fig. 7.2). Even so, it is apparent that there is a large natural diversity in the activity of the amino acid converting enzymes of starter cultures, thus allowing for potential flavour control and diversification in cheeses.

The activities of enzymes associated with lipid, lactate and citrate metabolism are other factors that could be included in a screening protocol for the selection of starter cultures. Many of these assays are outlined by Marilley and Casey (2004), but as in the case of adjunct organisms (see Chapter 8) the preparation of the cell-free extracts and the measurement of activity in a large number of organisms can be time consuming.

7.4.3 Attenuated starter cultures

The rationale behind the use of attenuated starter cultures is to reduce the acid producing capability of the cells while retaining activity of enzymes associated with the production of flavour compounds. Treated starter cultures are normally used as adjunct organisms for the accelerated maturation and controlled flavour development of cheese and are added in large numbers with the traditional starter culture (see Chapter 8). In this sense attenuated starter cultures cannot strictly be considered as starter organisms, though a number of treatments used in their production will be briefly covered here. A more detailed review of various attenuation treatments applied to starter cultures and their effect on ripening has been provided by Klein and Lortal (1999).

One of the more basic attenuated starter culture systems is the use of naturally occurring and easily isolated lactose-negative mutants that are unable to contribute to acid production but still retain an active proteolytic (peptidolytic) system. Cheese manufactured using these mutants as well as starter cultures that have lost their plasmid-encoded proteinase activity have been shown to increase the level of free amino acids in cheese and to reduce the level of bitterness. Starter cultures can also be treated with lysozyme, allowing the cells to survive the cheese manufacturing process, but on encountering the high salt levels present in the cheese curd the cells lyse, releasing their enzymes into the cheese matrix.

Heat or freeze shocking of starter cultures destroys or delays the acidification ability of cells without affecting proteinase and peptidase activity to any great extent. Generally, the addition of such treated cells to cheesemilk has a positive effect, with the reduction of bitterness the most frequently reported observation. Other treatments include solvent treatment of cells as well as spray and freeze drying, all of which tends to result in increased proteolysis and cheese flavour intensity. In all cases, however, the success of attenuated starter cultures is dependent on a number of factors, including production costs, regulatory controls and the effectiveness of the attenuated starter culture in the cheese medium, and at present their use in commercial cheese manufacture is still limited.

7.4.4 Cooperation with other cheese ripening agents

Many previous investigations into flavour development in cheeses are based on the elucidation of various pathways of specific starters and NSLAB; however, it is becoming apparent that combinations of organisms with varying enzyme complements and activities are required to achieve a balanced cheese flavour. This appears to be particularly relevant in proteolysis and amino acid metabolism as demonstrated by Ayad *et al.* (2001). Using milk as a medium, an industrial *Lc. lactis* strain with high proteolytic activity but limited amino acid decarboxylating activity in combination with a non-proteolytic wild-type strain of *Lc. lactis* with high decarboxylating activity was able to form a complete pathway for the formation of a specific flavour compound, in this case 3-methyl butanal. Cooperation between starter culture strains and NSLAB with amino

acid metabolism has also been demonstrated by Kieronczyk *et al.* (2003), suggesting that the flavour potential of starter cultures can alter depending on the type or concentration of other microorganisms in cheese (see Chapter 8).

7.5 Commercial starter cultures

In most modern dairy plants starter cultures are provided either as fresh culture (bulk starter) that is usually prepared from a frozen or freeze-dried inoculum supplied by an external specialist culture supplier, or as a concentrated frozen or freeze-dried culture for direct inoculation of the cheese vat (Limsowtin *et al.*, 1996). As described in section 7.1.2 starter cultures are supplied as undefined mixed or defined single strain cultures, but in both cases the strategy has been to develop a system that is resistant to attack by phage. Major international culture supply companies such as Chr Hansen (www.chr-hansen.com), Danisco Food Ingredients (www.danisco.com) and DSM Food Specialties (www.dsm-dairy.com) tend to supply concentrated blends of several defined strains for the direct inoculation of milk. The starter cultures are usually grouped according to the cheese type they are intended for and are supplied with information on acid production rates, gas production characteristics and general flavour attributes. However, the strain composition remains known only to the starter culture suppliers. On the other hand, companies that supply single-strain starter cultures for inoculation of bulk starter can be more specific in the type of starter culture supplied and the flavour attributes of each starter culture.

In most cases starter cultures that have been selected specifically for flavour control in cheese are added as adjunct cultures together with the primary acid-producing starter culture. Commercial lactococcal cultures for flavour control are usually lactose negative, although where lactose positive starter cultures are used, adjustment of inoculation levels limits their effect on acid production in the vat. Generally, the starter cultures are selected on the basis of their proteolytic and peptidase activities or for their ability to lyse, though their specific flavour effect can vary depending on the cheese variety, the cheese manufacturing plant and even the manufacturing conditions. They can also be marketed in low or high concentrations.

7.6 Future trends

7.6.1 High-throughput screening

As more emphasis is placed on selecting starter cultures for their ability to produce specific flavour compounds or for greater information on their range and activity of specific enzymes in addition to their acid-producing ability, a greater reliance will be placed on automated screening procedures. Where this involves enzymic analysis of large numbers of starter cultures using established spectrophotometric assays, there will be a need for rapid procedures to prepare

cell-free extracts or alternatively for the greater use of cell permeabilisation techniques. High-throughput screening will also involve the greater use of automated liquid handling systems, robotic microtitre plate transfer systems, microtitre plate spectrophotometers and automated data handling capabilities. Rapid automated systems using gas chromatography coupled to mass spectrometry (GC/MS) or direct-inlet mass spectrometry (DI-MS) have also been developed for headspace analysis of volatile flavour compounds produced by starter cultures. In one example of a DI-MS procedure described by Smit *et al.* (2004), starter cultures were screened for 3-methylbutanol production following growth in 2 ml culture vials arranged in a 96-well format. Under this system up to 1500 measurements per day could be performed.

Techniques such as the combinatorial methods used extensively in the pharmaceutical industry to screen large numbers of organic compounds may also enable much faster identification of optimum blends of specific compounds or starter cultures and/or adjunct combinations to achieve specific flavour profiles (Floyd *et al.*, 1996). However, in general, the high cost of high-throughput screening systems and the consequent pressure to operate them on a continual basis means that they are most likely to be used on a commercial basis either by larger starter culture supply organisations or by commercial laboratories.

7.6.2 Metabolic engineering

Metabolic engineering is concerned with redirecting metabolic fluxes through biosynthetic pathways without disturbing the overall cell physiology in order to form important flavour compounds, food ingredients or beneficial dietary components (Hugenholtz *et al.*, 2002). It involves inactivation of undesirable genes and/or controlled overexpression of existing or novel ones. One of the initial metabolic engineering studies in LAB was associated with diacetyl, an important flavour compound formed from pyruvate via α -acetolactate (Hugenholtz *et al.*, 2002). In order to increase the levels of diacetyl, glucose and lactose metabolism in *Lc. lactis* was redirected from the production of lactic acid to the production of α -acetolactate either by disrupting lactate dehydrogenase or by overproduction of NADH oxidase together with disruption of the gene encoding α -acetolactate decarboxylase which converts α -acetolactate to acetoin (Fig. 7.1).

In another example, L-alanine was produced from pyruvate by cloning the gene encoding for alanine dehydrogenase from *Bacillus sphæricus* into lactate dehydrogenase deficient *Lc. lactis* cells. The cells were then able to convert pyruvate in the presence of ammonia to alanine. Other compounds such as vitamins, sweeteners and polysaccharides can also be produced by starter cultures through metabolic engineering, with more products likely to be added to the list in the future. It is possible that metabolically engineered starter cultures with specific characteristics could be used directly in cheese manufacture providing they are able to produce lactic acid at sufficient rates, or alternatively

they could be included with the traditional starter culture as adjuncts. However, as discussed previously, the commercial success of metabolically engineered starter cultures will depend on whether they can consistently produce the targeted end product under industrial conditions, the cost effectiveness of the process, regulatory approval and market or consumer acceptance.

7.7 Sources of further information and advice

Cheese starter culture research, particularly with respect to their role in flavour development, is undertaken by numerous research centres around the world and within the major culture supply companies. The extent of interest in the area can be gauged from attendances at international symposia such as the Federation of European Microbiological Societies and Netherlands Society for Microbiology symposium on lactic acid bacteria held every three years, and the International Dairy Federation symposium on cheese held every four years. At the last symposium on lactic acid bacteria, held in The Netherlands in 2005, a review of the book of abstracts for posters covering flavour development by starter cultures showed that there were contributions from approximately 22 research laboratories representing 12 countries, while at the latest IDF symposium on cheese held in 2004 there were poster presentations covering starter flavour production from approximately 26 laboratories representing 18 countries. Of these research groups, there appear to be two that currently play a significant role in basic starter flavour research. These are the French group at Unité de Recherche de Biochimie et Structure des Protéins, INRA, 78352 Jouy-en-Josas, France, and the Dutch group at NIZO Food Research, Department of Flavour, Nutrition and Ingredients, PO Box 20 6710 BA Ede, The Netherlands. Information on commercial starter cultures can also be obtained directly from the major dairy culture supply companies such as Chr Hansen (www.chr-hansen.com), Danisco Food Ingredients (www.danisco.com) and DSM Food Specialties (www.dsm-dairy.com).

Much of the research on starter culture flavour development is published in a range of journals that include the *International Dairy Journal* (<http://www.sciencedirect.com/science/journal/09586946>), *Journal of Dairy Science* (<http://jds.fass.org/>), *Journal of Dairy Research* (<http://www.cambridge.org/uk/journals/>), *Le Lait* (<http://www.edpsciences.org/lait/>) and *Applied and Environmental Microbiology* (<http://aem.asm.org/>). Additional articles and relevant book chapters are listed in the references.

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8

Adjunct culture metabolism and cheese flavour

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8.1 Introduction to adjunct cultures

Cheese maturation under controlled temperature and often humidity conditions can be a slow process, varying from one to two months for a cheese such as Gouda to up to 12 months or longer for mature Cheddar. This substantially increases the overall cheese production costs; for example, one Australian Cheddar cheese manufacturer producing approximately 125,000 tonnes per year of which 30,000 tonnes is held in storage has estimated these costs to be \$AUD 10 per tonne per month. Consequently, any innovation that reduces maturation time without affecting cheese flavour and texture development would have a significant impact on the economics of cheese production, particularly for cheeses such as Cheddar that require additional time for further flavour development.

Adjunct cultures, the focus of this chapter, are microorganisms that are added to cheese, usually at the vat stage of manufacture together with the starter culture, in order to accelerate ripening and to assist in the development of a specific and consistent flavour profile. Control of the flavour profile allows larger manufacturers to produce a uniform flavour brand name while giving smaller speciality cheese producers the opportunity to develop a specific flavour unique to their product.

In this chapter adjunct cultures, their role in accelerating cheese ripening and their potential for imparting a consistent and specific flavour profile in cheese are reviewed. The chapter also covers procedures for selection, screening and characterisation of potential adjunct cultures in addition to an overview of their metabolism in the cheese matrix.

8.1.1 Cheese and non-starter lactic acid bacteria

The manufacture of most cheese varieties involves a number of similar basic steps (Fox *et al.*, 2000a). Milk is usually pasteurised to minimise the risk of pathogenic microorganisms being carried over into the cheese before cooling and the addition of starter culture, generally strains of *Lactococcus lactis* subsp. *lactis* or *Lc. lactis* subsp. *cremoris*, to initiate acidification where the milk lactose is converted to lactic acid. In cheese varieties that are 'cooked' to higher temperatures, such as Swiss and hard Italian varieties, starter cultures also include strains of *Streptococcus thermophilus* and various *Lactobacillus* spp. A coagulant and starter culture is added to the milk in order to form a gel. When the gel is cut or broken syneresis occurs, expelling the whey in a process that is also influenced by the heating regime applied to the curd (i.e. 'cooking'), by the rate of stirring of the curd/whey mixture and by the pH of the whey. Following whey drainage, the final step in the basic cheese manufacturing process is salting in which either salt (NaCl) is added directly to the curd particles or the cheese is immersed in a brine solution. This has a number of effects that influence cheese quality, including the control of microbial growth, microbial activity, enzyme activity and eventually flavour. Salting also contributes to curd moisture expulsion and influences cheese texture.

On completion of the manufacturing stage most cheese varieties undergo a period of ripening which can vary anywhere between three weeks to more than two years. During this process the residual lactose, citrate, fats and proteins that make up the cheese curd are broken down by numerous enzymes to give the characteristic flavour and texture of each cheese variety (McSweeney and Sousa, 2000). In most cheeses proteolysis is the key event in the development of cheese flavour and texture and is mediated by indigenous milk enzymes, milk coagulation enzymes, and enzymes from cheese starter cultures, adventitious non-starter lactic acid bacteria (NSLAB) and added secondary cultures. In the process caseins are hydrolysed to peptides and amino acids which contribute mainly to the background generic flavour of cheeses. Further enzymic degradation of peptides to amino acids and their subsequent catabolism or chemical transformation leads to the development of typical cheese flavour (Yvon and Rijnen, 2001).

8.1.2 Accelerated cheese ripening

The principal objective of accelerated cheese ripening has been to increase the rate of proteolysis and related events using either one or a combination of a number of different procedures. These include maturation of cheese at elevated ripening temperatures or the addition of exogenous enzymes, chemically, physically or genetically modified starter cultures, cheese slurries, enzyme-modified cheeses and adjunct cultures. The advantages and limitations of each of these techniques are extensively covered in a review by Fox *et al.* (2000b). For example, elevated maturation temperatures are practically simple and low cost to apply but tend to be non-specific and can lead to the generation of

unbalanced or off-flavours, whereas genetically engineered starter cultures can be easily incorporated into cheese to deliver specific flavour notes. The disadvantage of genetically engineered starter cultures is that their use may be restricted by legal barriers and consumer resistance.

There has been considerable interest in NSLAB as adjunct cultures, partly as a result of the high numbers that they achieve during cheese ripening and partly because of the possible relationship between NSLAB and flavour development in raw milk cheese. Raw milk cheeses tend to ripen faster and develop a more intense flavour than cheeses made from pasteurised milk, a characteristic that has been suggested to be the result of the incorporation of indigenous NSLAB from raw milk into the cheese. Furthermore, there is sufficient evidence to suggest that the addition of selected NSLAB to pasteurised milk cheese can improve flavour and accelerate ripening although occasional negative effects do arise. It should also be noted that while modified (attenuated) starter cultures have often been referred to as adjuncts, it is now generally accepted that adjunct cultures refer specifically to the non-starter lactic acid bacteria that are deliberately added to cheese for the express purpose of modifying the flavour profile.

8.1.3 Non-starter lactic acid bacteria

Non-starter lactic acid bacteria are contaminants that originate from either the raw milk or the factory environment and consist predominantly of *Lactobacillus paracasei* and *Lb. rhamnosus* as well as less common species such as *Pediococcus*, *Lb. brevis*, *Lb. fermentum*, *Lb. plantarum*, *Lb. coryneformis* subsp. *coryneformis*, *Lb. casei* and *Lb. curvatus*. Both the lactic acid starter culture and NSLAB are key agents in the degradation of milk protein during maturation and their proteolytic systems, particularly those of the *Lactococcus* starter cultures, have been extensively investigated. During cheese maturation a portion of the starter cultures, which are initially present at levels of up to 10^9 cfu g⁻¹, lyse thereby releasing various enzymes, including peptidases, into the cheese matrix. Conversely, the NSLAB increase from relatively low numbers (<100 cfu g⁻¹) in fresh curd to $\sim 10^7$ to 10^8 cfu g⁻¹ within a few months where their levels remain relatively constant. Due to the higher cell densities attained by the starter cultures they appear to contribute to between 10- and 100-fold greater levels of enzymes than the NSLAB. However, there is evidence to suggest that a succession of different NSLAB species and strains throughout ripening occurs and therefore contributes to an increase in cell turnover with possible associated autolysis of NSLAB. This would result in a higher NSLAB biomass than indicated from the viable cell counts, suggesting that in mature cheeses NSLAB enzymes would have a more significant role than the starter culture enzymes in flavour development (Crow *et al.*, 1995).

8.2 Adjunct culture types

8.2.1 Traditional adjunct cultures

Secondary organisms or cultures used in the production of Gouda and Swiss-type cheeses as well as mould- and smear-ripened cheeses can be considered as adjunct cultures, as they are deliberately added to cheesemilk in order to influence cheese flavour and texture during maturation without adding to the acidification. White surface-mould cheeses such as Camembert and Brie are traditionally made using a mesophilic starter culture, although the need for a more stable product in the marketplace has led to the incorporation of thermophilic streptococci or a mixture of streptococci and lactococci as part of the primary culture (Gripon, 2003). During maturation of white mould cheese made from raw milk, a complex microflora develops on the cheese surface commencing from day one with the growth of yeasts, usually species of *Kluyveromyces lactis*, *Saccharomyces cerevisiae* and *Debaryomyces hansenii* as well as the yeast-like mould *Geotrichum candidum*, although growth of this organism is limited by the salt concentration (Gripon, 2003). This is usually followed within a week by the appearance of the mould *Penicillium camemberti* which, on metabolising lactic acid and increasing the surface pH of the cheese, promotes the growth of acid-sensitive bacteria such as micrococci and coryneform bacteria, a high proportion of which are *Brevibacterium linens*.

White mould cheese manufactured using pasteurised milk develops a less complex surface flora and aroma and adjunct organisms including yeasts, corynebacteria, *G. candidum* and spores of *P. camemberti* are added with the starter culture to improve cheese quality. The *Penicillium* mould is responsible for much of the enzymic activity of the cheese during maturation, utilising the lactic acid in cheese for their growth and having some lipolytic activity. However, it is their active proteolytic system that is responsible for much of the change in flavour and texture. *Penicillium camemberti* produce an acid protease and a metalloprotease together with two exopeptidases, an acid carboxypeptidase with broad specificity, and an alkaline aminopeptidase, all of which contribute to the high amino acid levels present in mould-ripened cheeses. The increase in the surface pH through lactate utilisation and the production of ammonia by *P. camemberti* also leads to the establishment of a pH gradient from the centre to the surface of the cheese which contributes to softening of the cheese through its effect on calcium solubility and protein interactions.

Blue-veined cheeses, typically represented by varieties such as Stilton, Roquefort and Gorgonzola, traditionally use heterofermentative starter culture containing *Leuconostoc* and are not pressed following moulding. The production of CO₂ by the *Leuconostoc* and the loose structure of the curd encourages opening up of the cheese, allowing oxygen to enter and promote the natural growth of the mould *Penicillium roqueforti* which can also be added to cheesemilk as an adjunct culture. This organism is able to grow at lower O₂ levels and tolerate higher CO₂ levels than *P. camemberti* and is therefore particularly suited for growth in the internal cracks and holes of cheese. During

cheese maturation the major function of *P. roqueforti* is the degradation of milk proteins. The mould has a similar active extracellular proteolytic system to *P. camemberti* and also possesses two lipases with different specificities, resulting in a higher degree of lipolysis in blue-veined cheese.

Smear-ripened cheeses are produced from any rennet-coagulated curd that has a secondary (adjunct) culture surface layer consisting of various yeasts and bacteria (Bockelmann, 2003). Traditionally these adjunct cultures are added via an unpasteurised brine solution giving rise to various salt-tolerant yeasts and staphylococci. These organisms can also develop on salted pressed cheese curds which are stored at high humidity and brushed regularly with salt water (3%). The composition of the surface microflora will vary between factories and the type of cheese (i.e. semi-soft, semi-hard and hard) but is generally made up of *Corynebacterium* spp., *Brevibacterium* spp., *Arthrobacter* spp. and *Microbacterium* spp. In semi-soft and soft cheeses *Deb. hansenii*, *G. candidum* and *Staphylococcus equorum* are also present. The development of defined commercial secondary or adjunct cultures is not well advanced for smear-ripened cheeses, with *Deb. hansenii* and *B. linens* the most commonly available cultures. More recent studies using model systems have indicated that a complete minimal surface secondary starter could be made up by single strains of *Deb. hansenii*, *Staph. equorum*, *Arthrobacter nicotianae*, *B. linens* and *Corynebacterium ammoniagenes* (Bockelmann, 2003).

Swiss-type cheeses use mixed thermophilic lactic acid bacteria as the primary starter culture together with species of propionibacteria (usually *Propionibacterium freudenreichii*) as the secondary or adjunct culture. Propionibacteria are essential for the development of the characteristic flavour and eye formation in Swiss-type cheeses. They are inoculated into the cheesemilk at low levels and grow internally in the cheese to levels of up to $\sim 10^8$ to 10^9 cfu g⁻¹ where they metabolise the lactic acid produced by the starter culture to form propionate, acetate and CO₂.

Gouda cheese is manufactured using mesophilic lactic acid bacteria as the starter culture together with citrate-fermenting strains of *Leuconostoc lactis*, *Leuconostoc mesenteroides* subsp. *cremoris* and *Lc. lactis* subsp. *lactis* as the adjunct culture. These organisms are responsible for eye formation in the cheese as a result of CO₂ formation from the metabolism of citrate.

8.2.2 NSLAB adjunct cultures

The incorporation of adjunct cultures into the manufacture of cheeses such as Cheddar has usually involved isolation of the principal adventitious non-starter species from mature cheese, followed by growth of these organisms in an appropriate medium before addition back to the cheesemilk at high numbers with the starter culture. It was reasoned that as NSLABs were one of the key agents associated with Cheddar cheese ripening then the introduction of high numbers at the commencement of the maturation process would beneficially affect the rate of flavour development and the type of flavours generated.

Studies carried out in the 1930s indicated that cheeses made with different strains of *Lb. casei* generally exhibited increased proteolysis and scored higher in flavour than controls, although there were differences in the flavour profile between strains (Peterson and Marshall, 1990). Further work in New Zealand, also in the 1930s, indicated that cheese made from cheesemilk inoculated with pure strains or mixtures of several pure strains of lactobacilli isolated from Cheddar cheese was variable in its quality (Sherwood, 1937). More desirable flavours were usually evident in cheeses containing added cultures of *Lb. casei* and *Lb. plantarum* whereas the addition of *Lb. brevis* resulted in more objectionable flavours and poorer quality cheese. However, further studies indicated that even the desirable strains, when added at high inoculation levels, tended to produce cheeses with atypical Cheddar flavour. Interestingly, Sherwood (1937) observed that better control of flavour could be achieved by the use of mixed cultures of lactobacilli and that there may be a symbiotic relationship between cultures, issues that are the focus of many current adjunct studies. He also noted that in order to achieve full Cheddar flavour selected organisms, like lactobacilli, would need to be added to milk as its bacteriological quality improved with the more prevalent use of pasteurisation. Further details of this earlier work are covered in the review by Peterson and Marshall (1990).

Current adjunct studies have tended to concentrate on the development of specific selection criteria for adjunct cultures and investigations of their growth in the cheese matrix in addition to a better understanding of their metabolic pathways (particularly those associated with protein degradation) and metabolic cooperation with other cheese ripening agents. There have been numerous trials in which selected lactobacilli have been added to pasteurised cheesemilk and generally there has been an improvement in both flavour quality and intensity (Broome *et al.*, 1990; Crow *et al.*, 2001). There also appears to be variation between countries and cheese production plants in the species and strain of NSLAB that are best suited as adjunct cultures. While many of the trials have been with Cheddar cheese, lactobacilli have also been used as adjunct cultures in other cheese varieties such as Swiss-type cheeses and Danbo cheese. Again, these studies have shown that NSLAB adjunct cultures generally have a positive effect on cheese quality attributes, although this is dependent on the species and strain of organism used.

8.2.3 Other adjunct cultures

Brevibacterium linens is an aerobic halotolerant microorganism that is the major component of the flora of surface-ripened cheeses such as Limburger, Münster, Brick, Tilsiter and Appenzeller (Ratray and Fox, 1999). The interest in *B. linens* as a potential adjunct culture has been due to its ability to produce an extracellular proteinase and high levels of methanethiol, which is an important contributor to Cheddar cheese flavour (Dias and Weimer, 1999; Weimer *et al.*, 1999). The organism has been used as a cheese-ripening accelerant, either

indirectly where its extracellular proteinase or extracellular aminopeptidases are added to the cheesemilk, or directly in which viable cells or cell extracts are added to the curd or cheesemilk (Ratnayake and Fox, 1999). In Cheddar cheese, *B. linens* adjunct cultures have been shown to accelerate flavour development and to improve the flavour of 60% reduced fat cheese (Weimer *et al.*, 1997).

Various yeasts including *Deb. hansenii*, *Yarrowia lipolytica*, *G. candidum* and *Candida kefyr* have also been considered as candidates for accelerating cheese ripening. Yeasts are normally present as part of the surface microflora of mould-ripened or smear-ripened cheeses and can possess high proteolytic and lipolytic activities that contribute to textural change and the formation of various aromatic compounds. They are able to grow at low temperatures, in high salt concentrations and at low water activity. While there have been few studies on the use of yeasts as adjunct cultures, it appears that selected species added as a mixture can contribute to the development of a strong Cheddar flavour, although bitter and fruity flavours are evident when yeasts are inoculated individually (Ferreira and Viljoen, 2003).

8.2.4 Attenuated adjunct cultures

The objective of attenuation in starter cultures is to inhibit their growth and acid producing ability while maintaining their ability to contribute to cheese ripening (Klein and Lortal, 1999). In the case of NSLAB adjunct cultures that have been selected for their low acid-production activity, the principal objectives of attenuation have been to enhance their autolytic properties and to increase the release of intracellular enzymes into the cheese matrix. This has usually been achieved through the use of sublethal physical treatments such as freeze shock, heat shock, and spray drying. Typically, in pilot-scale freeze shock trials, lactobacilli are grown in an appropriate medium, harvested by centrifugation, washed, and resuspended to $\sim 10^9$ cfu ml⁻¹ before being subjected to one or two cycles of freezing at -20°C for 24 hours and thawing at 40°C (Madkor *et al.*, 2000). However, treatments do vary and cells may be held at -100°C or frozen for up to 4–5 weeks. The treated cells are then added to cheesemilk at levels of between 0.4 and 2%.

Heat shock treatment usually involves heating cells, grown under pH control in a broth medium or in reconstituted skim milk powder, in a continuous flow system at between 50°C and 70°C for 10–22 seconds. Heat-shocked cells have also been frozen at -20°C for 24 hours before use and the treated cells added to cheesemilk at levels between 0.1 and 4%. In another form of attenuation, adjunct cultures are spray dried after adjusting cells suspended in buffer to a solids content of 16–18% with maltodextrin and drying in a spray dryer at an inlet temperature of 189°C and an outlet temperature of 82°C to give a powder containing 10^6 cfu ml⁻¹ (Madkor *et al.*, 2000).

Most studies with attenuated adjunct cultures indicate increased autolysis in the cheese and improved flavour and aroma scores associated mainly with increased rates of amino nitrogen formation. Freeze shocking also tended to be

the more effective treatment for improved ripening in Cheddar cheese, although this was dependent on the species and strain of adjunct culture.

The use of attenuated adjunct cultures on a commercial scale is not widespread, probably because there are still a number of questions over the most effective attenuation procedures, the choice of suitable NSLAB species and strains, the extent of lysis required to give optimum results, and the metabolic relationship between adjunct cultures and the other cheese-ripening agents. However, the most critical factor that will determine the widespread use of attenuated adjunct cultures is economics and whether the cost of the large-scale preparation of attenuated adjunct cultures is justified by the gains achieved in ripening time and flavour development.

Attenuated starter cultures, while not strictly regarded as adjunct cultures, have also been investigated for their effect on cheese-ripening time and flavour development (Klein and Lortal, 1999). However, while similar attenuation treatments can be applied to starter cultures as well for the NSLAB, one novel approach has been the use of starter cultures that have spontaneously lost their ability to ferment lactose (Lac^-), a plasmid-encoded property. As proteinase production is also associated with plasmids, these variants are also usually proteinase negative (Prt^-). When $\text{Lac}^- \text{Prt}^-$ variants were added to milk together with the starter culture, the resulting cheeses tended to form higher levels of free amino acids during maturation, although the effect on cheese sensory properties was not as pronounced (Grieve and Dulle, 1983; Kamaly *et al.*, 1989).

8.3 Selection of adjunct cultures

The two basic requirements of NSLAB under consideration as adjunct cultures are that they provide a balance of beneficial ripening reactions in cheese and that they should also remain the dominant NSLAB in cheese throughout ripening (Crow *et al.*, 2001). Adjunct cultures are usually isolated from good quality cheese and, in order to reduce the possibility of contributing to flavour defects, are subjected to a rudimentary screening process before addition to cheesemilk at high levels as a single culture. This fundamental approach has resulted in variable results and the conclusion that a successful NSLAB adjunct culture system should be made up of a number of strains to provide a balance of flavours and a succession of strains throughout maturation. As there are a great number of NSLAB strains in maturing cheese and many possible combinations, any investigation based on cheese manufacturing trials could be an expensive exercise unless there is some form of prior selection process. Such a process would assist in eliminating any organisms that do not contribute to flavour development or contribute to specific defects in cheese. The potential variation in flavour outcomes was demonstrated by Weimer *et al.* (1997) when they showed a significant variation in the flavour profiles of 60% reduced fat Cheddar cheese made with different starter culture and adjunct culture combinations.

8.3.1 Isolation and identification of potential NSLAB adjunct cultures

Good quality mature cheese is usually the starting point for selection of potential adjunct cultures which in Cheddar cheese consist predominantly of species of lactobacilli. Cheese samples are usually emulsified in sterile trisodium citrate and diluted before plating onto *Lactobacillus*-selective agar (e.g. LBS or MRS agar) and incubating anaerobically at 30°C. The resulting isolates can then be identified using classical procedures that include colony morphology, thermophilic growth, utilisation of sugars and citrate, growth in litmus milk, absence of catalase, metabolic end-products produced and the lactic acid isomer produced. Also available are commercial test kits based on carbohydrate utilisation patterns such as the API 50 CHL system from API System S.A. More recently, various molecular profiling techniques that include pulsed-field electrophoresis, repetitive sequence PCR analysis, ribotyping, random amplified polymorphism DNA analysis, intergenic spacer region PCR amplification and amplified ribosomal DNA restriction analysis can be used to identify isolates (Marilley and Casey, 2004).

8.3.2 Characterisation of NSLAB adjunct cultures

The primary requirements of a NSLAB adjunct culture, when added to cheesemilk at low levels (10^2 to 10^5 cfu ml⁻¹), are that they do not contribute to acid production during cheese manufacture and that they grow rapidly to high cell densities (10^7 cfu g⁻¹ cheese) early in cheese maturation and maintain these levels in order to dominate the adventitious NSLAB (Crow *et al.*, 2001). It is also imperative that they do not produce any undesirable defects in the cheese. To assist in the selection process potential adjunct cultures can be screened for a number of specific features.

Basic selection criteria

As an indicator of their ability to grow under cheese environmental conditions any initial screening of potential NSLAB adjunct cultures usually involves an assessment of factors such as pH, salt and temperature for their effect on growth. For a NSLAB being considered as a Cheddar cheese adjunct culture the criteria may be an ability to grow at 10°C, pH 5.0 and a salt concentration of 4%; whereas for Gouda cheese it may be 13°C, pH 5.2 and a salt concentration of 5%. Various biochemical properties have also been assessed, including the potential to form biogenic amines, which are possible health risks, and the ability to racemise L(+)-lactate to D(-)-lactate that may contribute to calcium lactate formation in cheese. The ability to ferment citrate can be a problem with the formation of CO₂ and gas production in cheese, although citrate utilisation can also be a positive attribute in flavour formation, in which case the rate of citrate utilisation could be the critical selection factor. Glutamate utilisation has been used to screen adjunct cultures, since excessive utilisation in the later part of ripening appeared to correlate with poorer quality flavour in the mature cheese (Crow *et al.*, 2001). In this case it is possible that a high rate of

conversion of glutamate to α -ketoglutarate could indicate excessive amino acid metabolism and the resultant formation of unbalanced flavours.

Proteolytic activity

Proteolytic activity of potential NSLAB adjunct cultures is another function that can be assessed in an initial screening programme. During Cheddar cheese maturation the coagulant is normally the principal agent responsible for initial proteolysis. It contributes indirectly to cheese flavour development by producing large and medium-sized peptides that are further degraded by bacterial proteinases and peptidases to produce small peptides and free amino acids. The NSLAB, like most lactic acid bacteria, tend to be weakly proteolytic and although their proteolytic systems have not been studied as extensively as the lactococci, they are similar. They are known to have a complex set of intracellular enzymes that are involved in peptide hydrolysis and therefore have the capacity to contribute to peptide hydrolysis, amino acid formation and flavour formation (Williams *et al.*, 1998). The addition of NSLAB adjunct cultures to cheese generally results in an increase in smaller peptides and free amino acids, suggesting that peptidase profiling may be an important feature to include in a screening programme. In its simplest form this can be the determination of total free amino acids released in an assay where the adjunct culture is incubated in a milk protein solution pre-incubated with coagulant or trypsin. The assay can also be adapted so that a specific peptide such as the bitter C-terminal part of β -casein is used as the substrate in a measure of the debittering potential of adjunct cultures. Alternatively, an indicative assessment of peptidase profiles can be obtained by incubation of cell-free extracts with appropriate chromogenic substrates either of amidomethylcoumarin derivatives of amino acids in a fluorometric assay or of *p*-nitroanilide or β -naphthylamide amino acid derivatives in a colorimetric assay (Kunji *et al.*, 1996; Williams and Banks, 1997). In addition certain di- and tri-aminopeptidases can be assessed using peptide substrates and determination of the amino acids released using a colorimetric assay. Further details of the more significant peptidases that can be assessed and their indicative chromogenic substrates are shown in Table 8.1.

Peptidase profiling can also be carried out using PCR technology in order to demonstrate the presence or absence of genes coding for specific peptidases or peptidase variants present in the potential adjunct culture. This technique will become an increasingly attractive rapid screening procedure for the proteolytic system of lactobacilli with the increasing number of genome sequences that are being determined (see Chapter 10).

Autolytic potential

It has been shown that increased lysis of starter cultures and the release of intracellular enzymes into the cheese matrix influence Cheddar cheese flavour. At the same time it is also important to avoid excessive lysis, as optimum flavour formation probably depends on a balance of intact and autolysed cells

Table 8.1 Assessment of peptidase activities

Peptidase	Specificity	Indicative assay substrate
Aminopeptidase N (PepN)	X- [↓] -(X)n	Lys- (Leu-) (Arg-) <i>p</i> -nitroanilide
Aminopeptidase A (PepA)	Glu-/Asp- [↓] -(X)n	Glu- (Asp-) <i>p</i> -nitroanilide
Aminopeptidase L (PepL)	Leu- [↓] -X	Leu- <i>p</i> -nitroanilide
	Leu- [↓] -X-X	
Aminopeptidase C (PepC)	X- [↓] -(X)n	His- β -naphthylamide
Aminopeptidase X (PepX)	X-Pro- [↓] -(X)n	Gly-Pro- (Ala-Pro-) <i>p</i> -nitroanilide
Proiminopeptidase (PepI)	Pro- [↓] -X-(X)n	Pro- <i>p</i> -nitroanilide, Pro-AMC
Prolinase (PepR)	Pro- [↓] -X	Pro-Leu
Prolidase (PepQ)	X- [↓] -Pro	Leu-Pro
Pyrrolidone carboxyl peptidase (PCP)	Pyr- [↓] -(X)n	Pyr- <i>p</i> -nitroanilide
Dipeptidase (PepD)	X- [↓] -X	Leu-Leu
Tripeptidase (PepT)	X- [↓] -X-X	Leu-Leu-Leu

(Crow *et al.*, 1995). For example, lactose fermentation is dependent on cell membrane integrity, and excessive starter culture lysis may result in elevated residual lactose levels that are then used by adventitious NSLAB to form off-flavours. Autolysis of NSLAB has not been well studied, although there is some suggestion that the mesophilic lactobacilli may not lyse during cheese maturation. Other studies with thermophilic lactobacilli such as *Lactobacillus helveticus* have demonstrated autolysis of these organisms and an associated increase in amino acid levels and flavour scores both in Swiss cheese and in Cheddar when it was used as an adjunct culture. Consequently, there is some uncertainty over the importance of autolysis for NSLAB adjunct cultures and the role of NSLAB intracellular enzymes, including peptidases, in modifying the flavour of cheese. It is possible that NSLAB influence cheese flavour through other metabolic pathways such as amino acid metabolism.

The autolytic ability of adjunct cultures can be estimated simply by measurement of the decrease in absorbance when cells are suspended in buffer or clear growth medium. When time is not a limiting factor autolysis can also be monitored directly in cheese by determining the decrease in viable cells, release of DNA or release of intracellular enzymes.

Amino acid metabolism

Past studies into cheese proteolysis following the addition of starter cultures with genetically enhanced proteinase or peptidase activity or after addition of free amino acids to cheese curd indicated that the actual rate-limiting step in flavour formation is the conversion of amino acids to volatile aroma compounds. More recently the focus of cheese flavour research has been on amino acid metabolism, particularly the mechanisms involved in amino acid catabolism by lactic acid bacteria to compounds such as aldehydes, acids, alcohols, esters and thiols. The majority of these volatile aroma compounds are the result of the enzymatic breakdown of key aromatic (phenylalanine), branched chain (leucine)

and sulphurous (methionine and cysteine) amino acids as well as serine and threonine.

The key reaction in the conversion of these amino acids to flavour compounds is catalysed by an aromatic or branched chain aminotransferase that transfers the α -amino group of the amino acid to a keto acid acceptor, often α -ketoglutarate. The aminotransferases are more or less specific for the amino acid groups but have broadly overlapping substrate specificity and are located intracellularly. Under cheese maturation conditions the active transport of amino acids into intact but non-growing cells may be impaired; however, there is growing evidence to suggest that under carbohydrate starvation conditions, such as in maturing cheese, amino acid transport in non-growing lactococci remains active (Stuart *et al.*, 1999; Ganesan *et al.*, 2006). Although it is possible that the aminotransferases are released from lysed cells into the cheese matrix where they can directly act on amino acids, some of the enzymes involved in amino acid metabolism are susceptible to proteolysis when released from lysed cells, while other studies indicate that under cheese-like conditions key flavour compounds such as methanethiol are formed predominantly via an amino acid transamination pathway carried out primarily by whole cells (Dias and Weimer, 1999; Gao *et al.*, 1998; Ganesan *et al.*, 2004b; Ganesan and Weimer, 2004). This again questions the relative importance of lysis in the functioning of NSLAB adjunct and starter cultures. It also suggests that there may be an optimum balance of lysed to whole cells present in cheese for development of a balanced flavour profile. It is possible that insufficient lysis impedes amino acid formation while excessive lysis could restrict amino acid degradation.

The keto acids resulting from amino acid transamination either undergo spontaneous degradation or are enzymically degraded to the corresponding aldehydes, alcohols or carboxylic acids, although many of these pathways have not been fully investigated in lactobacilli, unlike lactococci (Ganesan *et al.*, 2004a, b; Ganesan and Weimer, 2004). Despite this, it is still feasible to screen potential adjunct cultures for specific enzyme activities (Smit *et al.*, 2002; Marilley and Casey, 2004). Some of the key enzymes associated with amino acid metabolism that can be assayed as part of a screening process for potential NSLAB adjuncts are outlined in Fig. 8.1. In some cases the ability to form certain compounds such as hydroxy acids from α -keto acids via a hydroxy acid dehydrogenase may be an undesirable feature as they do not contribute to flavour.

Volatile sulphur compounds derived from methionine, including methanethiol, dimethyl sulphide and dimethyl trisulphide, are important flavour components in a number of cheeses and can be generated through transamination reactions. These compounds can also be formed via cystathionine β -lyase, cystathionine γ -lyase or methionine γ -lyase, the activities of which could be another potential screening tool for adjunct culture selection, although generally substrate specificity by these enzymes for methionine is very low.

Serine dehydratase, the activity of which results in the formation of ammonia and pyruvate from serine, is another enzyme that could be considered in a

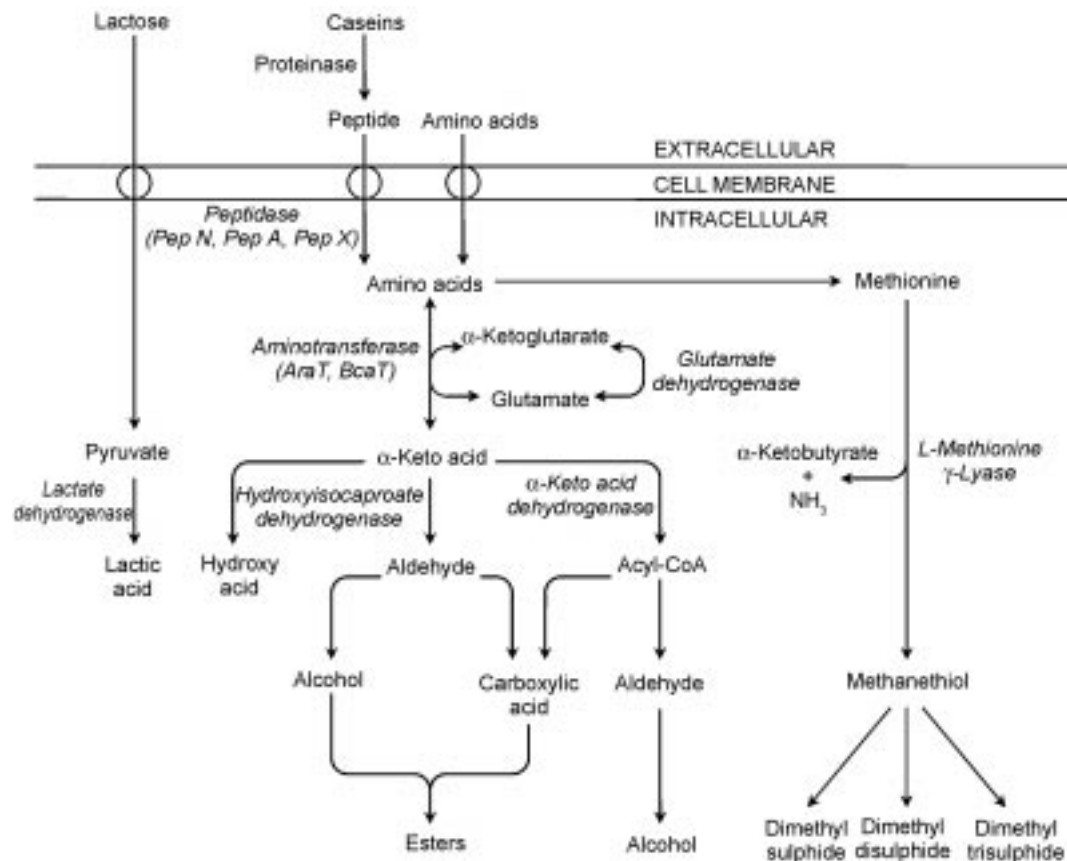


Fig. 8.1 Metabolic pathways and associated enzymes that can be assayed in an adjunct culture screening programme.

screening process, as pyruvate is a key compound in LAB metabolism through its involvement in $\text{NAD}^+/\text{NADH} + \text{H}^+$ regeneration and the formation of ATP, acetoin, diacetyl, 2,3-butanediol, acetate, formate and ethanol. In addition, various enzymes associated with lipid, lactate and citrate metabolism are further candidates for inclusion in an adjunct culture screening protocol, with many of the assays summarised by Marilley and Casey (2004).

The ability to clearly distinguish between various species and strains of lactic acid bacteria using enzyme assays makes this a powerful tool for selection of adjunct cultures although there are disadvantages in the time required to prepare cell-free extracts and the measurement of activity in a large number of organisms. With additional work to define procedures that will extract intracellular enzymes and the development of improved enzyme assay systems, many of these difficulties can be overcome. The use of genome sequence information will also aid in the rapid screening of strains with similar genetic potential (see Chapter 10).

Model and pilot-scale systems

In spite of the improved potential for selection of a successful adjunct culture through a planned screening process, there is still uncertainty over the performance of the adjunct culture under cheese manufacturing conditions and in the presence of the coagulant, starter culture and natural milk enzymes. A model cheese system can be a cost-efficient means of assessing potential adjunct cultures prior to any full-scale production trial, although again there is still no guarantee that model systems will accurately reflect adjunct culture performance in the cheese vat. Model systems vary considerably in their level of sophistication, with the more basic consisting of buffered solutions adjusted to the pH of cheese and containing salt at concentrations approximating that found in the moisture phase of cheese. Increasingly complex procedures for assessing the flavour potential of starter cultures and non-starter adjunct cultures under cheese maturation conditions have made use of cheese slurries based on aseptic processed cheese curd or unsalted curd or prepared directly from skim milk (Wijesundera *et al.*, 1997). These systems have been shown to replicate the ripening process of natural cheeses over a short time period; however, there are difficulties in maintaining aseptic conditions and uniform media composition. Semi-synthetic cheese media ranging from acidified (pH 5.25) sodium caseinate solutions that contain NaCl to those containing casein, lactate, lactose and a range of salts which attempt to simulate the aqueous phase of cheese have also been developed (Broome and Limsowtin, 2002). Model cheese systems, including the miniature cheese system used by Ur-Rehman *et al.* (1999) to assess the ripening properties of lactococci and the Ch-easy model developed at NIZO Food Research, The Netherlands, to study the effect of cheese-ripening agents on aroma formation, are alternative systems for assessing cheese-ripening agents. Pilot-scale cheese manufacturing trials that utilise up to 400 kg or greater of milk are also used to demonstrate the effectiveness of adjunct culture incorporation on cheese flavour development, although such trials can be expensive and are not suitable for the assessment of large numbers of adjunct cultures.

Growth of adjunct cultures within the cheese matrix

The successful application of adjunct cultures in cheese for maturation control is dependent on their ability to grow in the cheese matrix and the continued dominance of the adjunct culture over other adventitious NSLAB. Growth substrates must be available in cheese for the NSLAB and there have been many investigations into possible substrates and growth factors. Potential substrates include lactose, citrate, fatty acids, glycerol, lactate, amino sugars and glyco-protein carbohydrates in addition to nucleic acids. Various compounds associated with the milk fat globule membrane such as glycoproteins and glycolipids may also serve as possible energy sources. Several species of mesophilic lactobacilli have been shown to possess various glycosidases which can release sugars from glycoproteins (Fox *et al.*, 1998). With an increasing understanding of NSLAB growth in cheese it may be possible to incorporate utilisation of specific substrates in a screening process for selection of potential adjunct cultures.

Factory trials

Despite the use of sophisticated screening systems for potential adjunct cultures and assessment under model systems, including pilot-scale evaluation, there is still no guarantee that they will have a positive effect when used on a commercial basis. Each cheese manufacturing plant varies in the type of milk used, milk treatment, coagulant, starter culture, cheese type, cheese make recipe, cheese cooling regime, packaging and maturation conditions, all of which will affect the impact of the adjunct culture(s). The use of screening and assessment protocols may provide a guide, but ultimately the only true indication of the success of an adjunct culture will be when it is used under the conditions it is intended for. To date much of the information on the ability of adjunct cultures to influence flavour and aroma is from trials carried out under commercial conditions, and it is the larger culture supply companies that are in the best position to monitor the progress and success of adjunct culture use.

8.4 Adjunct culture metabolism in the cheese matrix

8.4.1 Cooperation of adjunct cultures with other cheese-ripening agents

The selection of potential adjunct cultures based on specific attributes may exclude organisms that still have a positive effect on cheese flavour when interacting with other ripening agents. Many previous studies describing the interaction between NSLAB and starter cultures have concentrated on either the inhibition or stimulation of growth, but there is growing evidence to suggest that there is also cooperation in specific metabolic pathways. Amino acid metabolism is a key process in the formation of many flavour compounds (see Chapter 4) and recent studies have indicated possible cooperation between NSLAB and starter cultures in the conversion of amino acids to aroma compounds (Kieronczyk *et al.*, 2003). Transamination of amino acids to an

α -keto acids requires another α -keto acid (often α -ketoglutarate) as an amino group acceptor. In turn α -ketoglutarate is formed from glutamate by glutamate dehydrogenase, but with most lactic acid bacteria the activity of glutamate dehydrogenase and the supply of α -ketoglutarate is limited. One approach to this problem is to incorporate a glutamate dehydrogenase positive (GDH⁺) adjunct culture that utilises the large quantities of glutamate that are present in cheese. The advantage of including a GDH⁺ *Lactobacillus* with a *Lc. lactis* subsp. *cremoris* starter has been demonstrated by Kieronczyk *et al.* (2003). In a proposed cooperation model the GDH⁺ lactobacilli initiate amino acid transamination to α -keto acids as well as the further partial conversion of the α -keto acid to hydroxy acids in a reversible reaction (Fig. 8.2). The starter culture then completes the process of potential aroma compound formation by converting the α -keto acids and hydroxy acids to carboxylic acids, probably via α -keto acid dehydrogenase as most lactococci of dairy origin do not exhibit decarboxylase activity. However, as any α -keto acid dehydrogenase is likely to be a multi-enzyme complex similar to the pyruvate dehydrogenase complex, it may be reliant on an intact cell to maintain integrity of the enzyme complex. Consequently, high cell numbers of both NSLAB adjunct and intact starter culture may be required for cooperation, a situation that usually only occurs early in the cheese maturation process before any substantial starter culture lysis. Alternatively, should starter cultures exist in a non-culturable but metabolically

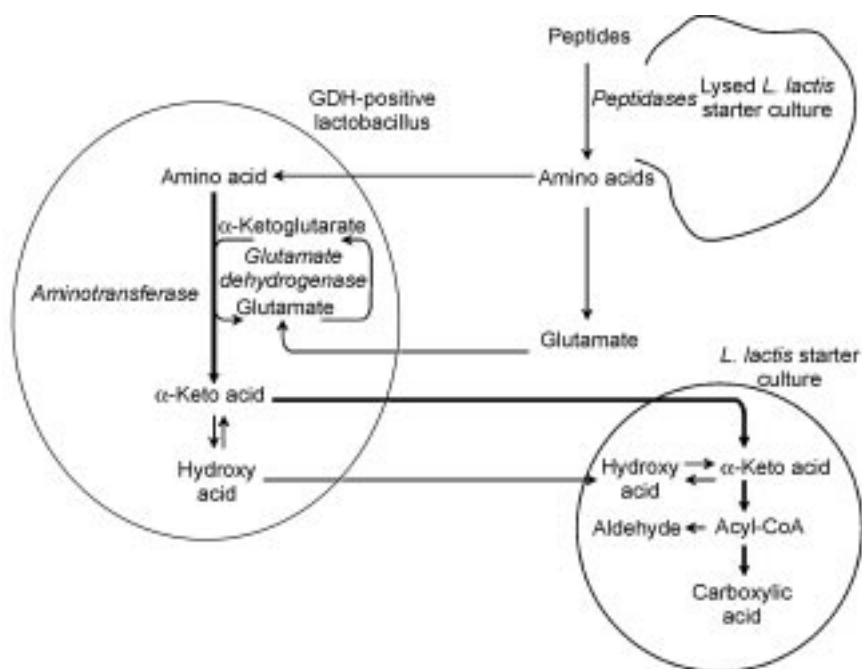


Fig. 8.2 Metabolic cooperation of starter and non-starter lactobacilli (based on Kieronczyk *et al.*, 2003).

active state rather than undergoing lysis, they may still be able to function in a cooperative manner for much longer in maturing cheese (Stuart *et al.*, 1999; Ganesan *et al.*, 2006). Cooperation has also been demonstrated between different *Lc. lactis* strains (Smit *et al.*, 2002). In this case it was found that while one proteolytic strain was able to degrade caseins and transaminate the resultant amino acids to the α -keto acid, it lacked a decarboxylase to further transform the α -keto acid to the aldehyde. The addition of a second wild-type *Lc. lactis* that lacked any proteolytic activity but had decarboxylase activity resulted in the formation of the aldehyde.

These examples of cooperation are a further demonstration that any screening protocol for potential adjunct cultures should ideally include a review of specific enzyme activities. Such a review would allow the selection of complementary adjunct cultures and match adjunct cultures to starter cultures in order to control specific flavour development in cheese.

8.5 Future trends

As the biochemical understanding of flavour development in cheese increases, and more information on the biodiversity of flavour compounds formed by lactic acid bacteria is generated, it is likely that there will be an increasing demand for adjunct cultures with specific enzyme activities. This demand will be based principally on their potential to decrease ripening times and more significantly for the formation of consistent and specific flavour profiles, including the creation of regional flavour characteristics.

It is anticipated that further research into amino acid and lipid metabolism by lactic acid bacteria will identify key enzymes and compounds that contribute to cheese flavour and aroma. While this information can then be used as part of a screening process for selection of potential adjunct cultures, the future emphasis will be on the development of technologies to maximise the numbers of organisms screened. There will also be increased pressure for the development of improved methods to assess the effect of larger numbers of adjunct and starter culture combinations on maturation rates and sensory characteristics in cheese.

Screening procedures can be time consuming and expensive, depending on the level of information required. To assist in the increased demand for selecting and assessing potential adjunct cultures, more reliance will be placed on the application of various new analytical tools that increase rates of strain identification, identification of key enzymes and identification of aroma and flavour compounds as well as assessment of culture combinations.

8.5.1 Genomics sequences

As the genomes of more lactic acid bacteria become available potential adjunct culture genes encoding enzymes associated with flavour-forming pathways can be identified quickly. Gene expression profiling assays such as microarrays can

then be utilised to screen organisms that possess key enzymes involved in the degradation of caseins and milk fats. It is also possible to identify genes encoding enzymes catalysing the formation of undesirable products (see Chapter 10).

8.5.2 Proteomics

Proteomics provides a means of identifying protein expression of an organism under varying conditions such as in the complex and constantly changing cheese matrix (Gagnaire *et al.*, 2004). The methodology provides information on the range of bacterial proteins, including enzymes, that are expressed under cheese environmental conditions, their bacterial source and how they vary during maturation. Cheese proteins are isolated from either a water-soluble extract or from the cheese aqueous phase extracted using hydraulic pressure before prefractionation by size exclusion chromatography. The protein solution is then separated using two-dimensional electrophoresis or by multidimensional liquid chromatography before characterisation by mass spectrometry, prior to peptide mass fingerprinting and a sequence homology database search. Where proteins are isolated from a water-soluble cheese extract, however, the method provides only a fingerprint of the enzymes released into cheese by lysed cells and does not provide information on the proteins of cells such as NSLAB adjuncts that may not readily lyse.

8.5.3 High-throughput screening

The automated enzymatic screening of large numbers of potential adjunct cultures using established spectrophotometric assays requires rapid procedures for the preparation of cell-free extracts or alternatively the development of cell permeabilisation techniques that make use of agents such as toluene, detergents or ethanol (see Chapter 7). In addition, automated liquid handling, robotic microtitre plate transfer systems and microtitre plate spectrophotometers combined with an automated data handling system can be incorporated into any high-throughput screening programme. Rapid automated systems using gas chromatography coupled to mass spectrometry (GC/MS) or direct-inlet mass spectrometry (DI-MS) have also been developed for headspace analysis of volatile flavour compounds produced by starter cultures (Smit *et al.*, 2004).

8.5.4 Metabolic engineering

As outlined in Chapter 7, metabolic engineering involves redirection of metabolic fluxes through biosynthetic pathways in order to produce important flavour compounds, food ingredients or beneficial dietary components (Hugenholtz *et al.*, 2002). The process involves inactivation of undesirable genes and/or controlled overexpression of existing or novel ones, with many of the initial metabolic engineering studies in lactic acid bacteria focusing on *Lc.*

lactis. In the future, metabolic engineering of NSLAB adjuncts may be an option for activation of specific enzymes associated with the production of flavour compounds or for compounds able to be further transformed by other lactic acid bacteria, although ultimately their success will depend on the cost-effectiveness of the process, regulatory approval and consumer acceptance.

8.6 Sources of further information and advice

There is a considerable basic research programme underway in a number of research institutions around the world into metabolic pathways of lactic acid bacteria, particularly those associated with amino acid metabolism. In most cases the objective is to gain a better understanding of potential control points in cheese aroma formation which in turn will lead to increased flavour intensity, increased flavour diversification, the development of more consistent flavour profiles and the control of off-flavours in fermented dairy products. There is also an increasing awareness that flavour and aroma control is dependent on the combined action of a range of adjuncts and starter cultures. Currently there are a number of research groups involved in understanding the basic mechanisms of flavour development, with two main groups at the forefront of this research. These are the French group at Unité de Recherche de Biochimie et Structure des Protéines, INRA, 78352 Jouy-en-Josas, France, and the Dutch group at NIZO Food Research, Department of Flavour, Nutrition and Ingredients, PO Box 20 6710 BA Ede, The Netherlands. Organisations such as NIZO Food Research Institute are also able to carry out screening assays of specific enzymes for clients wishing to evaluate their own range of potential adjunct organisms.

Information on the commercial application of adjuncts can be obtained directly from the major dairy culture supply companies. Chr Hansen (www.chr-hansen.com) supply a range of Flavor Control™ cultures developed specifically to enhance flavour and overall cheese quality without affecting the cheesemaking process. In cheeses such as Cheddar these cultures reduce ripening times while they also enhance flavour development in low-fat cheeses. Danisco Food Ingredients (www.danisco.com) also have a range of adjunct cultures under the CHOOZIT™ range developed from lactic acid bacteria and intended for use in semi-hard and hard cheeses. Their range of adjuncts consist of strains of *Lb. plantarum*, *Lb. helveticus*, *Pediococcus acidilactici*, *Lb. casei* and an attenuated *Lc. lactis* subsp. *cremoris* starter. Another major culture supply company, DSM Food Specialties (www.dsm-dairy.com), supply adjunct cultures under their DELVO-ADD® range. These organisms have been selected to develop additional flavours and/or textures in cheeses and include strains of *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lb. casei*, *Lb. helveticus*, *Lb. rhamnosus*, *Ln. mesenteroides* and *Sc. thermophilus*.

At present it appears that the use of commercial NSLAB adjunct cultures is not widespread; however, it is believed that adjunct cultures will play an increasingly important role in the development of specific cheese brands or for

developing consistent regional cheese flavours. Much of the information on the effectiveness of commercial adjunct cultures is derived from customer trials and therefore the results are usually confidential. Furthermore the results obtained are often not applicable to other countries and regions or even to other cheese factories, which means that individual factories wanting to achieve a certain flavour profile within a specified time will have to work closely with the adjunct culture supplier. It is also a reminder that while a range of screening procedures can be utilised to select potential adjunct cultures, the ultimate test will always be how the adjunct culture performs in a customer's cheese production plant. In addition it is important that a rigorous sensory evaluation system be implemented in order to gain the most value from any adjunct culture trial (see Chapters 17 and 18).

8.7 References

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9

Techniques for microbial species identification and characterization to identify commercially important traits

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9.1 Introduction

The metabolic activities of different microbial cultures account for the unique flavors of the more than 1000 varieties of cheese worldwide. The lactic acid bacteria (LAB) are the most important group of organisms in cheese production and ripening, with a few other organisms playing roles in some cheeses. Of the 12 recognized genera of LAB, only five – *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Leuconostoc* and *Enterococcus* – are believed to play a role in cheese flavor. Of the non-LAB, bacteria such as *Propionibacterium* and *Brevibacterium*, as well as molds such as *Penicillium* and *Geotrichium*, play important roles in certain cheeses. Identification, differentiation and characterization of cultures, as well as their commercially relevant traits, are therefore very important for cheese manufacturers. It can be argued that excellent cheese was being made before cheese bacteria were ever identified. Even today, many excellent cheeses are made, particularly in Europe, using undefined cultures without any knowledge of the individual organisms present. While this is true, manufacturers of these cheeses cannot control variability between batches and year-to-year. While this lack of consistency in quality is tolerated in some markets, it is not in many other markets such as the major American consumer markets. Lack of precise knowledge of the cultures involved and their important characteristics also limits the ability to improve cheese products and develop new varieties. The development of tools for identifying cultures and their traits important for cheese production and flavor has greatly improved the ability of cheese manufacturers to control the parameters needed for a consistent quality

product. This chapter provides an overview of bacterial speciation and the use of current tools for removing ambiguity from determining speciation. It also discusses tools for differentiating between different strains within a species, as it is specific strains within a species rather than whole species that are important for different characteristics in cheese production and flavor. Techniques for analyzing characteristics important for cheese production are also discussed, as well as the relevance to cheese manufacturers.

9.2 Techniques for microbial species identification

Lactococcus lactis is the most important species involved in cheese production worldwide and was the first of the LAB identified. Louis Pasteur (1857) first described this bacterium from a microscopic analysis of soured milk and 16 years later Joseph Lister isolated it in pure culture and called it *Bacterium lactis* (Lister, 1873). Further advances in phenotypic and biochemical characteristics of this bacterium resulted in its inclusion in the genus *Streptococcus* in 1909 (Löhnis, 1909) and subsequent hybridization and immunological studies in the 1980s resulted in the generation of the genus *Lactococcus* (Schleifer *et al.*, 1985).

This history of the evolution of the taxonomy of *L. lactis* over 100-plus years from Lister until 1985 is a good example of how all microbial taxonomy has evolved. Phenotypic and biochemical characteristics were the cornerstone of taxonomy until the additional nucleic acid hybridization, immunological, protein electrophoretic profiles and phage typing techniques were developed to supplement them and provide more sensitivity for speciation of microbes. In the last 25 years, DNA sequence analysis, particularly of the 16S rRNA gene, is the absolute standard of bacterial taxonomy and has removed much of the ambiguity that frequently occurred from relying solely on classical approaches. Carl Woese (1987) was one of the pioneers of this technology, which essentially classifies all microorganisms based on the nucleic acid sequence analysis of their 16S rRNA genes. Related microorganisms have higher sequence identities than unrelated organisms. The majority of researchers consider 16S rRNA sequences of 97% identity or higher to be within the same species. There are exceptions, however, as some bacteria have >97% sequence identity and are still sufficiently different to warrant a different species. This gene is referred to as a phylogenetic molecule as it can determine the phylogeny of microbes based on sequence similarity. For a gene, or other DNA region, to be used as a phylogenetic molecule it needs to be universally present to enable a universal comparison of microbes. It also needs to be easily obtainable such that its sequence can be determined. This essentially means that it needs to have at least two universally conserved sequence regions such that primers can be designed to target these conserved sequence regions and amplify the gene/DNA sequences bracketed by these primers using the polymerase chain reaction (PCR). The third requirement for an accurate phylogenetic characterization is that the gene, or

DNA region, be highly conserved. That is, its sequence should change very slowly over evolutionary time. Not all genes or intergenetic regions (non-coding DNA regions) change evenly over evolutionary time. DNA regions that are not essential for survival and competition of an organism will accumulate mutations much more frequently than essential DNA regions. This is especially relevant when an organism is exposed and adapted to very stressful conditions as DNA repair systems are less active and only essential DNA regions get repaired. Therefore, if a non-essential gene was used as a phylogenetic molecule, it could rapidly accumulate a substantial number of mutations in strains that are exposed to stressful conditions over long periods of time, relative to strains in less stressful environments. This would therefore not be an accurate measure of microbial phylogeny and could result in inaccurate taxonomy. The 16S rRNA gene is an ideal phylogenetic molecule as it is universally present, contains bordering universal sequences for amplification by PCR and is one of the most conserved genes in the cell.

Other loci are sometimes proposed as phylogenetic molecules, as the 16S rRNA gene is sometimes not sensitive enough to differentiate between very closely related species. The most common region to be employed for this purpose is the region between the 16S and 23S rRNA genes, termed the intergenic spacer region (ITS). This region is again universally present and is bordered by universally conserved sequences present on the 16S and 23S genes. However, it is generally not essential and is highly mutagenic, thus providing the necessary sensitivity to readily differentiate between closely related species (Gurtler and Mayall, 1999). In instances when these sequences may be subjected to uneven evolutionary pressures, they may not be reliable for discerning accurate phylogenetic relationships. However, because of their ease of use and greater sequence differences, they have found widespread acceptance.

Other DNA sequences are proposed as reliable alternatives for phylogenetic molecules. A small portion of the *recA* gene was proposed as it is universally present, plays important roles in the cell (for example, DNA recombination and SOS mediated DNA repair) and contains universally conserved sequences (Kullen *et al.*, 1997b). This has been used for the successful phylogenetic characterization of bacterial genera, such as *Bifidobacterium*, *Arthrobacter*, *Frankia* and *Acidothermus* (Kullen *et al.*, 1997b; Marechal *et al.*, 2000; van Waasbergen *et al.*, 2000). It was also used to phylogenetically sub-classify the *Lactobacillus casei* group, a very important dairy culture group of bacteria (Felis *et al.*, 2001).

A portion of the gene for the heat shock protein (HSP60) is also proposed as a useful phylogenetic marker for some bacteria (Kwok *et al.*, 1999). It was used to classify different *Bifidobacterium*, *Staphylococcus*, *Macrococcus* and *Heliobacter* species (Jian *et al.*, 2001; Kwok and Chow, 2003; Mikkonen *et al.*, 2004). As both the *recA* and *hsp60* genes are important to the well-being of cells, their use as phylogenetic molecules is scientifically strong as they are not subject to uneven evolutionary changes compared to DNA regions that have limited or no importance to the cell.

9.2.1 Classical speciation of dairy cultures

Classifying any unknown culture typically starts with a phenotypic analysis, usually Gram staining and microscopic analysis, an approach unchanged since the 1800s. This is especially useful for dairy cultures as they are composed of both cocci- and rod-shaped bacteria. Of the five genera of LAB generally associated with cheese, four are cocci (*Lactococcus*, *Streptococcus*, *Enterococcus* and *Leuconostoc*), although *Leuconostoc* cells often appear elongated under some conditions, and one (*Lactobacillus*) is rod-shaped. Following a phenotypic analysis, growth characteristics and biochemical tests provide further insights into their representative species (Table 9.1). Growth temperature can further subdivide the four cocci genera, as *S. thermophilus*, a thermophilic culture (the only *Streptococcus* species associated with cheese), and most enterococci can grow at 45°C. *S. thermophilus* can be further differentiated from enterococci by its inability to grow at 10°C or in the presence of 6.5% salt. Determining the species of an *Enterococcus* is challenging as it is a very diverse genus and phenotypic methods alone do not reliably differentiate between species (Devriese *et al.*, 1993; Kirschner *et al.*, 2001). However, only four species of *Enterococcus* (*faecalis*, *faecium*, *durans* and *hirae*) are typically reported to be associated with cheese out of more than 30 total species. Various proposals to combine some species and add new ones, such as by Naser *et al.* (2006), indicate uncertainty concerning the actual number of species in this genus. As recently as 1986, only four species were listed for this genus (Mundt, 1986). A listing for approved bacterial names, www.bacterio.cict.fr, currently lists 37 enterococcal species. Recently, a new species *E. italicus* was proposed, that is associated with some Italian-style cheeses (Grazia Fortina *et al.*, 2004). *E. durans* and *E. hirae* are members of the *E. faecium* group and this group is differentiated from *E. faecalis* by the inability to ferment sorbitol or grow in the presence of 0.4% potassium tellurite (Devriese *et al.*, 1993). *E. faecium* is differentiated from the other two species of this group by its greater sugar catabolic abilities: *E. hirae* and *E. durans* cannot ferment arabinose or mannitol, while *E. durans* is even further redundant in sugar catabolism, being negative for sucrose and melibiose. Table 9.2 summarizes a selection of useful diagnostic phenotypic characteristics for the cheese-associated enterococci.

Lactococcus lactis is the only species of *Lactococcus* usually associated with cheese and is the best characterized of all the cheese-related bacteria. It is also the easiest to identify using phenotypic characteristics: non-motile; homo-fermentative; grows at 10°C, but not at 45°C. However, as phenotypes, such as cell morphology, can alter under different situations, the misclassification of *L. lactis* isolates as *Lactobacillus* species has occurred (Stiles and Holzapfel, 1987). Within the *L. lactis* species two important subspecies, *lactis* and *cremoris*, are used extensively as starter cultures in the manufacture of most cheeses. These can be differentiated from each other by the inability of subspecies *cremoris* to grow at 40°C (the maximum growth temperature is 38°C) or in the presence of 4% salt. Subspecies *lactis* is further subdivided into the biovars *lactis* and *diacetylactis*, with the latter biovar having the ability to produce diacetyl from citrate.

Table 9.1 Comparison of selected characteristics of *Lactococcus lactis* with other cheese-associated lactic acid bacteria genera

Characteristic	<i>Lactococcus lactis</i>		<i>S. thermophilus</i>	<i>Enterococcus</i>	<i>Leuconostoc</i>	<i>Lactobacillus</i>
	<i>lactis</i>	<i>cremoris</i>				
Morphology	Coccus	Coccus	Coccus	Coccus	Coccus	Rod
Contains plasmids	Yes (many)	Yes (many)	Some (few)	Some (few)	Some (few)	Some (few)
Lactic acid type(s)	L	L	L	L	D	D, L, DL ¹
Growth at 10°C	+	+	—	+	+	± ²
Growth at 40°C	+	—	+	+	±	+
Growth at 45°C	—	—	+	+ ³	—	±
Growth in 4.0% salt	+	—	+	+	+	+
Growth in 6.5% salt	—	—	—	+	+	±
Growth in M17G	+	+	+	+	+	—

¹ Lactic acid type varies among species.

² Growth varies among species.

³ Exceptions have been reported.

Table 9.2 Selected phenotypic characteristics of cheese-associated enterococci¹

Characteristic	<i>E. faecalis</i>	<i>E. faecium</i>	<i>E. durans</i>	<i>E. hirae</i>
Growth at 10°C	+	+	+	+
Growth at 45°C	+	+	+	+
Growth in 6.5% salt	+	+	+	+
Growth in 0.4% potassium tellurite	+	—	—	—
Acid from melibiose	—	+	—	+
Acid from melezitose	+	—	—	—
Acid from sucrose	+	+	—	+
Acid from arabinose	+	+	—	—
Acid from mannitol	+	+	—	—
Acid from sorbitol	+	—	—	—

¹ Phenotypic exceptions have been reported for different species.

Lactococcus lactis is unusual in that it contains a large number of genes that are essential for growth in a milk environment on plasmids. This may be due to a relatively recent evolutionary adaptation to the milk environment, or it may be a response to a need for higher gene dosage of certain genes for better competitive abilities. From a practical viewpoint, this renders many of the essential traits needed by the starter culture unstable when they are cultured in different media. Following the first detection of plasmids in *L. lactis* (Cords *et al.*, 1974), the phenotypic instability of the traits needed for growth in milk is due to plasmid loss (Efsthathiou and McKay, 1976). However, a profile of their plasmids is strain specific and is often used to differentiate *L. lactis* strains. Plasmid profiles of *L. lactis* and other LAB are readily obtained in four hours or less (O'Sullivan and Klaenhammer, 1993).

While *Leuconostoc* is infrequently found in cheese, *Leu. lactis* was found as part of the non-starter lactic acid bacteria in an English Cheddar (Williams *et al.*, 2002). A similar study of the non-starter lactic acid bacteria in an American Cheddar did not find *Leuconostoc* (Swearingen *et al.*, 2001). They differ from *Lactococcus lactis* in that they are more salt tolerant, produce D- rather than L-lactic acid, and are heterofermenters, thus producing CO₂ from glucose metabolism (Hemme and Foucaud-Scheunemann, 2004).

The lactobacilli are a very heterogeneous group with the number of new approved species growing very rapidly. The listing for approved bacterial names at www.bacterio.cict.fr listed 88 species of *Lactobacillus* in 2003 (Coeuret *et al.*, 2003). Currently, it lists 131 species for this genus. Detection of lactobacilli in cheeses is relatively straightforward as they are the dominant rod-shaped LAB. Starter cultures frequently include lactobacilli, with *L. delbrueckii* subsp. *bulgaricus* and *L. helveticus* the most commonly used. Certain strains may also be used as flavor adjuncts. Regardless of addition of lactobacilli, they generally dominate during the ripening period. Species such as *paracasei*, *casei* and *curvatus* are frequently found, with the latter species associated with calcium crystal development in aged Cheddar cheeses. Speciation is frequently

accomplished using a battery of biochemical tests, generally using commercially available kits, such as the API 50 CH kit (BioMerieux, NC, USA) or the BBL Chrystal Gram positive ID Kit (Becton Dickson, MD, USA).

While phenotypic identification using commercial kits is frequently done for many different LAB, it is often not appreciated that the results are often incorrect. Phenotypic testing has always been subject to ambiguity and just because an API kit gives a result, it doesn't mean it is right. There are numerous examples of ambiguous identification or misidentification using commercial phenotypic kits for LAB. The use of an API 50 CH to identify 25 lactobacilli that were isolated from yogurts and cheeses resulted in ambiguous results for 16 of them (Andrighetto *et al.*, 1998). A study comparing API 50 CH kits with molecular methods using 30 type strains of lactobacilli found unreliable results with the kits and cautioned against relying solely on metabolic identification strategies (Nigatu, 2000). Misidentification of lactobacilli, especially closely related species, was also found in other studies with API 50 CH (Tynkkynen *et al.*, 1999). A recent study using the BBL Chrystal Gram positive ID kit for speciation of enterococci found that three strains identified positively by the kit as *E. casseliflavus* were actually two *E. faecium* and one *E. faecalis* (Jurkovic *et al.*, 2006). This clearly indicates that caution needs to be exercised with analyzing phenotypic data for identification purposes.

9.2.2 Molecular speciation of dairy cultures

While molecular speciation of microbes is now the norm in many fields, its widespread application to dairy cultures occurred much more slowly than in other fields, and even today is not universally applied. As discussed earlier, identification methods based on a sequence analysis of the 16S rRNA gene or other suitable phylogenetic molecule within the cell removes much of the ambiguity that classical phenotypic approaches are prone to. Technically it is very straightforward nowadays to obtain the 16S rRNA gene sequence of a culture. First a set of primers that target universally conserved regions of this gene are obtained. There are many proposed primers that work well for the amplification of all or segments of the 16S rRNA gene that work well for the LAB, such as the universal primers developed by Lane *et al.*, (1985). Following amplification and sequencing, they can be compared with the extensive databases available at the National Center for Biotechnology Information.

Species-specific probes were developed based on the variable regions of the 16S rRNA gene and these can speciate colonies via DNA hybridization. For many species of LAB, these short oligonucleotide probes provide definitive results. They can even separate at the subspecies level in some instances. Salama *et al.*, (1991) developed probes that target a region of the 16S rRNA gene that differed between *Lactococcus lactis* subsp *lactis* and subsp *cremoris* by 10 base pairs (bp). While these can subspeciate *lactis* or *cremoris* using hybridization, this can be challenging technically, given the close relationship between them. Recently, in my lab primers were designed bracketing this 10 bp region, and

following amplification via PCR, a single sequence reaction easily and unambiguously subspeciated 19 new *L. lactis* isolates.

The use of microbial genomics is providing a new dimension in identification methodologies for microbes and dairy LAB specifically. This enables primers to be designed that target genes unique to certain species or genera and greatly substantiate current methodologies. It will also allow companies to definitively track the use of their strains, even if different names are used. Every strain has its own unique nucleic acid polymorphisms and by analyzing the entire genomes a unique pattern of polymorphisms will uniquely identify the organism. By compiling a number of such polymorphisms, analogously to DNA testing of humans where 13 or more polymorphisms are used to positively identify an individual, DNA testing of cultures for individuality will also occur. While only a few genomes for the LAB are currently published, genomes for the majority of the commercially relevant species will be in the public domain before this book is published. This will largely be due to the publication of 11 new genomes by the Lactic Acid Bacterium Genome Consortium, which is discussed in Chapter 10.

9.3 Differentiation between strains within a species

The ability to rapidly confirm the identity of a strain or to differentiate between a number of isolates of the same species is very useful when working with cheese cultures. Traditional methods of solely relying on certain phenotypes are not sensitive enough if other strains also have the particular traits that are looked at. A good example occurred in my laboratory when a graduate student was developing a PCR-based DNA fingerprinting methodology for strain differentiation of LAB. He tested the technique with different *L. lactis* strains around the Department, and in one afternoon found that an *L. lactis* strain in another laboratory that was believed to be strain JS102 and was the subject of numerous theses and papers over 20 years previously was actually another strain. This clearly illustrates that a strain differentiating genetic fingerprinting technique would be very useful for routinely checking cultures for confirmation purposes.

Genetic fingerprinting of bacterial isolates plays a very important role in all fields of applied microbiology. Examples of DNA-based fingerprinting methods used for bacterial comparison purposes are plasmid profiling (Torre *et al.*, 1993; Morrison *et al.*, 1999), DNA hybridization (Erlandson and Batt, 1997; Gory *et al.*, 1999), ribotyping (Brosch *et al.*, 1996; McCartney *et al.*, 1996), pulse field gel electrophoresis (PFGE) (Klein *et al.*, 1998; Singh *et al.*, 1999) and PCR (van Belkum and Niesters, 1995).

9.3.1 PFGE DNA fingerprinting of cultures

Among the entire set of DNA fingerprinting methodologies available for bacteria, PFGE is the most definitive, as it provides the complete genome on one gel and enables any substantial DNA deletions or additions to be detected.

Because of this its reproducibility is beyond reproach. PFGE was originally developed by Schwartz and Cantor (1984) to enable large DNA fragments to migrate through an agarose gel and has undergone numerous technical updates. Today there are two types of pulse field strategies that are commonly used. The first involves equal pulse times of a constant electric current at various angles around the gel in such a geometry that the DNA fragments migrate in the desired direction. The second type involves pulsing of an electric current 180° with the forward pulse lasting longer than the reverse pulse, a process also referred to as field inversion gel electrophoresis (Smith *et al.*, 1991). To obtain a PFGE fingerprint, the genome is cut into relatively few (5–50) large fragments and separated using either of the two methods described above, although the cyclical electric pulse method is more common. The fragments are obtained by digesting the genome with rare cutting restriction enzymes, which generally have an 8 bp recognition site or a 6 bp recognition site, which are statistically rare for the particular genome. Because the DNA fragments are large, they cannot be manipulated in aqueous solutions or they would be sheared mechanically. Therefore, all manipulations, including DNA isolation and restriction, are carried out on cells embedded in agarose. The agarose pieces with the restricted fragments are inserted into a well in an agarose gel and separated by PFGE based on fragment size. The resulting pattern of DNA fragments is referred to as a restriction fragment length polymorphism (RFLP) and is highly characteristic of the particular organism. Because of the requirement of purifying DNA within an agarose matrix, the methodology takes several days to complete and is technically challenging. However, results are very reproducible and can differentiate at the strain level.

9.3.2 Ribotyping dairy cultures

Ribotyping has become quite common for identification and differentiation of dairy cultures. It is the only fully automated DNA fingerprinting system and therefore is very amenable to commercial facilities. A culture is put in the machine and eight hours later a ribotype appears. By comparing the ribotype band pattern to an extensive database of ribotypes, the identity of an unknown organism can be obtained. The convenience of the system cannot be overstated, which has resulted in its popularity. However, the system has limitations that are often not realized and it can be incorrect when used for strain identification purposes. To understand the limitations of ribotyping it is important to understand what is occurring within the riboprinter.

A ribotype is essentially an RFLP consisting of the restriction fragments from a particular genome, which contain rRNA genes. To obtain a ribotype for an organism, it must first be cultured to obtain enough cells for the procedure. Total DNA is then isolated within the riboprinter and is totally restricted using a restriction enzyme. The restricted (i.e. digested) fragments are subsequently separated using agarose gel electrophoresis and ultimately hybridized with a probe to the 16S rRNA. Following probe detection, restriction bands containing

copies of the rRNA genes are visualized and the pattern of the band sizes represents a ribotype. Within the ribotype pattern some DNA bands of low signal intensity are generally present. This is where the limitations occur, as many of these bands may not be reproducible. There are instances when these low intensity, or faint, bands can be reproducible, such as when two fragments of similar size co-migrate, thus increasing the intensity of some bands. However, many faint bands are often due to incomplete restriction of the genome. Reproducibility of total genome DNA restriction is extremely challenging, since different cultures will have different impurities that need to be removed during the DNA purification process and because of the large number of sites that need to be restricted. For this reason, the faint bands caused by incomplete DNA restriction can cause errors in the interpretation of a result. Using ribotyping for speciating a culture is therefore not a reliable approach. Besides this technical limitation, there is also the limitation of the sensitivity of the approach, as some genera of bacteria cannot be separated to the species level by ribotyping.

9.3.3 PCR based fingerprinting of dairy cultures

PCR was discovered by Kary Mullis during the 1980s (Mullis, 1990; Saiki *et al.*, 1988), and has since provided an array of fingerprinting applications. The original application of the PCR is to amplify a target sequence using two specific primers that bind both ends of the target DNA. While this feature has use as a bacterial identification method (Swaminathan and Feng, 1994; Hill, 1996), its reliability is not very high because only one band is generated. By targeting different sequences using different sets of specific primers in a multiplex-PCR reaction, its reliability is increased. Where primers and reaction conditions have been optimized, this technique has shown good use for fingerprinting purposes (Cormican *et al.*, 1995; Soumet *et al.*, 1999). Multiplex PCR has been used for detection of lactobacilli (Settanni *et al.* 2005), *S. thermophilus* (Mora *et al.*, 2003), *Leuconostoc* (Macian *et al.*, 2004), *Propionibacterium* (Meile *et al.* 1999), dairy enterococci (Goga *et al.*, 2003), *Lactococcus lactis* (Moschetti *et al.*, 2001) and *L. lactis* bacteriophage (Labrie and Moineau, 2000). Another strategy to increase the reliability of single-band PCR is to restrict the band with a restriction enzyme in order to obtain a restriction fragment length polymorphism (RFLP) of the band. By targeting the 16S rRNA gene, this strategy proved very amenable for differentiating bifidobacteria isolates (Kullen *et al.*, 1997a).

A novel approach, to use a two-primer specific PCR and get multiple bands for a genome fingerprint, was first to purify the DNA, totally restrict it into fragments using two different restriction enzyme ligate linkers (with compatible ends to the ligated fragments), and use primers complementary to the linkers to amplify all the different-sized restriction fragments (Vos *et al.*, 1995). This PCR-based fingerprinting system is called AFLP, which was chosen not as an acronym but because of its similarity to RFLP, given the bands obtained represent amplified restriction fragments. As discussed for ribotyping above, the

major limitation of this system is again that the total restriction of the genome is challenging and that partial restriction fragments will again give bands that are not reproducible. However, unlike in ribotyping the authors acknowledge this limitation and caution users about it.

9.3.4 Single primer PCR fingerprinting

PCR products using a single primer are generated through a primer binding to sites within a template that have full or partial homology to the primer and are positioned on opposite strands within a few thousand base pairs. Single primer PCR is essentially a survey of a genome for sites to which it shares full or partial homology. The homology between a primer and template, required to allow binding to occur, is dictated by the annealing conditions. The higher the stringency of the annealing conditions, the more homology is required. If a primer is used that targets repetitive sequences, high stringency conditions can be used, since multiple products will be obtained from loci having full homology to the primer. This form of single primer PCR fingerprinting is termed REP-PCR (Woods *et al.*, 1992) and has been developed for bacteria where REP sequences have been characterized. While REP-PCR is believed to be based on specific amplification of repetitive sequences, analyses of complete genome sequences indicate that this is incorrect and relies largely on arbitrary priming. This is consistent with the various band intensities that REP-PCR generates, which is similar to arbitrary primed PCR, which uses a primer of arbitrarily chosen sequence in a low stringency reaction to encourage binding to sites with partial homology.

The use of short arbitrary primers in a PCR for detecting polymorphisms in genomic DNA was first developed in 1990: random amplified polymorphic DNA (RAPD-PCR; Williams *et al.*, 1990) and arbitrarily primed PCR (AP-PCR; Welsh and McClelland, 1990). A third variation, DNA amplification fingerprinting (DAF; Caetano-Anolles *et al.*, 1991) was subsequently described. While all derivations are based on the same theoretical principles, RAPD-PCR is the most commonly used term.

Single primer PCR has been used to differentiate a wide variety of organisms including many of the LAB (Cancilla *et al.*, 1992; Cocconcelli *et al.*, 1995; Cusick and O'Sullivan, 2000; Czajka *et al.*, 1993; Boerlin *et al.*, 1995; Farber and Addison, 1994). When compared with other DNA fingerprinting systems, single primer PCR is generally very sensitive, being able to discriminate to the strain level, often comparable to or more sensitive than PFGE. When both techniques are employed greater strain diversity can be detected, as was demonstrated during the analysis of *Lactobacillus delbrueckii* subsp *lactis* strains isolated from Italian cheeses (Giraffa *et al.*, 2004). Given that single primer PCR is typically inexpensive, not technically demanding, requires no prior characterization of the organism, and uses very little template DNA, its use is limited only by the fact that it has been found susceptible to slight changes in experimental conditions, resulting in reproducibility problems. While these problems

can diminish confidence in RAPD or AP-PCR fingerprints (Gao *et al.*, 1996), they can be addressed and corrected from the final fingerprint. The strategy is to identify bands that may be subject to irreproducibility due to slight changes in reaction conditions from one day to another. This can be done by dividing reactions in three and running triplicate reactions each with a varied reaction. Using this strategy, changes in reaction conditions are deliberately introduced to allow the irreproducible bands to be detected, which are then not utilized in the fingerprint analysis. This is the basis of triplet arbitrary primed (TAP)-PCR, which runs the triplicate reactions on a gradient block thermocycler using three different annealing temperatures (Cusick and O'Sullivan, 2000).

9.3.5 Real-time PCR and dairy cultures

Real-time PCR allows quantitative analysis of a specific culture in a mixed culture situation. Therefore, it has the potential for monitoring the performance of individual starter culture strains during a fermentation.

Real-time PCR in its simplest form incorporates SYBR Green into the reaction, which will bind any double stranded DNA (dsDNA) amplicons that are generated and emit a fluorescent signal. Monitoring the fluorescent signal gives a measure of the accumulation of dsDNA in the reaction tube and, because the measurement is continuous throughout the reaction, rather than a one-time end point measurement of product as in traditional PCR, it reveals quantitative data on the target organism. This system was used to quantify *Lactococcus lactis* subsp *cremoris* during a mixed culture milk fermentation (Grattepanche *et al.*, 2005). A limitation of this type of real-time PCR is that the accumulation of any dsDNA in the reaction tube will emit the fluorescent signal and any non-specific products will also contribute to the signal. To have confidence in the quantitative results from this system, some confirmatory approaches are needed.

To address the limitations of the above system, a novel fluorescent probe technology was developed to enable the emitted fluorescence to come only from the specific target of the primers and not from any arbitrary priming that may be occurring (Heid *et al.*, 1996). This originally developed system consisted of a probe that hybridizes to a sequence between the two primers of the target region. The probe contained a 5' fluorescent dye and a 3' fluorescence quencher and was referred to as a TaqMan probe. The close presence of the quencher prevents the emission of fluorescence from the dye. During the annealing step the probe would anneal to its target as the primers are also annealing to theirs. As the *Taq* DNA polymerase extends the primer and approaches the probe, it releases the fluorescent dye using its 5' nuclease activity, thus causing the emission of fluorescence (Fig. 9.1). To ensure that the TaqMan probe remains bound to its target until the *Taq* DNA polymerase approaches it, the extension must occur at the same temperature as the annealing. As the annealing and extension temperatures are the same, it needs to be 60°C or more to ensure the 5' nuclease activity. This does make it more difficult for the low G+C LAB. However, with the advent of complete genome sequences, designing appropriate primers and

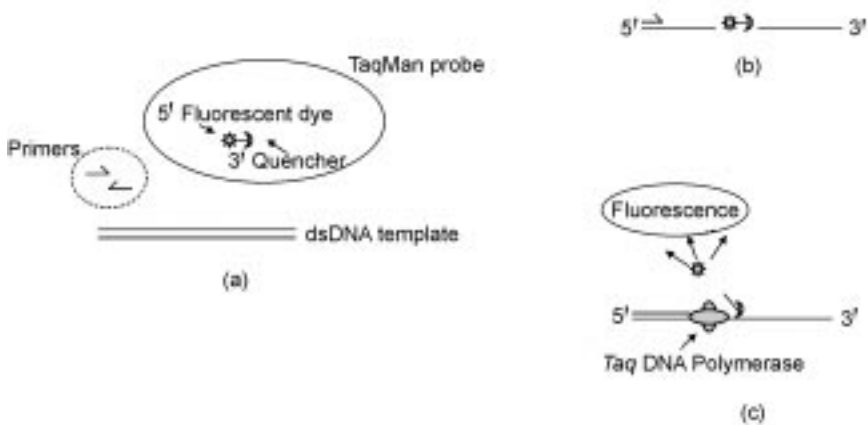


Fig. 9.1 Specific and quantitative real-time PCR using a TaqMan probe approach. A. Pertinent components in the reaction prior to denaturation. B. Illustrates the annealing of a primer to its target following denaturation and the simultaneous annealing of the probe to its target. C. During extension of the primer by *Taq* DNA polymerase, its 5' nuclease releases the fluorescent dye from the quencher, causing fluorescence.

probes is much more feasible. There are also slight variations of the TaqMan probe that do not require 5' nuclease activity and therefore do not have the 60°C requirement. One of these uses probes referred to as molecular beacons, which consist of two probes linked by a large ssDNA linker. Both the 5' and 3' probes are complementary to each other and at low temperatures, both probes bind together forming a stem loop structure that does not emit fluorescence due to the close proximity of the 3' quencher to the 5' fluorescent dye. However, following annealing of both probes to their respective regions on the target DNA (designed such that they are far apart), fluorescence is emitted as the quencher is now far away. This and other variations are reviewed by Espy *et al.* (2006).

9.4 Analysis of commercially important traits

Tremendous strides have been made over the years in understanding traits of cultures that are important for cheese manufacture. While a complete picture of all the traits required for production of a premium quality cheese is not yet available, there are a number of important established characteristics to use initially. For starter cultures, it is most desirable to have fast acid production and proteolytic systems that do not result in the generation of bitter peptides. This latter feature is extremely important as it directly impacts the quality of the cheese. Analytic methods for peptide release during casein breakdown primarily involve HPLC analysis and now frequently mass spectrometry where both size and amino acid composition can be discerned (Larsson *et al.*, 2006). These

analytic techniques allow potential ‘flavor’ profiles of different cultures to be predicted in the laboratory prior to picking cultures for starters or adjuncts.

A very important trait for commercial uses of cultures in cheese production is protection from the prevalent bacteriophage (phage) in commercial cheese plants. This is critical to enable optimal performance by the culture. Following screening for cultures that contain the desired characteristics for the cheese that is being manufactured, it is essential to address its phage susceptibility. To address this, cultures can be grown in the presence of isolates of phage species that are most prevalent. In the US, small isometric headed phage of the *Siphoviridae* family are the most prevalent in cheese plants. While testing phage collections against a culture gives a good idea of the susceptibility of a strain, it should also be supplemented by testing whey samples from the commercial plants where the culture is going to be used for any inhibitory effect on growth rate. Control whey samples treated with UV radiation to inactivate phage should be used to control for other inhibitors in the whey. Frequently, the most desirable culture selected for cheese production may be quite susceptible to phage. To combat this, suitable phage-resistant plasmids from other cultures can be introduced into the desired culture.

The dairy cheese environment has proved to be very suitable for the evolution of lactic phage. To combat this, certain strains have developed numerous ways to protect the cell. Understanding how these systems function has facilitated the protection of desirable starter cultures. The majority of the natural phage defense mechanisms that have evolved in *Lactococcus lactis* are plasmid encoded, allowing for their transfer into starter strains of interest. There are numerous reviews detailing phage resistance in *L. lactis*, including naturally evolved defenses as well as engineered systems (Sturino and Klaenhammer, 2004).

The enzymatic capabilities of specific cultures dictate the flavor characteristics of cheese. These enzymes include proteases, peptidases, aminopeptidases, aminotransferases, lipases and esterases. Screening cultures for desirable enzyme activities and other phenotypes is very time consuming. As the genes encoding individual enzymes are known, cultures can now be screened rapidly for enzyme profiles using DNA probes or PCR. These tools provide a means for screening new cultures for desirable characteristics in a high-throughput fashion.

9.5 Future trends

Technological advances for characterization and analysis of cultures over the last 20 years has been quite dramatic. Refinement of these technologies and development of new ones will clearly continue quite rapidly. In the dairy industry phenotypic approaches for culture identification and characterization are still often the sole technologies used. The last decade has seen the gradual introduction of molecular tools by some companies, not to replace classical approaches but to add an extra dimension to their analytical capabilities. This trend is likely to continue, especially as technologies continue to be refined.

Rapid tools for authentication of cultures, as discussed above, will likely grow in popularity, as they are invaluable components of any effective quality control system.

The availability of complete genomes of dairy cultures will likely accelerate the large-scale use of molecular technologies, as this will make it more feasible to design a technology for a specific purpose. Microarray technology is now being used to uncover genes that are important for different aspects of cheese production. Data availability is clearly growing exponentially and will uncover new traits that are important for cheese production and flavor. This will greatly enhance selection criteria for starter and adjunct cultures and will also reveal tempting approaches for improving existing cultures.

9.6 Sources of further information and advice

This chapter covers information on current technologies for identification and characterization of dairy cheese cultures. A number of reviews referenced in this chapter provide further details on specific topics covered. The review of Coeuret *et al.* (2003) provides further detail on phenotypic identification techniques as applied to lactobacilli; Hemme and Foucaud-Scheunemann (2004) provide further detail on phenotypic characterization of *Leuconostoc*; Sturino and Klaenhammer (2004) provide a detailed account of phage defense systems for lactococci; and Espy *et al.* (2006) provide an updated and detailed account of real-time PCR. Established companies exist that provide full-service molecular identification of unknown bacterial isolates based on a sequence analysis of the 16S rRNA gene. MIDI, Inc., Newark, DE (www.midi-inc.com) is one such company. While these companies do a good technical job of obtaining the sequence, their computerized analysis can sometimes be in error. This is frequently due to the presence of sequences in their databases that have not been 100% deciphered. However, a manual observation of the sequence analysis data can readily detect such errors. While there are numerous Internet sources, the American Society for Microbiology (asm.org) and the Society for General Microbiology (sgm.ac.uk) are excellent sources for updated news on all aspects of microbiology, including techniques for microbial characterization.

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10

Genomics and cheese flavor

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10.1 Introduction

Genomics has revolutionized the biological sciences. The invention of high-throughput DNA sequencing methods has evolved to provide entire genome sequences for microbes within hours. This rapid pace of production for genetic information has created a need to develop methods to visualize large amounts of data so that biologically relevant meaning can be merged with the genetic sequence information. This requires that genetics, biochemistry, computer science, and statistics become intimately intertwined so that the entire picture of the genome, proteome, and metabolome can be viewed.

Fermented foods research is a challenging application for genomics because it also requires the combination of the multiples with the addition of variability of production conditions, such as milk supply, starter culture combinations, nonstarter growth, and different ripening conditions. In spite of these challenges, research in cheese flavor has produced long lists of chemicals that are found in various cheeses over the past 50 years. However, the mechanisms for production of these compounds have remained elusive until recently, which was precipitated by consumer demand for low fat cheese varieties.

Use of genomics and functional genomics in cheese flavor production is rapidly becoming possible. The mechanistic answers to long-standing flavor questions are becoming available at a rapid pace, which has the possibility to transform our understanding of how cheese flavors are produced during aging. Access to genome sequence of lactic acid bacteria is the initial step in the effort to define the specific mechanisms of flavor compound production. Use of functional genomic tools, such as gene expression arrays and metabolomics, is beginning to provide details about starter culture and nonstarter culture

metabolism during cheese ripening. These results hold promise to provide insights into starter culture selection for production of specific flavors and accelerated aging programs.

10.2 Genomics

The rate at which new genome information is accumulating is staggering and challenging scientists' ability to collect, process, and understand these data. At the time of publication the number of finished and draft microbial genomes open to public use was over 900, with the total genomes available exceeding 2000. The challenge for food scientists is shifting from obtaining more genome sequences to the more difficult aim of using and understanding the impact of specific gene expression patterns to cheese flavor production in the laboratory and directly from cheese during ripening.

Production of a genome sequence is only the beginning of a new road to discovery. The heart of the discovery lies in the new fields of functional and comparative genomics, along with proteomics (Fields, 2000) and metabolomics (i.e. high-throughput biochemistry). The need to sequence large genomes, such as those of humans, has resulted in the development of instrumentation to generate genome sequence very fast. For example, the Joint Genome Institute (JGI; Walnut Creek, CA, USA) sequenced the genome of *Enterococcus faecium* in a single day (JGI, 2000a, b) in 2000, but today they can produce that same sequence in less than four hours. This organism is often multi-drug resistant and is commonly associated with food as a fecal contaminant. *Pseudomonas fluorescens*, a food-associated spoilage microbe, was also sequenced by JGI (JGI, 2000c) and the genome is freely available for analysis and application to the dairy industry. This sequencing capacity at the JGI led the Lactic Acid Bacteria Genome Consortium (Weimer and Mills, 2002) to sequence 11 genomes of bacteria involved in food fermentation and probiotic applications (Makarova *et al.*, 2006). However, the first lactic acid bacterium (LAB) genome reported was that of *Lactococcus lactis* ssp. *lactis* IL1403 (Bolotin *et al.*, 2001). Subsequently, a number of LAB genomes related to cheese production have been released (Table 10.1) (reviewed by Kok *et al.*, 2005). Companion to this are the bacteriophage genomes that have been completely sequenced (Table 10.2).

Comparative analyses between bacterial genomes are very interesting and hold great promise to provide new practical information because their genomes are very dynamic (Hughes, 2000). Gene duplication, translocation, inversion, deletion, and horizontal transfer are often found to facilitate genome rearrangement, which has already been found in lactococcal genomes (Campo *et al.*, 2002; Davidson *et al.*, 1995). Presumably, such rearrangements mediate rapid strain evolution and adaptation (Guédon *et al.*, 2000; Hughes, 2000). Comparing sequenced genomes is an excellent technique in beginning to understand genome plasticity and how it impacts the metabolism of organisms used for flavor production.

Table 10.1 Genomes available for public use or bacteria related to dairy applications. These organisms are a summary of cheese-related genomes hosted at NCBI. Only organisms related to cheese fermentations were included

Organism	Genome size (MB)	Plasmid content	Notable attributes	Classification	Comments
<i>Lactococcus lactis</i> ssp. <i>lactis</i> IL1403	2.37	0	Laboratory strain	Firmicute	Bolotin <i>et al.</i> , 2001
<i>Lactococcus lactis</i> ssp. <i>cremoris</i> SK11	2.4	5	Cheese making strain	Firmicute	Makarova <i>et al.</i> , 2006
<i>Lactobacillus casei</i>	2.5	0	NSLAB used as a flavor adjunct	Firmicute	Makarova <i>et al.</i> , 2006
<i>Lactobacillus helveticus</i> CNRZ32		0	NSLAB used as a flavor adjunct	Firmicute	University of Wisconsin and Utah State University; draft genome; reduces bitterness
<i>Lactobacillus plantarum</i> WCFS1	3.35	3	NSLAB used as a flavor adjunct	Firmicute	Kleerebezem <i>et al.</i> , 2003
<i>Lactobacillus rhamnosus</i> HN001			NSLAB used as a flavor adjunct	Firmicute	Fonterra; produces unique flavors
<i>Lactobacillus delbrueckii</i> ssp. <i>bulgaricus</i> 11842	1.86	0	Cheese and yogurt production	Firmicute	van de Guchte <i>et al.</i> , 2006; genome reduction
<i>Lactobacillus delbrueckii</i> ssp. <i>bulgaricus</i> BAA-365	2.3	0	Cheese and yogurt production	Firmicute	Makarova <i>et al.</i> , 2006; Small genome
<i>Lactobacillus brevis</i>	2.8	0	Limited metabolic capability	Firmicute	Makarova <i>et al.</i> , 2006
<i>Leuconostoc mesenteroides</i> ssp. <i>mesenteroides</i>	2.13	0	Sometimes found in cheese	Firmicute	Makarova <i>et al.</i> , 2006

Table 10.1 Continued

Organism	Genome size (MB)	Plasmid content	Notable attributes	Classification	Comments
<i>Oenococcus oeni</i> PSU-1	1.8	0	Wine fermentation	Firmicute	Makarova <i>et al.</i> , 2006; malolactic fermentation
<i>Oenococcus oeni</i> BAA1163	1.76	0	Wine fermentation	Firmicute	Université Bourgogne; malolactic fermentation
<i>Pediococcus pentosaceus</i> ATCC 25745	1.76	0	Cheese contaminant	Firmicute	Makarova <i>et al.</i> , 2006
<i>Streptococcus thermophilus</i> LMD-9	1.8	0	Cheese and yogurt fermentation	Firmicute	Makarova <i>et al.</i> , 2006
<i>Streptococcus thermophilus</i> NCRZ1066	1.8	0	Cheese and yogurt fermentation	Firmicute	Bolotin <i>et al.</i> 2004
<i>Streptococcus thermophilus</i> LMG18311	1.8	0	Cheese and yogurt fermentation	Firmicute	Bolotin <i>et al.</i> 2004
<i>Propionibacterium freudenreichii</i> ssp. <i>shermanii</i> CIP103027			Swiss cheese starter culture	Actinobacteria	Genoscope; flavor and carbon dioxide production
<i>Brevibacterium linens</i> ATCC9174	4.2	1	Used as a flavor adjunct in Cheddar cheese	Actinobacteria	Makarova <i>et al.</i> , 2006; extensive sulfur metabolism and proteolysis

Table 10.2 Complete bacteriophage genome sequences. These organisms are a summary of cheese-related genomes hosted at NCBI. All are dsDNA viruses that are members of the Siphoviridae family. Additional gene sequences for other phage are available, but they are not noted here. Only complete phage genomes are listed

Host	Phage	Genome size (bp)	Arrangement
<i>Lactococcus</i>	c2	22,172	linear
	Q54 ¹	26,537	
	P335	36,596	
	UL36	36,798	linear
	TP901-1	37,667	linear
	BK5-T	40,003	circular
<i>Lactobacillus plantarum</i>	Phi JL-1	36,674	linear
<i>Lactobacillus casei</i>	A2	43,411	linear
<i>Lactobacillus</i> spp.	Phi adh	43,785	circular
<i>Lactobacillus plantarum</i>	LP65	131,522	circular

¹ Not found in genbank, reported by Fortier *et al.* (2006).

The initial effort in LAB genomics has been to compare the structural features of these genomes. Comparative genomics is a field that is emerging from the flood of available genome sequence information in an effort to assess the link between genome structure and phenotypic characteristics and it provides understanding as to how organisms evolved. For example, Bolotin *et al.* (2004) compared the genome sequences of two *Streptococcus thermophilus* strains and concluded that this organism has undergone extensive genome reduction during its evolution (reviewed by Hols *et al.*, 2005). Similar conclusions were made from the comparison of *Lactobacillus bulgaricus* strains (van de Guchte *et al.*, 2006). Makarova *et al.* (2006) conducted the largest genome comparison to date for any set of organisms and found that all LAB evolved via genome reduction from a common *Bacillus* ancestor. This work has redefined the entire phylogeny of the LAB based on the entire genome sequence, opposed to relatively few phenotypic characteristics. Comparison of organisms that have not been sequenced is also possible by hybridizing the DNA from the unknown organism to genome arrays constructed based on the sequenced organism. In this case, a whole genome from organism A is fragmented into small pieces and hybridized to a genome array from the genome sequence of organism B. The amount of similarity between the two organisms is determined by the pattern of spots on the array. Weimer's group used different strains of lactococci to reveal many differences between individual genes among closely related laboratory and industrial strains with this technique (Table 10.3). The data indicate that industry 1 and MG1363 are the most closely related to IL1403 at 99% and 86% hybridization, respectively. This approach was particularly useful in determining specific prophage content between different strains (Table 10.4). The strains with the most similar prophage to content of IL1403 are SK11, industry 1 and FG2 at 62 (82%), 60 (82%), and 59 (81%) elements, respectively. This type of

Table 10.3 Genomic hybridization to determine strain relatedness in lactococci. The gene expression array was constructed as described by Xie *et al.* (2004) using a single gene probe per ORF as designed based on the *L. lactis* IL1403 genome sequence. The hybridization percentage was determined by dividing the total number of probes that hybridized by the total number of ORFs on the array (~2100). The percent hybridization indicates the similarity to IL1403 since that genome was used to create the array used in the hybridization

Lactococcal strain	% Hybridization
Industry 1	99
MG1363	86
FG2	73
SK11	71
LM0230	46
JLS450	40
Industry 2	37
ML3	35
Industry 3	26
Industry 4	24

analysis provides insight into genetic variability that will lead to metabolic differences without sequencing the entire genome of all the organisms of interest. Thus, this type of comparative approach is useful for starter culture strain selection to modify the production of flavor compounds.

The next logical step in comparative genomics is to conduct comparisons for metabolic capabilities that go beyond the genome structural similarities that have been studied so far. These comparisons are now possible using a number of bioinformatic tools. For example, direct comparison for sugar metabolism is possible between a starter culture and an adjunct or nonstarter organism (Table 10.5). Notice that fucose/rhamnose degradation is not possible in *L. lactis* IL1403, while *L. plantarum* has four genes involved in this ability. This provides a direct comparison of metabolic ability before adding the cultures together in cheese, so as to avoid unintended flavor compounds or to give the pairing a new capability. This step is directly applicable to cheese flavor as it will determine specific biochemical elements that represent the core metabolic pathways and abilities for a genus or species for use in cheese flavor modification.

10.3 Functional genomics

Gene expression arrays are the immediate next step for the functional use of the genome sequence. Essentially, expression arrays monitor how the cell communicates and regulates the biological functions. To make an expression array, small segments of DNA (i.e. probes that are homologous to the genome) are anchored to a glass slide or membrane. Subsequently, RNA is collected during the experiment and reverse transcribed into cDNA that is labeled with a

Table 10.4 Prophage content between various lactococcal strains as determined using genomic hybridization. *L. lactis* IL1403 has all of these prophage elements. The number 1 indicates presence of this gene in the respective strains. The total sum indicates the number of prophage in the respective strain that are similar to IL1403

Gene	Lactococcal strain										Annotation
	Industry 1	Industry 2	Industry 3	Industry 4	MG 1363	FG2	SK11	LM 0230	ML3	JLS 450	
pi101	1	1				1	1				pi1 protein 01, integrase
pi103	1						1		1		pi1 protein 03, transcriptional regulator
pi104	1					1	1			1	pi1 protein 04, transcriptional regulator
pi105		1				1	1			1	pi1 protein 05
pi107	1				1	1	1	1	1	1	pi1 protein 07
pi108		1	1			1	1	1			pi1 protein 08
pi109	1					1	1			1	pi1 protein 09
pi110	1	1			1	1	1			1	pi1 protein 10, transcriptional regulator
pi111						1	1			1	pi1 protein 11, recombinase
pi112	1					1	1		1		pi1 protein 12
pi116	1		1			1	1	1			pi1 protein 16
pi121	1				1	1	1	1			pi1 protein 21
pi122	1			1		1	1	1			pi1 protein 22
pi124	1					1	1				pi1 protein 24
pi125	1					1	1			1	pi1 protein 25
pi127	1			1		1	1	1		1	pi1 protein 27
pi128	1					1	1	1		1	pi1 protein 28
pi129						1	1	1		1	pi1 protein 29
pi130						1	1	1		1	pi1 protein 30
pi131	1					1	1				pi1 protein 31
pi132	1					1					pi1 protein 32
pi133	1	1						1		1	pi1 protein 33, terminase small subunit
pi134	1				1		1				pi1 protein 34, terminase large subunit

Table 10.4 Continued

Gene	Lactococcal strain										Annotation
	Industry 1	Industry 2	Industry 3	Industry 4	MG 1363	FG2	SK11	LM 0230	ML3	JLS 450	
pi135	1						1			1	pi1 protein 35
pi136	1			1		1	1				pi1 protein 36, prohead protease
pi137	1				1	1	1	1	1	1	pi1 protein 37, capsid protein
pi138	1					1	1	1		1	pi1 protein 38
pi139		1	1	1		1	1	1			pi1 protein 39
pi140	1	1				1	1				pi1 protein 40, tail component
pi141	1					1	1	1		1	pi1 protein 41, tail component
pi142		1	1			1	1				pi1 protein 42, small structural protein
pi143	1					1	1		1		pi1 protein 43
pi144				1			1	1			pi1 protein 44, tail component
pi146	1			1		1		1		1	pi1 protein 46, tail component
pi147	1				1	1	1				pi1 protein 47
pi201	1			1		1	1	1			pi2 protein 01, integrase
pi202	1					1	1				pi2 protein 02
pi203	1				1	1	1				pi2 protein 03
pi204	1					1	1				pi2 protein 04 hypothetical protein
pi205		1				1	1		1	1	pi2 protein 05
pi206	1				1	1		1			pi2 protein 06
pi207	1					1	1			1	pi2 protein 07
pi210	1		1			1	1	1			pi2 protein 10
pi211	1		1			1	1				pi2 protein 11, topoisomerase
pi212	1					1	1	1		1	pi2 protein 12, ssDNA binding protein

pi213	1				1	1	1	1			pi2 protein 13, replisome organizer
pi214	1						1				pi2 protein 14
pi215	1					1				1	pi2 protein 15
pi223	1					1	1	1			pi2 protein 23
pi227	1					1	1	1	1		pi2 protein 27
pi228	1				1	1	1		1	1	pi2 protein 28
pi229	1					1	1		1	1	pi2 protein 29
pi230	1			1			1			1	pi2 protein 30, terminase
pi231	1					1	1		1	1	pi2 protein 31
pi232	1	1				1	1				pi2 protein 32
pi233						1	1		1		pi2 protein 33, capsid protein
pi234						1	1	1			pi2 protein 34
pi237	1	1			1			1	1		pi2 protein 37
pi238	1							1	1		pi2 protein 38
pi239	1						1			1	pi2 protein 39
pi240	1				1		1			1	pi2 protein 40
pi242	1				1	1	1			1	pi2 protein 42
pi243	1		1		1	1	1	1			pi2 protein 43
pi244	1	1	1	1	1	1					pi2 protein 44
pi245	1				1	1	1	1		1	pi2 protein 45
pi246	1				1		1	1			pi2 protein 46
pi247	1				1	1	1	1	1		pi2 protein 47
pi248	1				1	1	1	1		1	pi2 protein 48
pi249				1	1	1	1	1		1	pi2 protein 49
pi252					1	1	1		1	1	pi2 protein 52, muramidase
pi301	1	1			1			1	1		pi3 protein 01
pi305	1		1	1	1			1	1	1	pi3 protein 05, muramidase
pi303	1		1			1					pi3 protein 03
Total	60	13	10	11	23	59	62	34	17	33	

Table 10.5 Metabolic comparison of sugar utilization between *L. lactis* IL1403 and *L. plantarum* WCSF1. Numbers indicate the total gene content related to the specific function listed

<i>L. plantarum</i>	<i>L. lactis</i>	Function
271	195	Carbohydrate metabolism
97	68	Carbohydrate biosynthesis
146	100	Carbohydrate assimilation
19	21	Polysaccharide degradation
14	11	Carboxylic acid degradation
21	18	Alcohol/aldehyde degradation
2	6	Uronic acids degradation
4	4	Aminosugar degradation
14	10	Polyol degradation
1	2	Xylose degradation
6	3	Arabinose degradation
7	6	Arabinose/ribose degradation
2	1	Sorbose degradation
4	0	Fucose/rhamnose degradation
10	8	Galactose degradation
5	4	Fructose/mannose degradation
8	4	Glucose, allose, gluconate degradation
65	37	Oligosaccharide assimilation

fluorescent tracer molecule. This is hybridized to the array and a fluorescent signal is determined on small individual spots that contain the homologous probe. Interpretation of these spots indicates the transcripts (i.e. proteins) that were present or absent due to the specific cellular treatment.

Visualization of these data provide an unprecedented view of the complex web of metabolic networks that lead to flavor compounds, growth, and response to the conditions of cheese making. Xie *et al.* (2004) described the first gene expression array using small oligonucleotide probes for use with LAB in a functional setting related to cheese conditions. This study determined the influence of stress on lactococci and found the common and unique genes modulated by acid, salt, and heat stress. It also demonstrated that gene expression arrays provide useful and reliable information about gene expression, as this study verified every known stress-linked gene in a single experiment in addition to finding new stress-related networks that were previously unreported. Use of this array in cheese during ripening provided evidence that the arginine catabolic pathway is active and stimulated by a variety of nutrients and co-factors throughout ripening to 45 days (Fig. 10.1).

Use of gene expression arrays provides a set of genes that are regulated during a specific condition and at a specific time. To be truly useful for flavor production this information must be translated into biologically meaningful information. This is a significant challenge, especially for cheese flavor compounds, that is being addressed using high-throughput biochemistry (e.g. metabolomics) and computer science (e.g. bioinformatics).

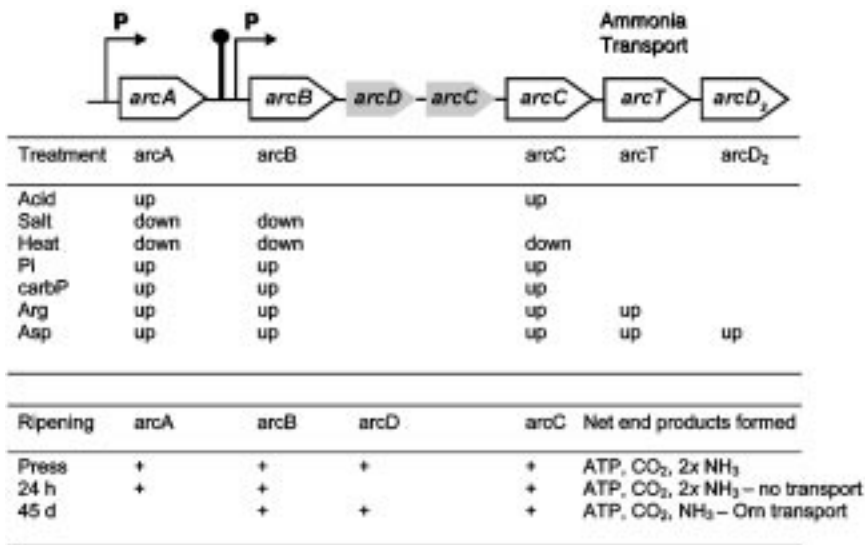


Fig. 10.1 Gene expression of the *arc* cluster for arginine catabolism. The treatments include abiotic stresses found during cheese making, nutrients and metabolic intermediates. Pi = inorganic phosphate, carbP = carbamylphosphate, Arg = arginine, Asp = aspartic acid. ‘Up’ and ‘down’ indicate induction and repression or gene expression, respectively. The ‘+’ indicates induction during cheese ripening.

Often, gene expression data need additional or supporting evidence to provide assurance that the metabolism extrapolated from the gene expression pattern is reliable. Ganesan *et al.* (2006) used NMR, gene expression analysis, and metabolic network maps to delineate the exact metabolic pathway used by lactococci to convert branched chain amino acids to branched chain fatty acids. This study demonstrates the usefulness of gene expression for flavor production, but it also found that the aminotransferase genes thought to catalyze the initial step of this transformation were not expressed in these conditions, rather another two of the possible nine genes were induced for this purpose.

Gene expression arrays are also useful to measure the impact of metabolic end products from flavor formation. For example, Pieterse *et al.* (2005) used microarray analysis to determine the impact of lactic acid exposure on *Lactobacillus plantarum*. Array analysis is also providing new tools to unravel the changes in metabolism due to nutrient availability. In this case, depletion of lactose in cheese during ripening is easy to measure, but the metabolic impact is very difficult. Stuart *et al.* (1998) determined that lactococci lose the ability to produce colonies after carbohydrate exhaustion, which is accompanied by production of methionine and serine into the medium – just as is observed during cheese ripening. Ganesan *et al.* (2006) extended this study using gene expression studies to confirm Stuart’s work and further demonstrate that the cells continue to transcribe RNA even without the ability to form colonies. This observation was used to demonstrate branched chain fatty acid production from amino acids

using gene expression arrays. The authors also demonstrated that this cellular state can last as long as three years without induction of the genes needed for cellular lysis. They proposed that ~0.001% of the starter culture lysed during this state. Hence, the culture appears to die because the culture cannot form colonies, yet the cells are intact and continue to metabolize peptides and amino acids to end products that impact flavor. The full impact of this metabolic state remains to be clarified during cheese ripening.

10.4 Bioinformatics and flavor

Working with DNA arrays requires immense data-handling capacity for analysis and visualization. A single DNA array with entire genome, even a small one, requires ~4500 individual spots of the DNA probes. A single experiment done with microarrays using two treatments (e.g. a single variable) and three replications results in 27,000 discrete data points for analysis. Statistical analysis is done using individual pair-wise comparisons, with a correction for the large number of multiple comparisons, to define the expression differences. Subsequently, these significant genes are associated with a biological function to determine the metabolic difference between the test conditions. Hence, visualization is a very important part of this process. To aid this process and to provide an ‘-omics’ warehouse for information, Karp’s group created a number of tools to bridge gene expression data and metabolism of small molecules (Paley and Karp, 2006). Metacyc (Caspi *et al.*, 2006) is a generalized inventory of metabolic pathways that is dynamic, as opposed to KEGG (see Section 10.6) that provides a static view of metabolism in a generalized fashion (Kanehisa, 2002), to provide a method to understand the metabolism that is running in the cell. Use of these bioinformatics tools provides a preview to the possible metabolism that may lead to flavor end products by individual LAB species. However, the specific biological conditions needed to induce production of these compounds are just beginning to emerge, especially as it relates to cheese flavor. Since these experiments are costly and time consuming, use of the predictive metabolic databases is a useful tool to direct the questions to study in order to improve flavor production.

Biocyc is a web-based database of over 160 organisms that allows complete customization of metabolic pathways for specific organisms (Karp *et al.*, 2005). This provides users with incredible predictive abilities to study the most important pathways for flavor production. Pathway Tools is the software suite that is used to create custom metabolic reconstruction databases for specific organisms based on the genome sequence. Databases are available for *L. lactis* IL1403 (biocyc.org; Notebaart *et al.*, 2006), *L. cremoris* SK11 (Fig. 10.2; www.biosystems.usu.edu), and *L. plantarum* WCSF1 (www.lacplantcyc.nl). These databases are available to query for specific compounds of interest. For example, once inside an organism database one can query for erythrose 4-phosphate and the database will return the metabolic pathway that is specific to

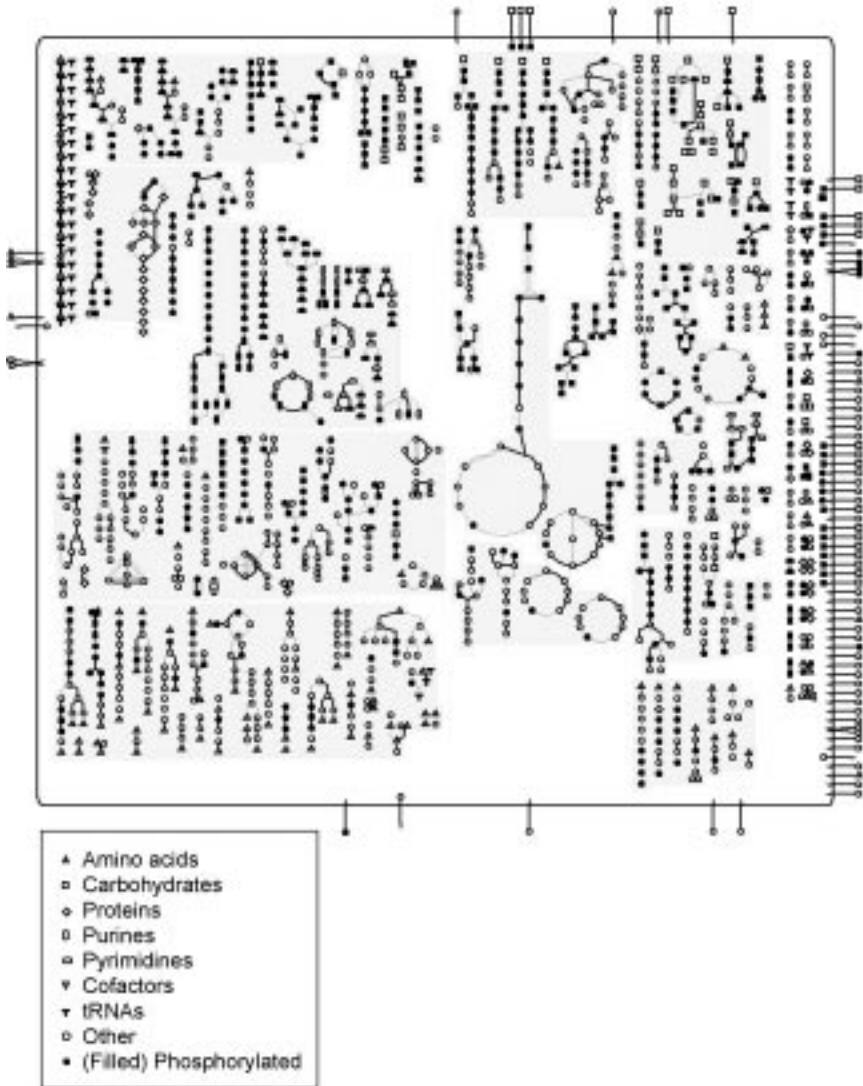


Fig. 10.2 The metabolic reconstruction map for *L. cremoris* SK11 (CremaCyc) created by Pathway Tools. Missing lines indicate missing genes in the genome, and hence incomplete metabolic pathways. This database to query the metabolic potential is available at www.biosystems.usu.edu.

that organism. Weimer's group created a tool as a plug-in to Pathway Tools that allows pathway interconnections to be displayed (Fig. 10.3). For example, a query with glutamic acid resulted in over 150 interconnections in *Lactococcus cremoris* SK11 clearly demonstrating the central role of this amino acid in lactococcal metabolism. This approach will be useful in understanding the system-wide interconnections that are used by LAB during growth and

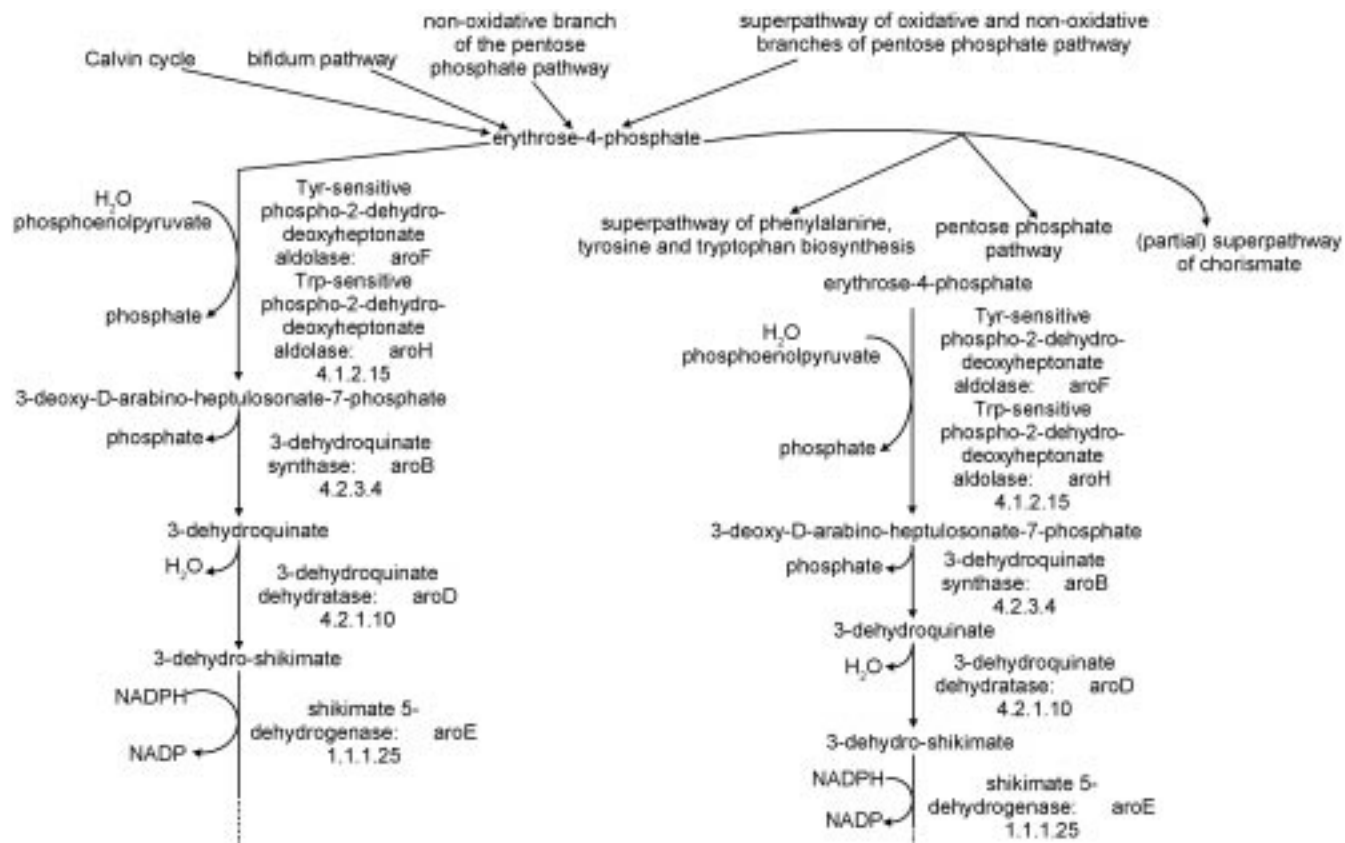


Fig. 10.3 Screen shot of the Pathway webbing tool for use with Pathway Tools organism databases. The query was erythrose 4-phosphate. Names of pathways leading into this compound indicate the metabolic pathways that produce this compound, while arrows leading away from this compound indicate routes of catabolism. This tool is available at www.biosystems.usu.edu.

metabolism during cheese ripening, so that processing conditions can be created that lead to production of a specific compound during ripening that is unique to the starter culture, nonstarter cultures, or the flavor adjunct.

10.5 Future trends

Genomics, proteomics and metabolomics hold great promise to uncover the metabolic mechanisms of flavor production during cheese ripening. To realize this potential is a matter of identifying and correlating the abiotic factors that lead to the complex biochemistry of LAB. Metabolic reconstruction tools are just beginning to provide biological insights that can be followed in the laboratory and during ripening. These insights will provide the entire picture of the metabolism in a single view so that modifications can be made for specific flavor end products. To do this more detailed experiments to uncover the mechanisms of genetic regulation in LAB are needed.

Additional bioinformatic tools to simulate cheese-ripening conditions for metabolic routes are needed. This will lead to inexpensive and fast methods to 'try' the experiment before it is conducted. Realization of the full impact of simulation tools based on '-omics' data will need a comprehensive database of gene expression, proteomic, and metabolomic data so that a complete picture of the entire metabolic system can be accessed during the simulation. This approach will provide a solid tractable basis to direct processing changes and substrate accessibility to provide the conditions conducive for flavor product formation. While difficult, this is a goal that is now achievable using genomics and functional genomics.

Strain-pairing programs have largely been based on phage resistance and fast acid production. Use of genomic information now allows more complex and flavor-directed strain-pairing programs to be developed. Use of genome hybridization will provide insights into proven industrial stains that have complex genetic components (i.e. plasmids and conjugation) for use in complex mixtures of strains to provide phage resistance, fast acid production, flavor production, and survival or lysis during ripening. Additionally, specific metabolic pathways that lead to unique flavor compounds can be added into the strain mixture with direct knowledge of the genes and enzymes involved in the conversion of interest.

Linking gene expression arrays to metabolomics provides a powerful tool for the study of flavor compound production. This approach allows the biochemistry of starter cultures to be directly linked to the processing conditions, milk type, and the ripening cheese conditions. This linkage is widely accepted for a few genes and molecules, but the specific parameters for exact control of flavor compound production are still missing for the vast number of compounds found in cheese. Now, cause-and-effect relationships can be established using the combination of genomics and metabolomics. This provides an exceedingly difficult challenge to assimilate all the data into a single database that can be visualized to make the needed conclusions and interconnections.

10.6 Sources of further information and advice

Biocyc (biocyc.org) – a collection of over 160 pathway databases for metabolic reconstruction of specific organisms.

GOLD (www.genomesonline.org) – provides current information about genome sequencing projects.

KEGG (www.genome.ad.jp/kegg/) – a suite of databases and software to simulate the metabolism of cells from its genome information.

Metacyc (metacyc.org) – a nonredundant metabolic encyclopedia of all the known pathways.

National Center Biotechnology Information (www.ncbi.nlm.nih.gov) – a genetic and bioinformatics resource within the National Institutes of Health that hosts GenBank files of genome sequences for public access.

The Institute for Genomic Research (TIGR; www.tigr.org/tdb) – a genome sequencing company that provides open source tools for genome analysis and access to genome information.

The Joint Genome Institute (www.jgi.doe.gov) – a genome sequencing facility hosted by the US Department of Energy that provides public access to draft and finished genomes.

The Sanger Institute (www.sanger.ac.uk) – a genome sequencing facility hosted by the Wellcome Trust Foundation that provides open source tools for genome analysis.

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Part II

Influence of ingredients, processing and physical and chemical factors on cheese flavour

11

The effects of milk, its ingredients and salt on cheese flavor

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11.1 Introduction

According to the Codex General Standard (FAO, 2003), cheese is defined as

the ripened or unripened soft or semi-hard, hard and extra hard product, which may be coated, and in which the whey protein/casein ratio does not exceed that of milk, obtained by:

- (a) coagulating wholly or partly the following raw materials: milk and/or products obtained from milk, through the action of rennet or other suitable coagulating agents, and by partially draining the whey resulting from such coagulation; and/or
- (b) processing techniques involving coagulation of milk and/or products obtained from milk which give an end-product with similar physical, chemical and organoleptic characteristics as the product defined under (a).

In addition to this global standard, individual countries have developed their own standards of identity of cheese as well. These standards, including the Codex standard for cheese, will continue to evolve depending on various factors, including economic, political and technological. While there may be subtle differences in these standards between countries, the basic principles of manufacturing cheese remain similar for any given variety.

A close examination of these basic definitions of cheese demonstrates that traditionally cheese truly consists of just a handful of ingredients: milk, which could be from various species; starter and other microorganisms for specific flavors; rennet; salt; potable water in some cheeses; color; preservatives. The

roles of some of these ingredients in flavor development in cheese, especially those of starter and other microorganisms, enzymes, including chymosin, and others, have been well defined and are discussed in other chapters. While cheese making utilizing these ingredients has been in existence for hundreds of years, spanning hundreds of different cheese varieties, in as many locations around the world, many technical developments have occurred in the dairy processing industry that have also permeated the cheese industry and have influenced the quality of cheese. These developments are in the form of processing techniques that directly impact cheese making and others that have enabled the development of novel ingredients that can be applied to cheese making. The term 'milk', for instance, has been expanded to include other milk-derived ingredients, each with unique characteristics and having the potential for cheese making. Examples include whey-derived liquids, buttermilk and concentrates. Not only have these developments influenced the economics of cheese making but they have also influenced flavor and texture quality of cheese and have enabled the development of new varieties of cheese.

Because the flavor of fresh cheeses is mainly the result of acid and flavor compounds generated by added bacteria within a very short time of cheese making, the discussion in this chapter will focus primarily on ripened cheese. The discussion will include the influence of the following:

1. Source of milk
2. Ingredients from milk and whey concentration processes
 - (a) vacuum condensing
 - (b) membrane concentration (ultrafiltration and microfiltration)
3. Salt (NaCl).

11.2 Source of milk

While the vast majority of cheeses around the world are manufactured from cow's milk, many varieties are exclusively made from milk of goats, sheep, water buffalo, or to a lesser extent camel, llama and perhaps other species as well (Table 11.1). Countries such as India and Italy are large producers of milk of water buffalo but only Italy produces relatively large quantities of cheese from this milk. Production of sheep and goat milk is prevalent in the Mediterranean countries, and in Greece in particular unique cheeses from sheep's milk can be readily found. Each of these milks possesses unique composition and flavor characteristics that are transferred to cheese and thus offer a distinctive quality to the cheese (Boyazoglu and Morand-Fehr, 2001).

Fat from goat and sheep milk typically contains larger proportions of short chain fatty acids (capric, caprylic and caproic fatty acids), medium chain and mono- and polyunsaturated fatty acids than does cow milk fat. These fatty acids give goat and sheep milk cheese a typically sharp and pungent flavor. The gross composition of milks from various species differs widely (Table 11.2). The composition of fat and proteins also differs, giving these components characteristic

Table 11.1 Examples of cheeses from goat, sheep and buffalo milk

Goat	Sheep	Water buffalo
Bougon	Akavi	Cheddar
Chabis	Broccio	Mozzarella
Couhe Verac	Cachcaval	
Poulligny Saint-Pierre	Feta	
Sainte-Maure	Halloumi	
Valency	Manchego	
	Pecorino Romano	
	Roquefort	
	Serra	

metabolic properties during cheese ripening. Ha and Lindsay (1991) reported that 4-ethyloctanoic acid was not present in cow milk cheese. Cow milk fat had low concentrations of 4-methyloctanoic acid but goat and sheep milk contained significant amounts of 4-methyloctanoic and 4-ethyloctanoic acids, which contribute to the typical flavor of goat and sheep milk cheese. Salles *et al.* (2002) also reported the importance of these compounds in goat milk.

The origin of some flavor compounds from sheep and goat milk is attributed to the feeding characteristics of these animals. Papamedas and Robinson (2002) reported that Halloumi cheese manufactured from a mixture of ovine and caprine milk contained higher proportions of terpenes such as α -pinene, β -pinene, copaene, thymol, α -caryophyllene, β -caryophyllene and δ -cadinene. These compounds were believed to have originated in the plants eaten by these animals. Thus, a blend of flavor compounds originating from milk components along with those from feeding habits of the animal determine the ultimate flavor profile of cheese produced from such milk.

In addition, for a given species, feed and geographical location also influence flavor. For instance, it is believed that the typical flavor of Parmigiano-Reggiano cheese comes not only from the ripening process, but also from the soil of the specific location in the Parma region where this cheese by Italian legislation can be manufactured (Kosikowski and Mistry, 1999). The continuous carryover of whey from one day to the next as starter for making cheese contributes to the development of flavor over its two-year ripening period.

Table 11.2 Composition of milk (%) from different species

Component	Cow	Goat	Sheep	Water buffalo	Camel
Fat	3.6	4.6	7.8	7.7	5.3
Casein	2.8	3.3	4.6	3.9	3.0
Lactose	4.9	4.4	4.7	5.0	3.4
Ash	0.7	0.8	0.9	0.8	0.7

Source: adapted from Kosikowski and Mistry (1997).

Milk from water buffalo generally contains more calcium and casein compared to that of cow milk (Table 11.2). This milk is very well suited for the manufacture of fresh Mozzarella, in which the typical flavor is the direct result of milk, unlike ripened cheeses where metabolism has to occur for flavor development. The manufacture of ripened cheeses such as Cheddar presents technological challenges because of the above-mentioned compositional characteristics. Acid development is generally very slow, rennet curd is firm and flavor development during ripening is slow (Kosikowski and Mistry, 1999). These problems have been addressed with the methods suggested by Czulak (1964), which include the reduction of calcium content by partial salting of whey prior to draining.

11.3 Concentrated milk

Cheese making is a process of removal of water from milk to induce flavor and texture development and ultimately for extending the life of milk in the form of cheese. The concept of concentrating milk prior to cheese making was introduced primarily for economics; instead of beginning with milk containing 88–90% water, if a portion of this water is removed prior to cheese making, the efficiency of cheese making would increase as there would be increased throughput per vat and increased yield. As these methods were adopted in cheese making, it was recognized that in addition to impacts on economics, the quality of cheese was also influenced. This led to the optimization of concentration procedures for cheese making, i.e., a balance had to be struck between economics and quality for optimizing cheese making conditions. Furthermore, the methods of concentrating milk have evolved over the past 50 years and introduced novel manufacturing opportunities. In the early years, concentration simply implied the concentration of milk through the removal of water under a vacuum. Later, with the development of permeable membranes for dairy processing, concentration was combined with selective separation. For example, ultrafiltration concentrates all milk components except the small water-soluble components such as lactose and soluble minerals. Microfiltration, depending on membrane pore size, will concentrate caseins and separate whey proteins from milk or simply remove bacteria without subjecting milk to pasteurization temperatures. These methods are thus capable of producing milks of distinctly different composition (Table 11.3).

Table 11.3 Composition of milk concentrates

	Control	Condensed	Ultrafiltration	Microfiltration
Total solids, %	13.00	16.93	15.09	14.77
Total protein, %	3.51	4.61	4.60	4.23
Casein, %	2.74	3.63	3.67	3.45
Ratio of casein to total protein	78.34	78.74	79.78	81.56
Fat, %	3.72	4.92	4.93	5.05

Microfiltration completely alters the proportion of casein to whey proteins by removing whey proteins from milk prior to cheese making. Further, all of these concentration techniques can be combined to produce concentrates of specific composition for use in cheese making. Because of the changes in the microenvironment of milk and ultimately cheese, the flavor development of cheese is also altered due to concentrated milk.

11.3.1 Vacuum concentration

Milk for cheese making may be directly vacuum condensed, or nonfat dry milk or highly condensed milk may be added as ingredients for standardizing milk to the desired protein and fat levels prior to cheese making. This is commonly practiced in circumstances either where there is a shortage of raw milk or when economics dictate the types of ingredients to use. In either case there are limitations for increasing the protein content of milk for cheese making with this method, because not only does the protein content of milk increase but the concentration of all milk components increases, including lactose and calcium. Academic research and industrial experience have demonstrated that with such methods, the total concentration of milk should not exceed 1.8:1 (i.e., the protein content of milk for making cheese should not exceed approximately 5.6%). This value coincides with over 8% lactose and 1.3% ash. With this level of lactose in the starting milk, there will be excessive residual lactose in the cheese, which results in continued excessive acid production during ripening. Research with the use of condensed milk for cheese making has focused on Cheddar cheese (Anderson *et al.*, 1993). Flavor of cheese manufactured with such milk is generally excellent regardless of milk concentration up to 1.8:1. At higher concentrations (i.e., >1.8:1), excessive lactose and minerals will cause slight sweetness and saltiness. In reduced-fat Cheddar cheese made from vacuum concentrated milk (>1.5:1 concentration), Anderson *et al.* (1993) noted excellent cheese flavor and flavor intensity during over two years of aging. This effect, which is not common in reduced-fat cheese, may be caused by the additional minerals present in concentrated milk as well as a low water activity. Methods suggested to reduce the lactose content include washing of curd with potable water during cheese making. This produces a bland flavor in cheese because of the removal of other flavor compounds as well with the wash water (Johnson *et al.*, 1998). It should be noted that commercial cheese makers have successfully used vacuum concentrated milk without any flavor problems. The concentration of milk is usually limited to approximately 1.25:1.

11.3.2 Membrane concentration

Membrane concentration has truly revolutionized cheese making since this technology was first introduced in the late 1960s in France (Mistry, 2002a). Many cheese plants in Europe use this technology for the commercial manufacture of cheese (Fig. 11.1). In addition, there have been numerous studies that



Fig. 11.1 Pavé d'Affinois, a new cheese developed with the ultrafiltration process.

have explored the impact of membrane technology on cheese making. Through this technology it is now possible to directly process milk immediately before cheese making or to also use prefabricated milk products in cheese making.

Membrane concentration applications in the dairy industry include reverse osmosis, nanofiltration, ultrafiltration and microfiltration, but the discussions here will focus on the latter two, which are already used commercially for cheese making. Milk produced by reverse osmosis is similar in composition to vacuum condensed milk and effects on cheese flavor are also similar (Agbevari *et al.*, 1983; Bynum and Barbano, 1985). Ultrafiltration of milk is generally conducted at approximately 50°C under pressure tangentially across an ultrafiltration membrane with a molecular weight cut-off of 10,000 to 100,000 daltons. Low molecular weight materials (i.e., water, lactose, and soluble minerals and vitamins) permeate through the membrane while the remaining components are retained to form a concentrate and used for cheese making.

Depending on cheese variety, the concentrate can be of low concentration (also known as protein standardization), medium, or high concentration (pre-cheese concept). Ultrafiltered milk possesses certain unique characteristics that have an impact on cheese making: viscosity, buffering capacity, and rennet coagulation properties (Mistry and Maubois, 2004). The latter two have a direct impact on flavor production in cheese.

During ultrafiltration of milk, proteins and colloidal salts are concentrated simultaneously and increase the buffer capacity, making it difficult to obtain the

desired pH even with the production of large amounts of lactic acid by the starter bacteria. A reduction in the rate at which pH drops allows lactic acid bacteria to grow to large numbers but also offers the potential for growth of spoilage and pathogenic organisms. The large amounts of lactic acid produced result in an acid-tasting product. The buffering capacity of ultrafiltered milk may be lowered by removing some of the colloidal salts by solubilization through the reduction of pH of milk to 5.6–6.0 during ultrafiltration or by diafiltration.

In ultrafiltered milk rennet coagulation occurs faster and curd is firmer. Firmness of rennet curd of unconcentrated whole milk as measured by a Forma-graph is approximately 8 mm after 40 min and that of 6% protein ultrafiltered milk is 58 mm. This occurs partly because of increased protein and calcium in the retentate and also because in ultrafiltered milk ($4\times$) hydrolysis of only 50% of the κ -casein is required for curd formation compared to 97% for unconcentrated milk (Dagleish, 1980).

Low concentration retentates (protein standardization)

Use of low concentration retentates (LCR) is a popular method because it is easily adaptable to most cheese varieties. Milk is ultrafiltered to a concentration of no more than $2\times$ (3.7–4.5% protein) followed by use of conventional cheese making procedures and equipment. This method is used for cheeses such as Camembert, Cheddar, Mozzarella and various others. Advantages of using this procedure include uniformity in milk composition from day to day, firm rennet curd and therefore lower losses of casein to whey, increased cheese yield (approximately 6% on protein basis), and improved cheese making efficiency (more cheese per vat). For Cheddar cheese, concentration of up to 1.6 – $1.7\times$ is commonly used. At higher levels the rennet curd is extremely firm and difficult to handle and fat losses to whey may be higher than desired. The moisture content of Cheddar cheeses made with this process decreases with protein content in milk because of rapid syneresis that is caused by coarser networks of protein gels. Employing standard procedures such as low-temperature cooking can increase the moisture content.

Medium concentration retentates

Milk is concentrated to 2 – $5\times$ prior to cheese making with or without diafiltration to adjust the mineral content and buffering capacity. High quantities of whey proteins are retained in the cheese and yield is also higher than with the LCR method. The changes in the physicochemical properties of milk discussed above are large enough to warrant use of specially designed equipment. Rennet curd, for example, is very firm and difficult to handle with conventional equipment. After various industrial trials commercial application of this method for cheeses such as Cheddar and Feta is limited.

Liquid pre-cheese

Milk is ultrafiltered to a concentration that is similar to the composition of the cheese being manufactured, followed by setting with rennet (Maubois *et al.*,

1969). Acid development takes place following treatments unique to the cheese variety being manufactured. No conventional cheese making equipment is required and of all the ultrafiltration methods, this method has the highest yield potential because of maximum whey protein retention in the cheese. This method is limited because it is not possible to achieve the composition of all cheeses by ultrafiltration. The process developed for this method was originally for Camembert cheese but it has also been applied to Feta cheese. Newer cheeses such as Pavé d'Affinois have also been developed using the liquid pre-cheese concept (Fig. 11.1) (Mistry and Maubois, 2004).

Characteristics of cheese from ultrafiltered milk

Depending on the level of concentration used, cheese made with ultrafiltered milk has a relatively higher concentration of whey proteins compared to conventional methods. Whey proteins undergo very little proteolysis during aging, thus flavor development is slow because of the increased concentration of these milks. Retarded proteolysis along with the high water-binding capacity of whey proteins also influences the texture of cheese. The high buffering capacity of such cheese also retards starter culture activity and proteolysis of casein. These effects become more pronounced as the whey protein concentration in cheese increases (cheese made from lower-concentrated ultrafiltered milk versus liquid pre-cheese concept). The impact of high mineral retention on cheese functionality and flavor is also of concern. Excessive calcium retention may lead to bitterness in fresh acid-curd cheeses. The latter occurs also because of increased buffering, which leads to high levels of starter cells. As indicated earlier, employing pre-acidification during ultrafiltration can control the mineral content of cheese.

Microfiltration of milk for cheese making provides the potential employing membrane of pore sizes ranging from 0.05 to 10 μm . This makes it possible to produce products of a wide range of characteristics for cheese making. Commercial applications of microfiltration for cheese making include processes for the removal of bacteria from milk, and standardization of casein of milk.

Removal of bacteria

Bacteria from milk can be effectively removed by a microfiltration process known as Bactocatch[®]. In this process raw skim milk is microfiltered at 35–50°C using a membrane of pore size 1.4 μm . The retentate contains bacteria and permeate is the bacteria-free milk, which can be blended with heated cream for standardization of fat. Bacteria removal efficiencies of 99.6–99.98% are reported.

This process is particularly suitable for manufacturing cheeses such as Swiss because of the possibility of removal of spores of *Clostridium tyrobutyricum* without using nitrates or excessively high heat. On the other hand, the extremely high efficiency of removal of bacteria by microfiltration presents situations in which the milk is stripped of non-starter bacteria and factors that are naturally present in milk and contribute to flavor development. French researchers have

demonstrated that under normal circumstances microfiltered milk is not ideal for eye formation in Swiss cheese because of the removal of non-starter lactic acid bacteria by microfiltration. This has been overcome by modifying the starter culture system. Specific heterolactic strains with mesophilic, thermophilic and propionic starters are recommended (Maubois, 2002). Similarly, in Camembert cheese making with microfiltration the addition of *Hafnia alvei* is required for typical flavor development. This bacterial species is able to produce volatile sulphur compounds from methionine in the cheese (Cousin, 1994).

Casein standardization

Using a microfiltration membrane of 0.1 μm pore size separation of casein and whey proteins is achievable. Casein content of milk is increased from 2.5% to 3.5% and whey protein content is decreased, hence cheese yield is increased. Depending on the actual concentration, the impact of this method due to buffering is similar to that of ultrafiltration as discussed above.

Milk protein concentrates

A blend of ultrafiltration, microfiltration, condensing and drying technologies enables the introduction of protein products commonly known as milk protein concentrates (MPC) (Mistry, 2002b). These products present novel technical possibilities in cheese making, and their impacts on cheese making are similar to those of ultrafiltration and microfiltration as discussed above with respect to buffering and its effect on acid production, inclusion of whey proteins and proteolysis during ripening. MPCs include dried products that range in milk protein content from 35% to over 85%. They can be applied to cheese making in either liquid or dried form.

MPCs have been introduced into cheese making, including natural and processed cheeses (Bhaskar *et al.*, 2001; Blazey *et al.*, 2001; Moran *et al.*, 2001a, b). Moran *et al.* (2001a) developed a continuous method for manufacturing processed cheese in which no base cheese is used but instead acidified milk is ultrafiltered and diafiltered to a concentration factor of $4\times$ to $7\times$, followed by evaporation up to 70% solids to give reduced-calcium pre-cheese. Processed cheese is made from this pre-cheese in the traditional manner using flavoring agents and emulsifiers, and the flavor of the cheese is similar to that of traditional cheese.

Microfiltration technology is used for the production of concentrates with a high micellar casein content of approximately 90% (Schuck *et al.*, 1994). When used in cheese making, increases in cheese yield are achievable (Caron *et al.*, 1997). Such powders also provide flexibility in usage regardless of the extent of heat treatment, because the κ -casein- β -lactoglobulin complex typically found in nonfat dry milk does not exist due to the removal of the β -lactoglobulin during microfiltration.

11.4 Influence of salt

The salt content of cheese depends on variety and ranges from 0.5% to over 5% in the moisture. Salt plays an important role in cheeses for flavor, texture and shelf life. For example, in low fat cheeses, the moisture content of cheese is higher relative to the full fat counterpart (Mistry and Kasperson, 1998). The salt-in-moisture content of low fat cheese is therefore reduced even though the actual salt content is similar. In full fat Cheddar cheese the moisture and salt content is 36% and 1.6%, respectively. In reduced fat Cheddar, they are 47% (or more depending on fat reduction) and 1.6%, respectively. Consequently, the salt-in-moisture contents of the two cheeses are 4.4% and 3.4%, respectively. Thus, inhibition of bacterial growth occurs to a lesser extent and lower fat cheese could readily develop flavor defects such as bitterness unless specific low proteolytic starter cultures are selected.

Salt influences the flavor of cheese in several ways: control of microbial growth, impact on enzyme activity, impact on water activity. Salt in cheese helps regulate pH of cheese and ultimately ripening and texture through the control of microbial growth. Salt is added to cheese in the dry form to curd, as in Cheddar cheese, or by immersing cheese in brine, as in Emmental. For cheeses such as Cheddar, salt is added when the lactose content has been reduced to less than 1% during the cheese making process. This added salt then retards the growth of lactic acid bacteria and helps control further acid development. It should be noted that at low levels of salt, some lactic cultures are stimulated (Irvine and Price, 1961). Thus, acid production at low salt levels will also result in high lactic acid bacterial cell numbers that may cause bitterness in cheese due to subsequent enzymatic activity (Guinee and Fox, 2004). It is therefore important that the salt-in-moisture levels in cheese be closely controlled. When inhibition of lactic acid bacteria occurs due to salt, salt-tolerant non-starter lactic acid bacteria are likely to continue the metabolism of lactose. The impact on activity of starter bacteria is mainly the result of reduced water activity.

Flavor in cheese is the result of numerous metabolic reactions that occur during ripening. Metabolism of caseins occurs to various degrees and leads to the formation of numerous flavor compounds. The role of α_{s1} - and β -casein is particularly important. The former undergoes proteolysis early in ripening to form various smaller peptides through the action of residual milk clotting enzyme in cheese. This activity is affected by the concentration of salt. Proteolytic activity is inhibited at higher levels of salt and stimulated at lower levels (6%, Guinea and Fox, 2004). On the other hand, lower levels of salt (5%) inhibit the proteolysis of β -casein through conformational changes. For example, Thomas and Pearce (1981) reported that more than 50% of the β -casein is degraded after 4 weeks of ripening with 4% salt-in-moisture but only 10% is degraded with 8% salt-in-moisture. Thus cheese with little or no salt tends to develop excessive acidity and bitterness during ripening because of unchecked lactic acid starter bacteria growth and enzyme activity. In Feta cheese, which has a relatively high salt content, its biogenic amine content is

low because of unfavorable conditions for amino acid decarboxylation due to low pH and high salt (Valsamaki *et al.*, 2000).

11.5 Future trends

Cheese making has evolved over the past centuries and will continue to do so in the future. Economics is the key driver for most new developments but in the process various phenomena also occur in the quality of cheese. As discussed above, new technologies such as membrane concentration of milk have provided opportunities for advances and automation in cheese making. In order to successfully employ these technologies, certain technical hurdles in cheese quality had to be overcome. Such occurrences will continue in the future as further technologies develop. The introduction of membrane technologies in particular has truly opened up new avenues for the development of novel ingredients from milk and whey through fractionation and concentration of these liquids. In addition to economics, impact of technologies on the environment will continue to be addressed, thus technologies for development of ingredients from products that were traditionally regarded as waste are likely to be developed, which will have an impact on sensory qualities of cheese. During the era of a surge on low fat cheese technology there were numerous developments in ingredients such as stabilizers and fat replacers. While the markets for low fat cheese seem to have stabilized, these technologies could reemerge in the future and require an evaluation of impacts of these ingredients on flavor. Finally, social reports from around the world seem to suggest that the demand for cheeses with sharp flavor has reduced among younger generations, but at the same time there also is a demand for newer products. Thus social aspects of cheese making for the development of optimally flavored products will need to be addressed.

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12

Physical factors affecting flavour of cheese

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12.1 Introduction

Directed cheese flavour development depends on retaining, adding or developing ripening agents in the milk and controlling their activity over time. Much of the research on cheese flavour development necessarily relates to microbiological and biochemical factors. However, those microbiological and biochemical factors are largely directed and limited by cumulative effects of physical factors during storage, transportation and processing of the milk, during subsequent cheese manufacture, and during ripening, storage and distribution. This is evident from the basic principles of cheese making.

Cheese making is the process of removing water, lactose and some minerals from milk to produce a concentrate of milk fat and protein. Cheese was likely developed as a means to preserve some of the nutritional components of milk. Or, perhaps more accurately, cheese happened during failed attempts to preserve milk. Implicit to these humble beginnings are three physical factors that are fundamental to cheese technology, namely, time, temperature and acidity (pH). Indeed, most physical and chemical properties of cheese can be described, to a large extent, as functions of time, temperature and acidity. This chapter describes the cumulative and interactive effects of physical factors on the principal determinants of cheese flavour and flavour perception, namely cheese microbiology, chemical composition, biochemistry and ultrastructure. The focus is on direct and indirect effects of physical factors during the early stages of cheese manufacture on subsequent development of cheese flavour. Optimizing physical factors during ripening is described in other chapters.

For the purpose of this discussion, physical factors include physico-chemical factors such as pH, buffer capacity (BC) and water activity (a_w). The focus is on

physical factors relevant to cheese making, not necessarily physical properties. Many physical properties of milk are well known, but not all are directly relevant to development of cheese texture and flavour. For example, surface tension, thermal conductivity, viscosity and density of milk have relevance to milk processing, including cheese manufacture, but are not very interesting for discussion of cheese flavour. Moisture (and a_w) and oxidation–reduction (redox) potential are of direct interest to cheese flavour and are discussed in the context of the relevant dynamic physical factors that apply during cheese making. Other relevant physical factors are physical history of the milk, size and geometry of curds and cheese, pressure history, physical aspects of curd handling treatments, and environmental factors such as exposure to copper and light.

12.2 The general relationship between cheese composition, structure and flavour

Of particular interest to any discussion of the effects of physico-chemical factors on cheese are temperature and pH history of the milk and curd. Given cheese milk of a particular composition, it is the pH and temperature history that principally determine cheese composition and ultrastructure, and these in turn determine the basic cheese texture and to a great extent direct development of cheese flavour. Therefore, much of the discussion in this chapter is predicated on the principle that typical texture in a young cheese is prerequisite for development of typical cheese flavour (Adda *et al.*, 1982; Green and Manning, 1982; Lawrence *et al.*, 1983, 1984, 1987).

Perhaps most influential in this regard, is the early work of Lawrence and co-workers who described the basic structure of cheese with reference to apparent changes in the structure of the casein micelle (Lawrence *et al.*, 1983, 1984, 1987):

The structural unit in the protein matrix of Swiss or Gouda is essentially in the same globular form (10–15 nm in diameter) as in the original sub-micelles in the cheese milk. The structure of the casein sub-micelles is retained in these two cheese varieties even though the 6-casein has been converted to para-6-casein by the coagulant. In contrast, the protein aggregates in [the more acidic] Cheshire are much smaller (3–4 nm) and are apparently in the form of strands of chains, that is, the original sub-micellar protein aggregates appear to have lost almost all their identity. As expected most of the protein aggregates in Cheddar vary in size between those found in Gouda and in Cheshire, that is, between 4 nm and 10 nm. (Lawrence *et al.*, 1983)

This apparent relationship between cheese structure and the pH history of the cheese implies structural differences at the micelle and sub-micelle levels, but those changes have yet to be described in precise terms, although pH-induced

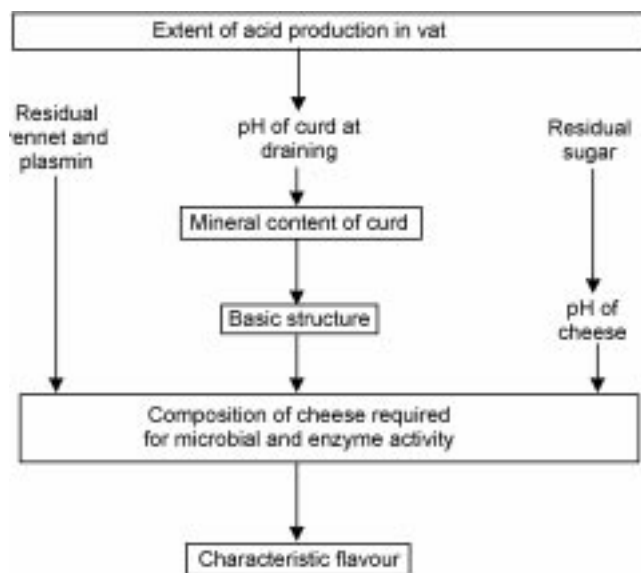


Fig. 12.1 The main factors that determine the basic structure and flavour of a particular cheese variety. Redrawn from Lawrence *et al.* (1984), with permission.

changes to the internal and surface properties of the micelle have been extensively investigated (van Vliet *et al.*, 2004).

It follows that, with respect to cheese quality, the objectives of cheese making are as follows:

1. To obtain the optimum cheese composition with respect to moisture, acidity (pH), fat, protein and minerals (especially calcium).
2. To establish the correct ultrastructure of the cheese.
3. To establish the optimum ripening conditions.

Objectives 1 and 2 are achieved by varying initial make procedures, most of which are different means to control the rate and extent of acid development and the rate and extent of moisture release. These principles are illustrated in Fig. 12.1 (Lawrence *et al.*, 1987).

Also largely based on these principles is Table 12.1, which describes cheese families grouped according to type of coagulation and procedures used for pH and moisture control. Table 12.2 gives composition of some cheese varieties, also grouped according to type of coagulation and procedures used for pH and moisture control. Any attempt to classify cheese on the basis of a few variables is necessarily simplistic and will allow many varieties to fall between the categories. Nevertheless, the general categories in Table 12.1 will serve as a reference point for subsequent discussions in this chapter. Also included for general reference is Table 12.3, which illustrates the effects of selected processing conditions on several process control parameters.

Table 12.1 Some properties of cheese categorized according to type of coagulation and procedures used for pH and moisture control¹

Category	Examples	Coagulation ²	MNFS ³	pH ⁴	Calcium ⁵	Ripening
Acid and acid-rennet coagulated	Fresh: cottage, quark, cream; ripened: Valençay, Harzer	Predominantly acid coagulation at pH 4.6–4.9; rennet: 0 1200 IMCU/1000 l 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, mould and/or smear ripened
Heat-acid coagulated	Cooking cheeses: paneer and channa, ricotta, requeson; cream: mascarpone	Whey proteins co-precipitate 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
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 flavour
Soft ripened	Feta, Camembert, Blue	Rennet+++; culture+++; ripening time+++; cutting at pH < 6.5	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

Table 12.1 Continued

Category	Examples	Coagulation ²	MNFS ³	pH ⁴	Calcium ⁵	Ripening
Mesophilic washed.	Gouda, Edam, Colby, Havarti, Montasio and many others	Rennet++; culture++; ripening time++; cutting at pH < 6.6	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 to remove lactose	500–700	2 weeks–12+ months
Mesophilic unwashed	Cheddar, Provolone	Rennet++; culture++; ripening time++; cutting at pH < 6.6	52–60%; a_w 0.940–0.960; controlled by cooking, curd handling, 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
Thermophilic	Swiss and hard Italian types	Rennet+; culture+; ripening+ or none; cutting pH 6.6–6.5	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

¹ Representative data from various sources is given to define broad ranges and trends only.

² '+', symbols indicate amounts of rennet and culture and ripening time relative to other categories.

³ Moisture in non-fat substance.

⁴ Minimum pH reached during manufacture or during first days of ripening.

⁵ mm kg⁻¹ non-fat-solids

Source: compiled from Hill (1995) and other sources.

Table 12.2 Typical composition (% by weight) of some cheese varieties

Type	Cheese	Moisture	Protein	Fat	Total CHO	FDM	Ash	Ca	P	Salt	% Salt in moisture	pH ¹
Acid coagulated	Cottage (dry curd)	79.8	17.3	0.4	1.8	2.1	0.7	0.03	0.10	nil	nil	5.0
	Creamed cottage	79.0	12.5	4.5	2.7	21.4	1.4	0.06	0.13	1.0	1.3	5.0
	Quark	72.0	18.0	8.0	3.0	28.5		0.30	0.35		0.0	4.5
	Cream	53.7	7.5	34.9	2.7	75.4	1.2	0.08	0.10	0.7	1.4	4.6
	Neufchatel	62.2	10.0	23.4	2.9	62.0	1.5	0.07	0.13	0.8	1.2	4.6
Heat-acid coagulated	Chhana	53.0	17.0	25.0	2.0	53.2				nil	nil	5.4
	Queso Blanco (acid)	55.0	19.7	20.4	3.0	44.8		0.28	0.24	3.0	5.5	5.4
	Ricotta (from milk)	72.2	11.2	12.7	3.0	45.7				0.5	0.7	5.9
	Ricotone (whey and milk)	82.5	11.3	0.5	1.5	2.9				0.5	0.6	5.8
	Queso Blanco (rennet)	52.0	23.0	20.0		42.0				2.5	4.8	5.8
Unripened-rennet coagulated	Queso de Freir	52.4	23.0	19.5		41.0				3.0	5.7	5.8
	Italian fresh cheese	49.0	28.0	16.0		31.4				nil	nil	6.5
	Camembert	51.8	19.8	24.3	0.5	50.3	3.7	0.39	0.35	2.1	4.1	6.9
Soft ripened high acid	Feta	55.2	14.2	21.3		47.5	5.2	0.49	0.34	3.0	5.4	4.4
	Blue	42.0	21.0	29.0	2.3	50.0	5.1	0.53	0.39	3.5	8.3	6.5
	Colby	40.0	25.0	31.0	2.0	51.7	3.4	0.68	0.46	1.8	4.5	5.3
Semi-hard washed	Gouda	41.5	25.0	27.4	2.2	46.9	3.9	0.70	0.55	2.0	4.8	5.8
	Edam	41.4	25.0	27.8	1.4	47.6	4.2	0.73	0.54	2.0	4.8	5.7
	Fontina	42.8	24.2	25.5		44.6	3.3			1.2	2.8	5.6
	Havarti (Danish)	43.5	24.7	26.5		46.9	2.8			2.2	5.1	5.9
	Munster	41.8	23.4	30.0	1.1	51.6	3.7	0.72	0.47	1.8	4.3	6.2
Hard cheese low-temp.	Cheddar	36.7	24.9	33.1	1.3	52.4	3.9	0.72	0.51	1.8	4.9	5.4
	Manchego (Spanish)	37.9	28.1	26.9		45.2	3.6			1.5	3.9	5.8
	Provolone	40.9	25.6	26.6	2.1	45.1	4.7	0.76	0.50	2.2	5.4	5.4
	Mozzarella	54.1	19.4	21.6	2.2	47.1	2.6	0.52	0.37	1.0	1.9	5.3
Hard cheese high-temp.	Parmesan	29.2	35.7	25.8	3.2	36.5	6.0	1.18	0.69	3.0	10.3	5.4
	Romano	30.9	31.8	26.9	3.6	39.0	6.7	1.06	0.76	3.0	9.7	5.4
	Swiss	37.2	28.4	27.4	3.4	43.7	3.5	0.96	0.60	1.0	2.7	5.6

¹ pH at retail.

Source: adapted from Hill (1995) and other sources.

Table 12.3 Depiction of particular processing conditions, assuming other factors do not change, on pH at draining, minimum pH occurring in the cheese during early stages of curing, calcium retained in the cheese, the rate of syneresis, the moisture in the non-fat substance (MNFS) and the amount of calf rennet activity retained in the cheese. The figure is intended to show trends that apply to most rennet-coagulated cheese within normal ranges of moisture content and percent fat in the dry matter. UE = Unknown effect, but likely small. NE = no effect. NA = not applicable.

Variable	Draining pH	Minimum pH	Ca ²⁺	Syneresis	MNFS	Rennet retention
Milk protein/fat	UE	UE	UE	—	—	UE
Pasteurization intensity	—	—	—	—	—	—
CaCl ₂ : amount added	—	—	—	—	—	—
Starter: amount added	—	—	—	—	—	—
Pre-ripening time ¹	—	—	—	—	—	—
Rennet: amount added	—	UE	UE	—	—	—
Renneting temperature	NE	UE	UE	—	—	UE
Curd size	NE	UE	UE	—	—	—
Stirring intensity	—	UE	UE	—	—	UE
Cooking intensity	—	UE	—	—	—	—
Time until draining ²	—	—	—	NE	—	—
Wash water amount	NA	—	—	—	—	—
Wash water temp.	NA	—	UE	—	—	—
Time until salting ³	NA	—	—	NE	—	UE
Salt: amount added	NA	—	—	—	—	UE
Pressure in the moulds	NA	—	UE	—	—	UE

¹ Time between adding culture and adding rennet.

² Total time between cutting and draining.

³ Total time between draining and salting; mainly applicable to Cheddar and American varieties. Source: after Walstra *et al.* (1987), Hill (1995) and various other sources.

12.3 General aspects of acidity in cheese

The principal and interacting physical, chemical and biochemical events taking place during the first 24–48 h of cheese making (especially up to the time of demoulding) are development of acidity, moisture reduction (concentration of protein and fat), and physical manipulation of the curd. Of these, acidity as monitored by pH is the most primary physico-chemical parameter. As already noted, pH largely determines the basic structure of cheese at the level of the

casein micelle matrix and its resulting functional properties, including hardness, friability, meltability and stretchability. The pH is critical to the growth and survival of spoilage and pathogenic microorganisms in cheese and as such strongly influences its safety and shelf life. Lactic acid contributes 'lactic' flavour, which is especially important to unripened cheese where it may be a dominant flavour. Lactate is also a critical substrate for microbial ripening agents in all ripened cheeses. For a given cheese where the basic process with respect to physical curd treatments is defined, pH is the most useful process control tool.

The pH of fresh milk is 6.6–6.8. The titratable acidity (TA) of milk (titration to pH 8.3) is 14–21°N (mM NaOH L⁻¹), averaging 17°N. The approximate distribution of the average TA of 17°N is 5.7, 0.9, 1.0, 7.8 and 1.7°N for casein, serum protein, colloidal inorganic phosphate, dissolved inorganic phosphate and other compounds (including citrate salts), respectively (Walstra *et al.*, 1999). Of particular interest to cheese making are changes in the so-called pseudo-equilibrium between colloidal and dissolved calcium phosphates and between dissolved calcium phosphates and various ions, especially Ca²⁺, as they are affected by heating, acidity, addition of Ca²⁺, milk concentration (e.g., by evaporation, reverse osmosis or addition of milk solids), and protein fortification (e.g., by ultrafiltration or direct addition of milk proteins).

Briefly, the principal effects of direct relevance to cheese composition and structure are as follows.

- Acidity reduces the negative charge on the micelles (micelle pI is 4.6) and increases the solubility of the milk salts. Citrates are completely soluble at pH near 5.5. Colloidal calcium phosphate, which is mainly located in the casein micelle, is completely soluble at pH 5.0. This means that the micelle is mostly demineralized at pH 5.0.
- Heating in the range 60–80°C causes some calcium and phosphate to form insoluble colloidal phosphates and associate with the casein micelles. Formation of insoluble colloidal phosphates also releases some H⁺ and slightly reduces the pH. For cheese making this means that heating reduces the dissolved calcium and phosphates available to promote rennet coagulation.
- Milk concentration (e.g., by standardizing with non-fat milk solids) also increases the colloidal phosphates associated with the casein micelles and slightly reduces the pH. However, of greater significance to cheese making are decreased rennet clotting time due to higher casein concentration and increased buffer capacity of the concentrated milk plasma.
- The pH and buffer capacity and pH of cheese and whey and other fermented dairy products are also influenced by depletion of citrate, production of carbon dioxide with associated carbonic acid and production of other acids such as acetic acid by heterofermentative starters (Emmons and Beckett, 1984a; Emmons and Tuckey, 1967).

The contributions of the principal buffer components to the TA of milk are described above. More interesting for cheese makers are the effects of the milk

buffers during acidification in the pH range of cheese making. During acidification, milk exhibits a buffer maximum, dB/dpH , of $\sim 15 \text{ mEq L}^{-1}$, near pH 7.0, which is near the third pK_a of phosphoric acid. Another and larger maximum, dB/dpH , of about 30 mEq L^{-1} , due to milk proteins and solubilization of colloidal calcium phosphates, occurs at pH 5.1–5.2 (Lucey *et al.*, 1993a; Walstra *et al.*, 1999). The maximum near pH 5.1 is an important natural ‘barrier’ that probably accounts for the evolution of sweet rennet coagulated cheese, of which an essential characteristic is that the minimum pH at any time in the pH history of the milk and cheese is ~ 5.0 .

During neutralization after acidification, the maximum buffer capacity occurs near pH 6.3, probably due to partial dissolution of calcium phosphates (Lucey *et al.*, 1993a,b, 1996). The important implication for cheese making is that increased pH during ripening, which is important for texture and flavour development in most ripened cheese varieties, can be achieved with fewer mEq of base than the mEq of acid required for acidification.

12.4 pH and the type of coagulation

The type of coagulation is the first level at which pH fundamentally influences the type and activity of ripening agents that do (or do not) develop flavour in cheese. Because of the globular shape of casein micelles, milk gels formed by aggregation (coagulation) of native casein are generally described as particle gels. However, the micelles are not rigid spheres, but dynamic particles in the sense that dynamic internal properties, such as pH dependence of the solubility of calcium salts, and surface properties, such as cleavage of κ -casein by rennet or flattening κ -casein during acidification, affect gelation and gel properties (van Vliet *et al.*, 2004). Milk gels for cheese making are formed by acid coagulation and acid-rennet coagulation at pH < 5.2 , usually at pH < 4.9 and by rennet coagulation, which may be assisted by acid development, but is mainly induced by renneting, usually at pH 6.5–6.7. A third type of coagulation for cheese making, heat-acid precipitation, is by acidification of hot milk, which results in a precipitate rather than a gel.

12.4.1 Acid and acid-rennet coagulated varieties

Acid coagulated cheese is nearly synonymous with fresh cheese, including varieties such as quark and related varieties, cream cheese and cottage cheese. However, some unripened rennet coagulated varieties, such as Italian ‘fresh cheese’ and Latin American Queso Blanco, are also called fresh cheese (Table 12.1). Acidification is normally by lactic acid bacteria, but direct acidification alone or combined with culture is also done. Fermentation is usually mesophilic, but thermophilic fermentations are used for some ripened acid coagulated varieties (Spreer, 1998). As the name implies, fresh cheese is consumed within days after manufacture without ripening, but there are also ripened acid

coagulated varieties, the best known of which are some goat-milk varieties such as Valençay from France and Kochkäse, a type of processed cheese made from ripened Quark.

The distinguishing characteristic of these varieties is that they are made from a predominantly acid gel where gelation may or may not be assisted by rennet. Levels of rennet are typically in the range 0–20 ml per 1000 L of milk, corresponding to 0–1220 International Milk Clotting Units (IMCU) per 1000 L of milk. For comparison, addition of 73,200–122,000 IMCU of rennet per 1000 L of milk is typical for cheeses that are predominantly rennet coagulated. Several workers have studied acid-rennet gelation (Dalglish and Horne 1991; Lucey *et al.*, 2000; Roefs *et al.*, 1990; Schulz *et al.*, 1999; Schulz-Collins and Senge, 2004; Tranchant *et al.*, 2001). From these reports several observations are relevant to the chemistry and structure of acid and acid-rennet coagulated cheeses.

- Due to essentially complete demineralization of the casein micelles near pH 5.0, Ca and phosphate contents are low (Tables 12.1 and 12.2).
- Gel firming rate and firmness increase with rennet addition up to about 500 IMCU per 1000 L of milk. For comparison, rennet concentration used for cottage cheese manufacture is typically about 1800 IMCU per 1000 L.
- Low renneting decreases clotting time and increases the gelling pH due to the higher isoelectric point of the para- κ -casein relative to κ -casein (Roefs *et al.*, 1990, Schulz-Collins and Senge, 2004). Both the local viscosity minimum at pH 4.95 and the final viscosity maximum at pH 4.45 are not affected by low renneting (Schulz-Collins and Senge, 2004). In practice, regardless of rennet addition or not, stirred varieties such as quark, bakers' cheese and cream cheese are set to pH < 4.6, typically pH 4.5 near the final viscosity maximum, while cut varieties (cottage cheese and its relatives) are cut at pH near 4.8 (Emmons and Beckett, 1984b; Emmons and Tuckey, 1967; Emmons *et al.*, 1957; Jelen and Renz-Schauen, 1989; Schulz-Collins and Senge, 2004; Sohal *et al.*, 1988).
- Acid-rennet gels relative to acid gels have higher elastic modulus (G' 800 versus 20 Pa), higher fracture stress (300 versus 100 Pa), and higher permeability and syneresis (Lucey *et al.*, 2000; Roefs *et al.*, 1990; Schulz-Collins and Senge, 2004).
- Finally, acid-rennet gelation profiles have a shoulder or local maximum in the consistency curve near pH 5.5 and a minimum near pH 5.2, both of which shift to a higher pH with increasing rennet concentration (Tranchant *et al.*, 2001). Several workers have reported similar results, suggesting that the gel structure is changing from a predominantly rennet to a predominantly acid gel in the region near pH 5.2 (Lucey *et al.*, 2000; Roefs *et al.*, 1990; Schulz *et al.*, 1999).

Dominant flavour compounds in acid and acid-rennet coagulated cheese, including lactic acid, diacetyl, acetaldehyde, formic acid, propionic acid and butyric acid, are primarily determined by culture selection (Emmons and Beckett, 1984a; Farkye, 2004a,b). Single or multiple washing treatments can be applied to reduce acidity and lactose to increase the pH up to 5.2 and inhibit

further acid development. Herbs, dried fruits and other flavourings may be blended into or applied to the surface of fresh cheese moulded in various shapes. For cottage cheese, flavour and texture of the final product is strongly influenced by the components and processes used to prepare the cottage cheese dressing (Emmons and Tuckey, 1967; Farkye, 2004a; Rosenberg, 1993).

A common flavour defect in quark is bitterness caused by rennet, especially in quark produced by ultrafiltration prior to fermentation where Ca retention is high (Jelen and Renz-Schauen, 1989). Rennet activity is high at the low pH of quark and its retention is increased by low pH at draining (Guinee and Wilkinson, 1992). In cottage cheese, high cooking temperatures (up to 55°C) deactivate rennet, destroy the mesophilic culture and reduce non-starter bacteria.

In ripened acid coagulated varieties, high moisture encourages rapid microbial growth and development of intense flavours (Schulz-Collins and Senge, 2004). Low pH favours initial growth by yeasts, which utilize lactic acid and facilitate the growth of bacteria and white and or blue moulds. Mould growth can also be encouraged by rubbing wood ash on to the surface to reduce acidity. Several acid coagulated and surface ripened cheeses, including Valençay, which has a rind of ash and blue mould, are described by Masui and Yamada (1996). Low Ca (50–350 mM kg⁻¹ of cheese solids non-fat) and high moisture of 70–80% moisture in the non-fat substance (MNFS) (Table 12.1) create a weak casein matrix that, apparently, forms a creamy texture with neutralization caused by mould growth and assisted in some cases with ash or added carbonates. While mould-ripened acid coagulated cheeses are traditional, there is little published on the biochemistry of ripening these varieties. Masui and Yamada (1996) noted rind formation, substantial dehydration and firmer texture to be encouraged by ripening at low relative humidity (80%). Apparently, the high cheese moisture content allows rapid bloom development at lower RH than is required for other mould-ripened varieties, such as Brie. In their review of acid curd varieties, Schulz-Collins and Senge (2004) describe a general process for ripening acid curd, where acid Quark, apparently made without rennet, is salted, neutralized with NaHCO₃ or CaCO₃, moulded into a log or other shapes, and inoculated with white moulds or *Brevibacterium linens*.

12.4.2 Heat acid-coagulated varieties

Ricotta (Italy) and its many relatives, Channa and Paneer (India), some varieties of Latin American white cheese and other cheese varieties are made from sweet whey (pH 6.0 minimum and preferably > 6.3), milk, or blends of milk and sweet whey; see review by Farkye (2004b) and Tables 12.1 and 12.2 for a full description. Heat treatment of milk and whey to 85–90°C for 5–20 min before acidification (final cheese pH 5.2–5.8) coagulates both casein and denatured whey proteins, which can then be recovered as a floating (skimmed off) or a sinking curd, which is separated from the whey by draining. Acidification can be achieved using any organic acid, but lactic and citric acids are most common (Siapantas and Kosikowski, 1973; Torres and Chandan, 1981a, b). For sinking

and pressed curd varieties, slow acidification with dilute acid increases curd flocculation during acidification, increases the rate of drainage, and makes the cheese less pasty and more sliceable (Parnell-Clunies *et al.*, 1985a).

Moisture is high (55–80%) due in part to the high water-holding capacity of whey proteins. For both floating and sinking curd varieties, moisture can be reduced somewhat by holding the curd in the hot curd–whey mixture after coagulation. For sinking curd, moisture reduction can be achieved by stirring out during draining, and pressing. Fat is occluded in the protein matrix (Kalab and Modler, 1985; Kalab *et al.*, 1988), but fat recovery is low and variable (55–85%) (Hill *et al.*, 1982; Parnell-Clunies *et al.*, 1985b, c).

A significant flavour determinant is the acidulant (Siapantas and Kosikowski, 1973). For example, more lactic acid flavour can be obtained by acidifying with fermented skim milk. Otherwise, flavour is acquired mainly via further processing, such as boiling Paneer in sweet syrups (Chandan, 1992) and frying heat-acid Queso Blanco. Residual lactose, which is roughly 3% and varies directly with cheese moisture, ensures colour and flavour development during cooking (Hill *et al.*, 1982). Frying and cooking properties of heat-acid varieties are created by their high concentrations of whey proteins, which decrease cheese meltability and increase water retention. Relative to Paneer and Channa, which contain no added NaCl, heat-acid Latin American white cheese generally contains salt-in-moisture (SM) > 5%, which provides adequate microbial stability to allow addition of flavouring condiments such as herbs and spices (Chandan 1992). Torres and Chandan (1981b) reported that heat-acid Queso Blanco can be ripened by adding lactobacilli or lipases to the salted curd before pressing.

There are also traditional ripened varieties of heat-acid precipitated curd. The best known is Mizithra, a type of whey (ricotta) cheese that is cured with dehydration, further dried, and consumed as a grating cheese. Similar to ripened acid coagulated varieties, ripening of Mizithra is accompanied by and depends on dramatic moisture reduction from ~70% to 40%.

12.4.3 Rennet coagulated cheese

Most ripened cheeses are predominantly coagulated by rennet (chymosin) or other aspartic proteases used to substitute for rennet (Stepaniak, 2004). Therefore, most of the work on cheese flavour development and most of the discussion in subsequent sections of this chapter are devoted to rennet coagulated varieties. Rennet coagulation is usually with simultaneous lactic acid fermentation, but coagulation and setting take place at pH 6.5–6.7 before substantive pH decrease. The evolution of high pH renneting of raw uncooled milk was probably directed by the high buffer capacity of milk at pH near 7.0, which allows substantial acid development with minimal pH change. Sections 12.3.3 and 12.3.4 describe, mainly, the influence of acid development after gelation on cheese composition, structure and potential for ripening and flavour development. The following sections focus on rennet coagulation with other parameters important in cheese making.

12.5 Effects of pH history on cheese composition, structure and functionality

For most rennet coagulated varieties most of the acid development occurs after coagulation. The summaries of pH values at critical processing steps form pH profiles (e.g., histories) typical of particular varieties. Table 12.4 includes typical pH profiles for several rennet coagulated varieties and, for comparison, a pH profile for cottage cheese. The effects of several variables on the draining pH and the minimum pH, which are important process control values for all rennet coagulated cheese, are illustrated in Table 12.3. With respect to minimum pH obtained at any time during cheese making and ripening, most rennet coagulated varieties can be categorized in three groups: (1) fresh varieties with little or no acid development and minimum pH 5.8–6.5; (2) soft ripened varieties with minimum pH < 5.0; and (3) varieties with minimum pH > 5.0.

12.5.1 Fresh varieties with little or no acid development and minimum pH 5.8–6.5

Due to high pH, rennet coagulated varieties with little or no acid development do not melt when used in stir fry or other cooked recipes. Varieties that are consumed fresh with minimal salting are also vulnerable to growth of spoilage and pathogenic organisms (Bruhn, 1986; Genigeorgis *et al.*, 1991a,b; Razavilar, 1997). Flavour determinants in fresh rennet coagulated varieties include (Bruhn, 1986): (1) amount and type of culture; (2) cooking or not, especially as cooking affects moisture content; (3) milling before pressing or not; and (4) amount of salt. Another factor is storage time, especially for Hispanic cheeses, which typically have extended best-before periods (up to 60 days) during which time the bacteria, yeast and mould populations increase (Bruhn, 1986). The resulting flavours, especially yeasty flavours, have become typical of these varieties.

12.5.2 Soft ripened varieties with minimum pH < 5.0

This category includes some brined cheeses such as Feta, white bloomy cheeses such as Camembert and Brie, and Blue mould varieties. Acidification is accomplished by the addition of mesophilic starter cultures. Demineralization and low minimum pH and the resulting firm and brittle texture (at least initially) are determined by extended draining and incubation in the forms at room temperature. Demineralization, especially for traditional blue and feta varieties, is further encouraged by: (1) a relatively large amount of starter culture; (2) extended ripening period before renneting, often beyond 60 min; and (3) extended setting time, sometimes as long as 90 min beyond the 20–30 min required to obtain a curd suitable for cutting. Allowing for some traditional variations and modern innovations, soft-ripened varieties are more demineralized and reach a lower minimum pH relative to other mesophilic varieties (cooking less than 40°C). The pH, at least for traditional varieties, is 4.3–4.7 on the day following manufacture, and, in the case of Feta, remains low during

Table 12.4 The pH versus time profiles for several cheese varieties¹

Operation	Swiss type		Gouda		Cheddar MNFS 53%		Cheddar MNFS 57%		Feta		Cottage	
	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	6.60	35		60	6.55	30	6.55	75	6.50	60	6.50
Cut	45	6.55	70	6.45	90	6.50	75	6.50	115		300	4.80
Drain or dip into forms	150	6.35	100		210	6.20	195	6.3	130	NA	360	
Milling	NA	NA	NA	NA	360	5.40	315	5.45	NA	NA	NA	NA
Pressing	165	6.35	130		420	5.35	390	5.40	NA	NA	NA	NA
Demoulding	16 h	5.30	8 h	5.40	24 h	5.20	10 h	5.20	24 h	4.6	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 mon	5.6	6 mon	5.6	24 mon	5.50	4 mon	5.3	6 wk	4.4	2–14 d	5.2

¹ Times in minutes unless otherwise stated.

curing. Syneresis is induced by acid development after forming and by brine salting with 60–70% moisture-in-non-fat-substance (MNFS) (Table 12.1).

In addition to lactic acid bacteria, the cultures are white moulds (*Penicillium camemberti* and *P. candidum*) for Brie and Camembert and blue moulds (*P. roqueforti*) for Blue varieties. However, mould growth is normally preceded by growth of yeasts such as *Geotrichum candidum*, which are more acid tolerant, and followed or accompanied by growth of bacteria such as coryneform bacteria (Karahadian and Lindsay, 1987; Le Gräet *et al.*, 1983; Spinnler and Gripon, 2004). This generally recognized three-phase transition (yeast–mould–bacteria), which is associated with increasing pH, depletion of lactate and lactose, and accumulation of NH_3 , was demonstrated for Camembert-type cheese made from pasteurized milk inoculated with *Kluyveromyces lactis*, *G. candidum*, *P. camemberti* and *B. linens* (Leclercq-Perlat *et al.*, 2004a,b). *K. lactis* grew rapidly between days 1 and 6, *G. candidum* grew exponentially between days 4 and 10, and rapid growth of *B. linens* and *P. camemberti* began after day 6 when the pH of the rind was close to 7. The interior of the cheese reached pH 7.0 after 45 days.

A similar three-phase transition takes place in Blue varieties, but proceeds more quickly in the interior where the salt content is lower than at the surface (Cantor *et al.*, 2004). For so-called ‘stabilized’ soft ripened cheese, low amounts of rennet, low acid forming cultures, and earlier salting and cooling are used to reduce rennet proteolysis and determine minimum pH near 5.2 (Lawrence *et al.*, 1987). Higher pH reduces demineralization, diversity of microflora and rate of proteolysis by the moulds, and helps maintain spreadable (but not runny) texture during an extended shelf life.

12.5.3 Varieties with minimum pH > 5.0

Other than soft ripened varieties, and a few exceptions such as Cheshire, most rennet coagulated varieties have a minimum pH > 5.0; cheeses with pH values between 5.1–4.90 appear to be in a critical region with respect to acidic texture and flavour characteristics. For Cheddar and American varieties such as Colby, acid defects such as sour and/or bitter flavour, acid-cut colour (mottled or bleached), and soft, pasty body are traditionally associated with excessive acidity at any stage in processing and often with a minimum pH < 5.0 during the first few days of ripening (pH < 5.05 according to van Slyke and Price, 1949).

Three procedures have evolved to produce rennet coagulated cheese with minimum pH > 5.0. First, for mesophilic washed cheese with moisture greater than 39% (Table 12.1), part or all of the whey is removed and replaced with water to leach lactose and lactic acid from the curd. This washing treatment, combined with syneresis controlled by the temperature and time of cooking and by the temperature of the wash water, determines residual lactose available for fermentation by LAB and ultimately the minimum pH of the curd.

Second, mesophilic unwashed cheeses (Table 12.1) are characterized by: (1) maximum cooking temperature of 39°C; (2) substantive reduction of pH, moisture and lactose and texture development in a process called ‘cheddaring’,

although a similar process is used for provolone and other *pasta-filata* varieties; and (3) except in some *pasta-filata* varieties, moisture content < 40%. Lower moisture permits removal of sufficient lactose by syneresis to avoid the necessity of washing. Moisture is controlled by cooking temperature and time, stirring out after draining, cheddaring, amount of culture, and salting treatments. A mesophilic unwashed cheese that 'breaks all these rules' is Cheshire. Cheshire and its relatives are acidified quickly with large amounts of culture, cooked to only 31–34°C, and fermented overnight to obtain pH near 4.8 before hooping (Lawrence *et al.*, 1983; Robinson and Wilbey, 1998). The result is a cheese intermediate between Cheddar and Feta with respect to calcium retention, texture and flavour.

The third strategy is high-temperature cooking to remove lactose via rapid syneresis. Thermophilic varieties (Table 12.1) typically have a slow initial rate of acidification with little or no ripening before renneting, are cooked at temperatures of 45–55°C relative to 32–39°C for mesophilic varieties, and are drained at relatively high pH, especially Swiss types. High pH and high temperature at draining contribute to higher levels of Ca and firmer and stronger curd, relative to mesophilic varieties (Section 12.5).

12.5.4 Effects of pH history on retention and activity of ripening enzymes

Here we discuss only the effects of pH history during cheese manufacture on their retention and activity. Other than enzymes derived from microorganisms, the most important cheese-ripening enzymes are lipoprotein lipase (LPL) and plasmin, which are endogenous to milk, and the coagulant(s). Because lipase is mostly inactivated by pasteurization and by the higher cooking used for thermophilic varieties, its role in cheese ripening is minimal except in mesophilic varieties made from raw milk (Collins *et al.*, 2003).

The milk alkaline protease, plasmin, is bound to casein micelles in milk at levels ranging from 0.1 to 0.7 mg L⁻¹ and the ratio of plasmin to its zymogen, plasminogen, ranges from 50:1 to 2:1 (Stepaniak, 2004). The plasmin system in milk also includes plasminogen activators such as urokinase and inhibitors of both plasmin and plasminogen activators (Barrett *et al.*, 1999; Crudden *et al.*, 2005; Upadhyay *et al.*, 2004). Plasminogen is also activated by heating during cheese making (Kelly, 1999; Kerjean *et al.*, 2001). Plasmin hydrolyzes β -casein releasing γ -caseins and proteose peptones; it also hydrolyzes α_{s2} -casein and to a lesser degree α_{s1} -casein (Farkye and Fox, 1992). As might be expected for an alkaline protease, plasmin activity is higher at higher pH (Ma and Barbano, 2003; Watkinson *et al.*, 2001).

Dupont and Grappin (1998) measured distribution of plasmin and plasminogen between curd and whey for acid coagulated (10 h fermentation at 42°C with LAB to pH 4.52) and rennet (10 h incubation at 42°C with 50 mg chymosin L⁻¹). Plasmin and plasminogen retention values in the curd as a percentage of their initial concentrations in the milk were 17% and 7%, respectively, in acid curd, and 59% and 55%, respectively, in the rennet curd. This is in

agreement with Grufferty and Fox (1988) who reported that plasmin and plasminogen dissociate from micelles at $\text{pH} < 4.6$. However, the effect of pH on dissociation of plasmin from the micelle within the pH range of rennet coagulated cheese is less clear.

Dupont and Grappin (1998) also determined plasmin and plasminogen contents of several acid coagulated, mesophilic and thermophilic varieties. Their data, summarized in Table 12.5, confirm that curd drained at $\text{pH} < 4.6$ retains little plasmin or plasminogen. The relative order among cheese types for total plasmin + plasminogen content was soft ripened $<$ mesophilic = thermophilic. This probably suggests that, although soft ripened cheeses are dipped into the forms at a relatively high pH, extensive syneresis during acid development to $\text{pH} < 5.0$ causes some dissociation of plasmin from the micelles. It also suggests that variability in retention of total plasmin + plasminogen among rennet coagulated cheese is small. The relative order for plasmin activity is mesophilic $<$ soft ripened $<$ thermophilic. As Dupont and Grappin (1998) suggest, this result can probably be explained by thermal activation of plasminogen during pasteurization of the soft ripened varieties and during cooking of the thermophilic varieties.

The principal enzymatic milk coagulants used in cheese making are chymosin, recombinant chymosin, bovine pepsin and microbial proteases. In addition to their role in milk coagulation, enzyme coagulants are important for cheese ripening, especially in mesophilic varieties such as Cheddar and Gouda. Chymosin is most active against caseins at pH near 5.0 and mainly hydrolyzes α_{s1} -casein (Harboe and Budtz, 1999; Stepaniak, 2004). Reported retention values of rennet activity as a percentage of total activity added to the milk in specific cheese varieties include 6% for Cheddar (Holmes *et al.*, 1977), 15% for Gouda (Stadhouders and Hup, 1975) and 55% for Camembert (Garnot *et al.*, 1987). Retention values of microbial coagulants derived from *Mucor pusillus* and *M. miehei* in Cheddar cheese were 3% and 1.8%, respectively (Holmes *et al.*, 1977). Chymosin retention in Gouda cheese was increased by higher pasteurization temperature (Stadhouders and Hup, 1975).

In model casein systems, it was demonstrated that chymosin association with para- κ -casein is decreased by competition between chymosin and caseins for association with para- κ -casein, by increasing pH, ionic strength and concentration of substrate, and slightly by lower temperature in the range 10–40°C, suggesting that the association involves both electrostatic and hydrophobic interactions (Dunnewind *et al.*, 1996; Larsson *et al.*, 1997; Roos *et al.*, 1998, 2000). These results confirm and help explain the observed effects of cheese making parameters, especially pH, on rennet retention in cheese:

- Rennet retention is increased by lower renneting pH (Garnot *et al.*, 1987; Holmes *et al.*, 1977; Stadhouders and Hup, 1975). Rennet retention in freshly coagulated curd increased from 28% at pH 6.6 to 86% at pH 5.2, but retentions of coagulants derived from *Mucor pusillus* and *M. miehei* were not affected by coagulation pH (Holmes *et al.*, 1977).

Table 12.5 Plasmin and plasminogen activity in commercial cheeses determined by ELISA

Cheese type	Commercial name	Milk: pasteurized (P) or raw (R)	Plasmin ($\mu\text{g g}^{-1}$)	Plasminogen ($\mu\text{g g}^{-1}$)	Plasmin + plasminogen ($\mu\text{g g}^{-1}$)	Plasmin: plasminogen ratio
Fresh cheese	Petit Suisse	P	0	0	0	0
	Boursin	P	0.09	0.27		0.333
Soft ripened	Brie	P	10.41	3.96	14.37	2.629
	Camembert	R	11.36	5.75	17.11	1.976
	Camembert	P	10.20	1.98	12.18	5.152
	Blue de Bresse	P	8.45	2.70	11.15	3.130
	Munster	P	8.18	5.81	13.99	1.408
	Mean		9.72	4.04	13.76	2.859
Mesophilic	St Nectaire	R	8.40	16.68	25.08	0.504
	Tomme de Savoie	R	2.99	17.01	20.00	0.176
	Reblochon	R	4.47	11.24	15.71	0.398
	Raclette	P	10.29	18.12	28.41	0.568
	Cantal	R	2.67	18.53	21.20	0.144
	Morbier	R	2.81	12.00	14.81	0.234
	Gouda	P	3.65	22.73	26.38	0.161
	Mean		5.04	16.62	21.66	0.312
Hard cheese	Emmental	R	21.94	3.18	25.12	6.899
	Comté	R	17.97	4.52	22.49	3.976
	Comté	R	16.59	6.05	22.64	2.742
	Comté	R	16.89	4.88	21.77	3.461
	Beaufort	R	14.08	4.16	18.24	3.385
	Mean		17.49	4.56	22.05	4.093

Source: adapted from Dupont and Grappin (1998).

- Retention values in Cheddar cheese at cutting, draining and pressing were 30, 7 and 6%, respectively (Holmes *et al.*, 1977).
- Considering that the renneting pH is relatively constant for most pasteurized and mesophilic varieties, the most important parameter influencing retention of chymosin activity in these varieties is pH at draining (Lawrence *et al.*, 1987). In Camembert cheese chymosin activity decreases during acid development in the forms until the pH is near 6.0 and remains constant or increases until draining is complete at 24 h (Garnot *et al.*, 1987). In the same experiment, *Mucor miehei* protease activity remained constant after hooping.
- Milk concentration by ultrafiltration decreased rennet retention in Camembert (Garnot *et al.*, 1987) and Cheddar (Green *et al.*, 1981).

12.6 Redox history

The oxidation/reduction (redox; E_h) systems important in fresh milk include $\text{Fe}^{2+}/\text{Fe}^{3+}$, $\text{Cu}^+/\text{Cu}^{2+}$, dehydro-ascorbate, riboflavin and lactate/pyruvate (Walstra *et al.*, 1999). The E_h of fresh oxygen-free milk is about 50 mV, mainly due to ascorbate. Under normal storage and handling conditions, dissolved oxygen increases the E_h to 150–300 mV (Beresford *et al.*, 2001; Walstra *et al.*, 1999). Decreasing the pH and increasing the temperature produces free sulfhydryl groups on the milk proteins or amino acids, thereby causing a reduction in the milk E_h . Fermentation by lactic acid bacteria (LAB) lowers the E_h to –100 to –200 mV, depending on species and starter culture mixture (Beresford *et al.*, 2001; Walstra *et al.*, 1999). The importance of low E_h (–150 to –300 mV) to production and stability of flavour compounds in ripened cheese is well established, especially for Cheddar, in which the production of volatile sulphur compounds is associated with typical Cheddar flavour and dependent on low E_h (Adda *et al.*, 1982; Beresford *et al.*, 2001; Green and Manning, 1982; Urbach, 1995).

There are few data on changes in E_h during cheese making. According to Green and Manning (1982), the E_h decreases during setting, rises during cutting, cooking and draining, decreases during cheddaring, rises again during milling, and finally decreases during pressing and ripening. After 6 weeks of ripening, the E_h of Cheddar cheese acidified with δ -gluconolactone without an added starter culture was +315 mV, relative to –150 to –250 mV for normal Cheddar cheese (Green and Manning, 1982; Manning, 1974). Cheese acidified without a starter culture lacks H_2S and methanethiol. Kristoffersen (1985) demonstrated that production of volatile sulphur compounds in Cheddar cheese decreases with increasing heat treatment of the milk, probably due to incorporation of whey proteins into the curd blocking proteolytic sites for bacterial enzyme hydrolysis. Similarly, Urbach (1995) notes that reduced production of methanethiol in Cheddar cheese made from ultrafiltered milk is likely due to inhibition by increased whey protein retention in the cheese which blocks hydrolysis sites causing a reduction in methionine access to the starter culture.

12.7 Temperature history

Temperature effects on milk properties and cheese making properties commence as soon as the milk leaves the udder. Bulk cooling, which is the norm in most jurisdictions, selects for growth of proteolytic and lipolytic psychrotrophic bacteria and inhibits LAB, so that cheese making without added culture must use fresh uncooled milk. During cheese making, formation of the casein gel and subsequent syneresis of cheese curd are dependent on electrostatic and hydrophobic interactions, which are strengthened by decreasing pH and higher temperature. Because acid development by the culture is also dependent on temperature, the temperature/time profile is the primary tool available to the cheese maker to control cheese pH and moisture. The principal effects at each stage of cheese making are described below.

12.7.1 Gelation and cutting

Higher temperature during rennet gelation (e.g., 37°C for some hard cheeses such as some Swiss-type varieties versus 31°C for most other varieties) increases the initial porosity of the gel and the initial rate of syneresis after the curd is cut (Dejmek and Walstra, 2004). Syneresis is also encouraged by smaller curd size at cutting and more vigorous agitation (Dejmek and Walstra, 2004). To avoid breaking fresh cut curd, agitation is normally delayed (healing time) to allow the curd to firm up with initial syneresis. Higher temperature at cutting requires reduced healing time because warmer curds firm up faster and have a greater tendency to aggregate. Aggregation is also encouraged by cooking too quickly after cutting.

12.7.2 Cooking

The primary purpose of cooking is to control the retention of moisture, lactose, lactic acid and minerals in the curd. The rates of syneresis and diffusion of solutes are affected by milk composition, especially protein/fat ratio, cooking temperature, the extent and rate of acid development, and the time/temperature profile. Optimum and uniform cheese composition (retention of moisture, lactose, lactate, milk salts, rennet) from day to day and seasonally is achieved by maintaining a constant time/temperature profile, and selecting or adjusting the amount of starters to obtain the optimum pH at specified stages, especially at draining (Lawrence *et al.*, 1984, 1987). Cooking too soon or too quickly is perceived by cheese makers to cause 'case hardening' that slows syneresis due to reduced porosity at the surfaces of the curd particles.

Because the culture is mostly retained in the curd, acid development takes place more quickly in the curd relative to the whey, lactic acid diffuses from the curd to the whey, and lactose is depleted more quickly in the curd (Czulak, 1981; Czulak *et al.*, 1969; Fox *et al.*, 1990). This probably implies that lactose in the whey diffuses into the curd to replace lactose fermented in the curd (Czulak, 1981; Czulak *et al.*, 1969; Lawrence *et al.*, 2004), although it is not clear if this

diffusion is substantive while substantive syneresis is taking place. Lewis (1974) concluded that lactose diffusion into the curd is likely to be small because the concentration gradient before draining is small, the diffusion coefficient for lactose is small and the outflow of whey from the curd would hinder diffusion of lactose into the curd. Czulak *et al.* (1969) and data reported by Robinson and Wilbey (1998) also suggest that in spite of higher acid development in the curd relative to the whey, the pH remains higher than the pH of the surrounding whey because its buffer capacity is higher. However, it is the author's experience that curd pH is 0.0–0.1 units lower than whey pH depending on time after cutting and rate of acid development. This is consistent with comments by Lewis (1974), who suggests that curd pH is a better indicator of recent acid production than the whey pH.

Cultures are selected according to the temperature treatments used during cheese making. Mesophilic and thermophilic cultures have optimum temperatures for growth of 20–30°C and 37–45°C, respectively (Tamime, 1990). Generally, cooking temperatures are higher than the optimum growth temperatures of the cultures, so higher cooking temperatures increase the rate of syneresis but reduce the rate of acid development. For most acid and acid-rennet coagulated varieties (Table 12.1), acid development and setting take place at temperatures near the optimum for mesophilic cultures. Cooking after cutting is or is not applied as required to adjust moisture. In cottage cheese, for example, cooking temperatures up to 55°C firm the curd and reduce moisture. Destruction of the mesophilic culture during cooking as in cottage cheese helps ensure that further acid development will not occur after creaming. For soft ripened varieties there is little or no cooking and most of the acid development takes place in the forms at temperatures less than 30°C, so syneresis is determined mainly by pH. For Cheddar most of the acid is developed before salting and moulding. Cooking time and temperature along with culture activity principally determine curd pH, buffer capacity, and rennet retention at draining. In most washed varieties, cooking importantly influences moisture content, but acidity and lactose retention depend more on washing and culture selection than on cooking time and temperature.

Thermophilic varieties (Table 12.1) are uniquely dependent on high cooking temperatures, which normally, especially for varieties that are pressed under the whey, result in high pressing temperatures. Kerjean *et al.* (2001) identified the following consequences of high cooking temperatures in the range 50–55°C for Emmental cheese:

1. Calcium retention and buffer capacity are increased due to reduced solubility at the higher cooking temperature and high draining pH (see Lucey *et al.*, 1993a).
2. Hydrolysis of β -casein by plasmin is increased due to activation of plasminogen and deactivation of inhibitors to plasminogen activators (see Section 12.3.4 and Somers and Kelly, 2002).
3. Reduced hydrolysis of α -casein due to thermal denaturation of chymosin (note that some chymosin probably survives in cheese cooked at least as high

as 52°C (Hynes *et al.*, 2004), but its importance to ripening of cooked cheese is probably minimal).

4. Thermophilic lactobacilli grow during pressing at high temperatures in Emmental and produce flavour compounds by secondary proteolysis.
5. Potential pathogenic bacteria and other non-thermoduric bacteria that may influence flavour do not survive.

12.7.3 Other temperature-related effects

For cheese that is drained in the forms, especially cheese with minimum pH > 5.0, the time and temperature of draining and subsequent salting and cooling are critical to pH control and subsequent activity of bacterial and other ripening agents. For Cheddar cheese, which retains substantial amounts of lactose after hooping, the time and temperature of pressing and the temperature during the first days of ripening importantly influence the extent of acid development and the minimum pH in the cheese. More rapid cooling is particularly critical for high moisture Cheddar. High temperature during pressing of thermophilic varieties importantly determines the synergistic growth of the mixed thermophilic rod and coccus cultures, and, as noted above, increases plasmin activity.

12.8 Moisture

Primary determinants of cheese moisture are cheese composition, pH history (Section 12.3), temperature history (Section 12.5) and salting treatments. Availability of moisture for microbial growth and enzyme activity is critical with respect to cheese safety, spoilage and ripening, so there is an obvious relationship between ripening time and moisture content for particular cheese varieties (Table 12.1). Cheese moisture ranges from >80% for some fresh cheese to <30% for some hard-ripened varieties. Still, cheese moisture *per se* is not the best predictor of moisture related properties of the cheese. Because cheese fat has minimal influence on biological and enzymatic ripening agents, percent moisture-in-non-fat-substance (MNFS) is a better indicator of potential quality in a young cheese than absolute moisture. Similarly, percent salt-in-moisture (SM) is a better index of the influence of salt on cheese ripening than the absolute salt content. The concentration of salt in cheese moisture is also the principal determinant of the a_w of cheese. Both absolute and SM values for salt content for some varieties are given in Table 12.2.

12.8.1 Water activity

Water activity (a_w) along with pH is a major determinant of survival and growth of microorganisms in cheese. The approximate minimum a_w values for growth of bacteria, most yeasts, osmophilic yeasts and moulds are 0.92, 0.83, 0.60 and 0.75, respectively (Fox *et al.*, 2000). Very approximate ranges for a_w of various

cheeses are given in Table 12.1. The a_w of fresh or young cheese is generally in the range 0.985–0.997 and can be estimated from the molal or percentage concentration of salt in the moisture of the cheese (Equation 12.1) (Marcos and Esteban, 1982):

$$a_w = 1 - 0.00565(\text{g NaCl}/100 \text{ g cheese moisture}) \quad 12.1$$

Equation 12.1 predicts somewhat higher than experimental a_w values because fresh and young cheeses also contain other solutes, including lactose, lactate (which on a weight basis lowers vapour pressure four times as much as lactose), Ca^{2+} , and soluble phosphate and citrate salts of calcium. Because the amounts of all of these solutes other than NaCl are influenced by pH, the a_w of fresh and young cheeses can be estimated more accurately (30 samples, $R^2 = 0.95$, accuracy $\sim 0.005a_w$ units for 87% of the samples) as a function of NaCl and pH (Lopez *et al.*, 1990) (Equation 12.2):

$$a_w = 0.9719 - 0.0044(\text{g NaCl}/100 \text{ g cheese moisture}) + 0.0041\text{pH} \quad 12.2$$

In ripened cheese, a_w is further reduced (0.940–0.980) by the accumulation of low molecular weight solutes due to hydrolysis of lipids and proteins, utilization of water in hydrolysis reactions, and water loss due to evaporation, at least for rind-ripened varieties (Beresford *et al.*, 2001). Ruegg (1985) gave an equation to estimate a_w of both young and mature cheeses based on pH, ash, salt and the non-protein nitrogen (NPN) (Equation 12.3):

$$a_w = 0.945 - 0.0056(\text{NPN}) - 0.0059(\text{NaCl}) - 0.0019(\text{ash} - \text{NaCl}) \\ + 0.0105\text{pH} \quad 12.3$$

where all concentrations are expressed in g component per 100 g of cheese moisture. The a_w within a cheese varies with pH, salt and moisture gradients. For example, a_w is reduced on the surface of cheese with dry rinds (Fox *et al.*, 2000).

12.8.2 Moisture in the non-fat-substance (MNFS)

The a_w of cheese along with its pH is a useful indicator of the general types of microbial activity that can be expected in a certain variety, especially in drier and long-ripened varieties. However, for young cheese, a_w varies within a relatively small range and is generally too high to substantially impact microbial growth. Therefore, MNFS along with SM is a more useful index of the ripening potential of a young cheese than a_w . MNFS values for some selected cheeses are given in Table 12.1.

For most soft-ripened varieties MNFS is determined mainly by the time, temperature and rate and extent of acid development in the forms, and by salting. For most varieties with minimum pH > 5.0, MNFS is controlled mainly by pH at draining and cooking treatments. Moisture content is also substantially increased by longer renneting time (Johnson *et al.*, 2001), cutting time and larger curd size, and reduced by stirring out after draining, salting and pressing. Other

conditions being kept constant, MNFS increases with increasing fat-in-dry-matter (FDM) because fat inhibits syneresis (Lawrence and Gilles, 1986). This is of commercial interest because optimization of yield efficiency requires adjustment of FDM based on variations in the cost of milk components. The necessary adjustments to the process to correct for the resulting effect on MNFS are difficult to implement in large mechanized operations.

MNFS is also affected by milk treatments that increase recovery of whey proteins in the cheese, such as heat treatment in excess of normal pasteurization ($>75^{\circ}\text{C}$, 15–16 s), pre-concentration of milk by ultrafiltration or reverse osmosis, and direct addition of denatured or native milk and/or whey protein concentrates to cheese milk (Banks *et al.*, 1994; Harjinder and Waungana, 2001; Jameson and Lelievre, 1996; Law *et al.*, 1994; Rippel *et al.*, 2004; Zisu and Shah, 2005). These strategies, especially ultrafiltration, have been successful for some soft-ripened varieties, especially Feta, and for some fresh varieties. For most sweet curd varieties (minimum pH > 5.0) problems with texture, functionality and impaired flavour development persist (Jameson and Lelievre, 1996; Kelly, 1999; Lelievre and Lawrence, 1988; Somers and Kelly, 2002). Rippel *et al.* (2004) demonstrated that whey proteins at typical concentrations did not inhibit plasmin activity and that plasminogen activation was stimulated by both native and heat denatured β -lactoglobulin and α -lactalbumin. Nevertheless, there is a considerable body of evidence that native whey proteins inhibit proteolysis of β -casein and α_{s1} -casein and that denatured whey proteins inhibit plasmin (Harjinder and Waungana, 2001; Jameson and Lelievre, 1996; Rippel *et al.*, 2004).

Optimum MNFS depends on expected date of maturity. This applies generally among varieties, but also as a ripening control tool within varieties. For example, given typical SM values and ripening temperature less than 10°C , yield and quality of Cheddar intended to be marketed as mild at 3–4 months are optimized by MNFS in the range 55–57%. For aged Cheddar (≥ 12 months) the optimum MNFS is 52–54%. Higher MNFS in Cheddar cheese is achieved by targeting a slightly higher pH at each stage of manufacture, reduced cooking and cheddaring times, and less stirring out after draining (Table 12.4). The optimum MNFS with respect to balancing yield and quality is also dependent on other factors that affect the activity of ripening agents. For example, the MNFS of Cheddar cheese can be safely increased if other measures such as earlier salting, reduced pressing temperature and lower curing temperatures are implemented.

12.9 Improving cheese flavour by controlling physical factors

Determination of cheese flavour development requires the cheese maker to manage, by art or by science, interacting physical factors. Much progress has been made by standardizing each of many steps of the cheese making process in response to observed effects on particular cheese attributes or on overall cheese quality. Occasionally, a major change is implemented to achieve a particular objective, such as pasteurization to eliminate pathogens, removing two-thirds of

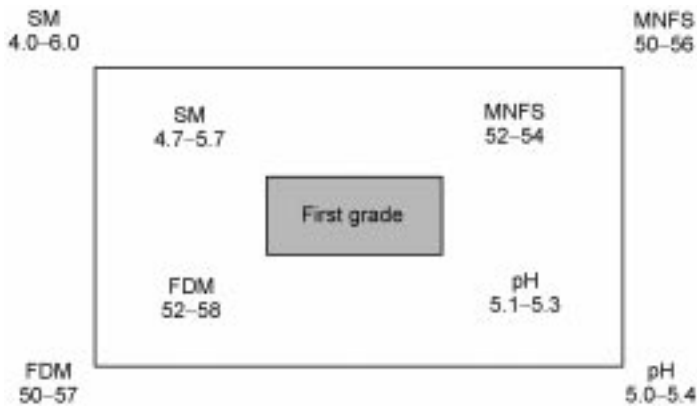


Fig. 12.2 Suggested ranges of salt-in-moisture (SM), moisture-in-non-fat-substance (MNFS), fat-in-dry-matter (FDM), and pH for first-grade and second-grade Cheddar cheese. Redrawn from Lawrence *et al.* (2004).

the fat to make a low fat cheese, or supplementing cheese milk with milk protein concentrates. Such changes have domino effects on other processing parameters and cheese attributes, so the incremental and iterative process of standardizing each step has to be repeated. For example, we are still learning about the impact of pasteurization on cheese making.

Considering the interaction effects among acid development (pH and buffer capacity), temperature profile, salt uptake and diffusion, syneresis etc., it is immediately obvious that any model to predict and control cheese quality will be complex. The most successful attempt to do this is the application of compositional grading (CG) of young Cheddar as a means to predict its ripening potential and eventual quality. Gilles and Lawrence (1973) proposed a Cheddar cheese grading system based on defined ranges of MNFS, SM, FDM and pH as measured at 14 d after manufacture. Figure 12.2 is a grading chart based on that model as presented in Lawrence *et al.* (2004). Other studies (Fox, 1975; Lelievre and Gilles, 1982; O'Connor, 1971; Pearce and Gilles, 1979) suggested similar quality ranges. Several observations can be made from these studies.

- The relationship between grade and composition varies between cheese plants and probably between regions, so individual plants should establish their own CD standards (Lelievre and Gilles, 1982).
- Composition within the suggested zones is not a good predictor of quality. Other factors such as starter and non-starter organisms, critical processing variables such as pH at draining, and variations in enzyme activities become dominant when pH, MNFS and SM are within appropriate limits (Fox and Cogan, 2004; Lawrence *et al.*, 2004).
- The acceptable range of FDM is large, probably because relatively little lipolysis occurs in Cheddar cheese (Fox and Cogan, 2004; Gilles and Lawrence, 1973). This is commercially important because it allows substantial variation of milk fat in response to variations in the cost of milk components.

- Also large is the apparent acceptable range of SM. According to the CG model suggested by Lawrence *et al.* (2004), the SM for premium Cheddar lies within a 1% range between 4.7 and 5.7%. As a ratio of the midpoint of this range, the acceptable variation is 20% (1.0/5.2) compared to only 4% for MNFS. This may be due to poor salt distribution in and between large commercial blocks of Cheddar. Although salt content within individual cheddared and milled curds (chips) is reached within 24–48 h, it was demonstrated that equilibrium within blocks was not reached in 4 months (see review by Guinee, 2004). This suggests that salt distribution to ensure uniformity within and between blocks and vats could improve quality control and permit definition of a narrower range of SM in the CG model. It may also permit the use of targeted SM values to minimize salt content for dietetic reasons or to effect more or less proteolysis.
- Calcium content is inversely related to chymosin retention and proteolysis, especially of α_{s1} -casein. However, Ca is also a covariate with pH, so including Ca in addition to pH does not improve compositional grading (Lawrence *et al.*, 2004).

It is interesting that CG models have not been developed for other varieties. This may be partly because the principal determinants in Cheddar are more stable within 2 weeks after manufacture, relative to brine- or surface-salted varieties. Experience with American varieties, especially with reduced-fat American varieties such as American Mozzarella, suggests that SM less than 4.0 is associated with gas formation in the package (ballooning) and bitter flavours. In any case, it seems reasonable to suggest that even in the absence of defined CG models, consistent SM, MNFS and pH values, at least within individual plants, will improve control of cheese texture and flavour for all varieties and will help determine and implement processing changes as required in response to major process changes, such as reduced fat. Achievement of such consistency requires defined and consistent timing, temperature, curd composition (lactic acid, lactose, moisture, Ca, salt) and physical properties (pH, buffer capacity, curd size, texture and rheology) at each step in the process. Some relationships among process variables and cheese properties are illustrated in Table 12.3.

12.10 Sources of further information and advice

Several recent reviews extend the discussion of principles discussed in this chapter. The third edition of *Cheese: Chemistry, Physics and Microbiology* (Fox *et al.*, 2004) describes many aspects of cheese physics and chemistry from multiple perspectives. Aspects related to acidity, pH and buffer capacity were reviewed earlier by Fox *et al.* (1990). Physical, chemical and biochemical aspects of cheese as they relate to cheese rheology and texture were reviewed by Lucey *et al.* (2003). Beresford *et al.* (2001) reviewed effects of physical and chemical properties on cheese microbiology, and the role of salt in cheese was reviewed by Guinee (2004).

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13

Flavorant–matrix interactions and flavor development in cheese

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13.1 Introduction

This chapter considers the factors that influence flavor development wherein cheese acts as a multiphase matrix for the generation and release of flavor compounds. Both volatile and non-volatile flavorants in cheese combine to create the unique flavor profile of different cheeses. Cheese can be considered a system containing pools of flavorants partitioned amongst the main matrix constituents: milk fat, protein and a saline aqueous phase. Flavorants are distributed throughout the cheese matrix as a function of several factors including the hydrophobicity of the flavorant, the cheese composition, the physical state of the milk fat, and the pH and ionic strength of the aqueous phase. Additionally, many flavorants are sufficiently reactive or have sufficient affinities to bind with milk protein moieties or structures through hydrophobic, ionic and covalent interactions. With perhaps the partial exception of physical entrapment of flavorants within solidified milk fat, most flavorants within the cheese matrix are relatively motile. Excluding aging-induced matrix changes (e.g. proteolysis), given sufficient time at a constant temperature, the flavorants will reach a state where the rate of flux between phases, for example between the milk fat and aqueous phases or between bound versus unbound states with a protein, reaches equilibrium. With such equilibrium states, temperature plays a major role in determining the degree and rate at which equilibrium is reached. Cheese poses an especially challenging matrix to understand because of the dramatic changes in the solid fat index over temperatures typical of storage, ripening and consumption. Taken individually or collectively, flavorant–matrix interactions play an

integral part in defining and controlling cheese flavor. This area of research has produced numerous, substantive works, but is primarily addressed in the context of phase partitioning.

13.2 Experiencing cheese flavor

To better relate the influence of the cheese matrix on flavor, one must first understand some basic components of flavor perception. First, numerous reactions occur during the course of experiencing cheese flavor. Even perceiving odor from the cheese surface requires that a sufficient mass of volatile compounds is released from the cheese matrix and is inhaled into the nasal cavity, thus allowing flavorant interactions with olfactory neurons. Once the cheese is imbibed, the process of mastication further releases flavorants as the product is chewed, mixed with saliva and, under most circumstances, warmed to approach body temperature. While non-volatile flavorants interact with taste receptors, air present in the oral cavity, throat and nasal passages allows volatiles to reach the olfactory bulb ortho- and retronasally. Flavorants must be released from the cheese matrix at a specific rate and to a specific extent to accurately represent the expected cheese flavor character. More thorough descriptions of the physiology of flavor perception are contained in sensory evaluation texts (see Meilgaard *et al.*, 1999). Although the intent of this chapter is not to repeat such coverage, the reader is encouraged to examine these details to better bridge the gap between the physical chemistry of flavor compounds and the physiology of flavor perception. Several chemical phenomena are worth considering when relating and predicting the potential sensory impact of a flavorant as influenced by the food matrix. Most fundamental of those phenomena is the concept of phase partitioning.

13.3 Phase partitioning

A basis for understanding how the physical and chemical properties of cheese matrix may influence flavor perception is based on the physical chemistry of flavorant partitioning in multiphasic systems (see Walstra, 2003). As a function of factors such as relative hydrophobicity, net and localized charge distribution, and molecular weight, flavorants partition into the different phases of cheese. Albeit a gross oversimplification, consider cheese as a biphasic system: milk fat dispersed in an aqueous medium. When a flavor compound is introduced into the system, it will begin to diffuse into and out of each phase. Based on the affinity of the compound, it will typically begin to preferentially accumulate in one phase over another, ultimately reaching equilibrium if the diffusion is allowed to continue undisturbed by a change in solvent phase character or system temperature. The average translational kinetic energy (U) of a molecule (or small particle) is characterized according to Equation 13.1:

$$U = \frac{1}{2}mv^2 = \frac{3}{2}k_B T \quad 13.1$$

where m is the mass, v is the velocity in meters per second, k_B is Boltzmann's constant and T is the temperature in kelvin. Solving the equation for v , one can surmise that velocity of the particle or molecule is dependent on temperature and inversely related to mass. A further development of this relationship describes the movement or diffusion of a particle or molecule through a solvent; D (m^2s^{-1}), the diffusivity constant, is characterized according to Equation 13.2:

$$D = k_B T / 6\pi\eta_S r \quad 13.2$$

where η_S is the viscosity of the solvent and r is the radius of the particle. These two equations describe the influence of three major intrinsic effectors in cheese flavor perception: solvent viscosity, flavorant mass and system temperature. As a further extension of these relationships, the balance of this chapter will examine two flavorant phenomena, air-liquid and liquid-liquid partitioning.

13.3.1 Air-liquid phase partitioning

For the purposes of this discussion, air-liquid partitioning will refer to the movement of volatile compounds from the cheese matrix into the gaseous phase, allowing one to detect or smell the compound. Certainly, the treatment of the cheese matrix as a simple, homogeneous liquid phase is inaccurate. However, some free liquid water exists in cheese, as well as liquid milk fat. Additionally, under the conditions of mastication, the cheese is further 'liquefied' as it is mixed with saliva, thus the treatment of the cheese phase as having at least some liquid properties is not entirely inaccurate. Most descriptions of air-liquid volatile partitioning properties are treated in a simplified manner with a defined homogeneous, continuous liquid phase, such as water or oil. While far from accurately depicting the myriad of complex interactions in a cheese matrix, there are phenomena that manifest in such simple models as to find application in authentic food systems. One such phenomenon is referred to as an air-liquid partitioning coefficient (K_{al}). This empirical attribute is defined by the behavior of volatile compounds in a biphasic system consisting of known volumes of air and liquid phases, where the volatile compound concentrations are allowed to reach equilibrium between the two phases. In most instances, the more specific terms air-water (K_{aw}) or air-oil (K_{ao}) partition coefficients are used, reflective of systems utilizing a hydrophilic or hydrophobic liquid phase. K_{al} is determined by the following relationship (Equation 13.3):

$$K_{al} = \frac{[X]_{\text{air}}}{[X]_{\text{liquid}}} \quad 13.3$$

where $[X]$ refers to the concentration of a flavor compound in either the air or liquid phase; each is expressed in any equivalent unit of measurement, e.g. ppm, moles, etc. Conducted under standard conditions, K_{al} values for numerous compounds are readily available in the literature as a means of comparing their relative volatility. Such values are available for many volatile compounds where

either oil or water is used as the liquid phase to more accurately represent a particular food medium. The K_{al} value provides a basal index of predicting the mass of a volatile compound available to the olfactory system. Although this fundamental platform of volatile characterization has great value, shortcomings of K_{al} values are obvious when comparisons are made to an actual food system and volatile release during consumption. Solvent–solute interactions as influenced by such factors as temperature, liquid phase properties (e.g. ionic strength), interfacial area, and equilibrium rate (related to release rate) become areas of further research and exploration. Also of significance in actual food systems are the myriad of molecule bonds (covalent, ionic, hydrogen, etc.) between the volatile compound and constituents in the food that may impede the motility or availability of a compound to exhibit volatile behavior. Certainly, solvent properties such as polarity and viscosity can also be altered by the presence of food constituents, such as proteins or starch complexes, that further influence solvent–solute interactions.

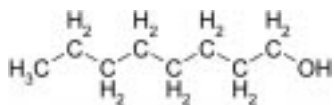
13.3.2 Liquid–liquid partitioning

Liquid–liquid partitioning is the physical phenomenon of how a solute will disperse itself between two immiscible phases (Sangster, 1989). The partition coefficient (P) is a measurement of equilibrium concentration in one phase divided by the other, typically hydrophobic (octanol) and aqueous phases (Equation 13.4):

$$P = \frac{[\text{solute}]_{\text{octanol}}}{[\text{solute}]_{\text{water}}} \quad 13.4$$

P is typically reserved for 1-octanol and water partition coefficients, while K is often used for other solvent pairs. Due to the wide range of partition coefficients, a log base 10 scale ($\log P$, $\log K$) is often used. Partitioning has been studied for over 100 years in a variety of systems. Nernst identified some of the guiding principles in 1891. According to Nernst, the partition coefficient (P) is a constant for a single molecular species partitioned between two phases. Partitioning follows Henry's law as a linear relationship occurs between the concentration of each phase, so that the slope is constant and equivalent to the partition coefficient (Rodis *et al.*, 2002). Partitioning can be treated by classical thermodynamics as an equilibrium process, where the tendency of a solute to move from one phase to another is a measure of its activity. Partitioning is therefore also related to other activity functions, such as partial pressure, osmotic pressure and chemical potential (Leo *et al.*, 1971).

The solvent most often used in partitioning studies, 1-octanol, is a straight chain alcohol with a hydrophobic hydrocarbon chain and a hydrophilic end group:



The interactions arising from 1-octanol's structure approximate the environment of living tissue more closely than that of a non-polar solvent, such as methane or hexane (Sangster, 1997). Many of the reference data available on partitioning are from 1-octanol models. Log P is used for pharmaceutical development, determining the fate of environmental contaminants and toxicology of substances (Tanii and Hashimoto, 1982). A high log P value indicates the tendency of flavor compounds to accumulate into the lipid phase.

Partitioning is complicated by ionization and dimerization. In such cases, a simple single molecular species cannot be considered as accurately depicting partitioning properties (Sangster, 1997). The pH has a direct influence on log P for compounds capable of disassociation, such as free fatty acids, as increases in the ionized form will favor the aqueous phase. Cornell *et al.* (1970) demonstrated the effect of pH on partitioning of ionizable antioxidants between butter oil and water. Propyl gallate maintained a constant partition coefficient (~ 1.3) from pH 3.0 to 5.5. Above pH 5.5, the partition coefficient decreases and levels off again above pH 8.0 to be ~ 0.1 . This tendency correlates to propyl gallate's pK_a . At a low pH, propyl gallate is almost completely in the protonated form. But at a higher pH, propyl gallate disassociates to its ion form, which would highly favor the polar solvent.

As temperature changes, several factors contribute to changes in log P values. Temperature also changes log P . According to Leo *et al.* (1971), insufficient data exist to 'attempt any useful generalizations' about the relationship between log P and matrix temperature changes. However, some of the controlling factors are elucidated, such as partitioning itself and the temperature effect on partitioning as a function of entropy and enthalpy in a system. Free energy from entropy is a direct function of temperature ($T\Delta S$), while temperature also impacts enthalpy. As temperature rises water becomes less polar and, in effect, a better solvent for non-polar compounds (Sangster, 1997). The change in water may lead to both higher enthalpy (less energy in bonding) and higher entropy (less orderly arrangement of water surrounding solvated hydrophobic groups). Since increases in enthalpy and entropy can offset each other in total free energy, the final change in log P is not intuitive. Compilations of data from several studies led Southall *et al.* (2002) to conclude that the poor solubility of non-polar compounds in water is due to entropy at room temperature and due to enthalpy in hot water as it approaches its boiling temperature. Changes in polarity of the hydrophobic solvent and the solute may also affect partitioning. Secondary effects on the observed partitioning can be due to changes in the level of solvents dissolved in complementary phases as well as density changes with temperature (Leo *et al.*, 1971). Data on temperature dependent partitioning trends in 1-octanol and water are limited. The hydrophobic effect, which is a key controller of phase partitioning and protein folding, varies substantially with temperature and solute properties (Southall *et al.*, 2002).

Trends in partition coefficient values based on molecule size and chemical group types led scientists to develop mathematical models to predict partition coefficients. The Hansch and Fujita π -system, the Leo and Hansch f -system and

Rekker's revised f -system are all based on additive group activity coefficients with various corrective factors for certain molecular structures (Hansch and Fujita, 1964; Hansch and Leo, 1979; Rekker and Mannhold, 1992). Non-polar groups (e.g. methylene groups) have positive values, indicating a 1-octanol preferring influence, while polar compounds (e.g. oxygen or nitrogen containing groups) have negative values, which have an aqueous phase preferring influence. A predicted $\log P$ for 1-butanol is illustrated below according to Rekker and Mannhold (1992):

Fragment		Constant
CH ₃		0.724
CH ₂	0.519 $\times$ 3 groups =	1.557
OH		-1.448
Predicted $\log P$		0.833

Rekker and Mannhold (1992) made extensions to their fragmental constant models to accommodate differences for an aliphatic hydrocarbon ($\log K_{\text{AHC}}$) lipid phase. For example, $\log K_{\text{AHC}}$ of 1-butanol is -0.83 , indicating that, unlike in 1-octanol systems, 1-butanol prefers the aqueous phase to that of a much more non-polar hydrocarbon phase.

$\log P$ group activity models are a specific application of the universal function for activity coefficients (UNIFAC). UNIFAC models can be extended to other solvent pairs. With the assumption that olive oil would behave similarly to thermodynamically defined triolein, Rodis *et al.* (2002) predicted partition coefficients of antioxidants between olive oil and water. However, experimental and predicted results were generally precise to the nearest order of magnitude. It appears that interaction parameters of natural lipids and antioxidants are more complex than a simple 1-octanol and water system or UNIFAC derived models. Carey *et al.* (2002) showed that although octanone and ethyl octanoate have similar $\log P$ values, their volatile flavor release behavior from a lipid cloud emulsion was substantially different. Southall *et al.* (2002) concluded that there are limitations to the assumptions of additivity models to determine partitioning among diverse solutes and phases.

Partitioning properties between 1-octanol and water have been studied extensively, with numerous experimental $\log P$ values recorded in the literature. Although 1-octanol and water $\log P$ values may provide partitioning tendencies, thorough experimentation in cheese systems is needed.

13.3.3 Partitioning in lipid-containing systems

Archer *et al.* (1994) demonstrated the effect of partitioning between aqueous and lipid phases on chemical reactions and metabolic potential, usually from microbes in cheese, albeit such effect is limited. Since microorganisms grow in the aqueous phase or at the oil/water interface, antimicrobial agents that migrate

completely into the lipid phase do not inhibit growth in fat-containing foods (Lubieniecki-von Schelhorn, 1964; Archer *et al.*, 1994). Archer *et al.* (1994) related diacetyl's partition coefficient and growth inhibition in a water-in-oil emulsion. In an aqueous medium, *Salmonella typhimurium* grew at concentrations up to 1.1 mM diacetyl, but no growth was seen at 1.2 mM or higher concentration. When the experiment was repeated with sunflower oil added to the medium, growth was again seen at concentrations in the aqueous phase of up to 1.1 mM, as calculated from the initial diacetyl concentration minus the portion that was predicted to partition into the oil volume. At ≥ 1.2 mM of diacetyl in the aqueous phase, growth was inhibited. Under model conditions, partitioning as a predictable factor for microbial activity was demonstrated. However, the authors noted that results would vary in a real food item, where migration of a preservative through solid or semi-solid foods would be slower than in a liquid, preservative-protein interactions may occur, and the salt content will modify partitioning due to its effect on water polarity.

The phenomenon of phase partitioning is important to reactions in biological systems. In cheese, many investigators propose that the starter culture must lyse to gain access to the substrate in the matrix for hydrolysis by intracellular enzymes. However, the availability of substrate is not solely dependent on lysis, but also on liquid-liquid partitioning. Factors including solidified lipids, surface-active agents, reversible and non-reversible binding, dissolved salts and kinetics of diffusion (viscosity effects) may have an impact on substrate availability and need to be considered in these studies. A full understanding of how these effects contribute to substrate availability is lacking (Seuvre *et al.*, 2000).

Some hypothesize that volatiles primarily penetrate liquid fat, and that solid fat will have little flavorant absorption capacity (van Boekel and Lindsay, 1992; Roberts *et al.*, 2003). As such, temperature is an important parameter to observe in partitioning studies where a phase change exists in one or more of the solvents. Small changes in temperature around the melting point of fat have been shown to cause large deviations in the air-oil partition coefficient; however, this phenomenon has not been observed in all cases (Bakker, 1995). Surprisingly, there are very few reports testing the hypothesis that solid fats are inert to solubilizing effects. Lubieniecki-von Schelhorn (1964) reported slow diffusion of preservatives into crystallized fat, but method details were limited. McNulty and Karel (1973) demonstrated that *n*-hexanol's transfer rate out of oil decreases with increases in solid fat content, while overall partitioning into the aqueous phase increases. However, this increase is less than what would be expected if solid fat were considered completely inert. Maier (1975) reported that solid lipids (e.g. trilaurin) sorb compounds, but much less so than liquid lipids (e.g. tributyrin). Liquid lipids sorb flavor compounds at a rate corresponding to Fick's law of diffusion, which states that the diffusion rate is inversely proportional to the thickness of the medium (Walstra, 2003). Sorption for solid lipids reaches the maximum much quicker than liquids, suggesting that sorption is a surface phenomenon. In a cloud emulsion flavor release study, Carey *et al.* (2002) reports no significant difference ($p > 0.05$) between aroma compound volatility

in solid trilaurin or liquid tricaprylin systems. The presence of only 0.5 g lipid kg^{-1} of liquid cloud emulsion made significant reductions in the headspace concentration for several compounds. This study suggests that small amounts of fat, in solid or liquid forms, dissolve or interact with volatiles to limit their release into a third air phase. Roberts *et al.* (2003) report that reductions in volatile adsorption are not seen until at least 50% of the lipid content is solid. Solid fats undergo continuous rearrangement and polymorphism of crystal structures, which may influence interactions with diffusing compounds (Walstra *et al.*, 1999).

Contradictory results in research on the role of surfactants in partitioning are reported. Guyot *et al.* (1996) reported decreases in the partition coefficients in paraffin oil and water systems when an emulsifier was included. Emulsifiers may block aroma compounds from diffusing into lipid phases or they may interact directly with the volatile within the aqueous phase. In systems without any lipids, Landy *et al.* (1996) reported that emulsifiers (sodium caseinate or sucrose stearate) reduced the volatility of ethyl butyrate, but with oils, the emulsifiers did not affect the air–liquid partitioning coefficient of ethyl hexanoate. Also the level of dispersion did not influence the volatility of ethyl butanoate. Increasing the liquid–liquid surface area from 1.6×10^{-3} to $10 \text{ m}^2 \text{ ml}^{-1}$ didn't change the volatility, suggesting that esters aren't adsorbed at the interface. In agreement with Landy *et al.* (1996), Carey *et al.* (2002) reported an emulsifier effect in lipid-free systems; however, with a constant level of lipids, the type, amount or particle size (surface area) of emulsifiers did not have a significant impact on flavor release. In flavor release studies, mass transport across an emulsion interface is the rate-limiting step, but its effect beyond this is questionable (Harrison *et al.*, 1997).

13.3.4 Non-lipid component effects on binding

Many researchers have investigated binding of flavor compounds to proteins and carbohydrates. Such binding impacts flavor release and may influence substrate availability, but studies show that with oil as low as 1%, the impact of non-lipid binding is minimal in comparison to lipid interactions (Seuvre *et al.*, 2000; Roberts *et al.*, 2003).

Salt content (NaCl and others) affects $\log K$ due to its interaction with water. When anions or cations are hydrated, the interacting water forms either a more hydrophilic or a more hydrophobic structure depending on the ion's relative lyotropic strength. The impact causes apolar substances to 'salt-out' or 'salt-in'. Likewise, polar substances also encounter a more or less favorable interaction in the aqueous phase (Walstra, 2003). Lubieniecki-von Schelhorn (1964) reported that sodium chloride decreased the solubility of sorbic and benzoic acid in the aqueous phase, while sucrose had no effect. McNulty and Karel (1973) demonstrated that addition of sodium sulfate increased partitioning of 1-octanol into an oil phase, due to an increase in water's polarity.

Influence of the milk fat globule membrane on partitioning and substrate availability is debated (Foda *et al.*, 1974; Roberts *et al.*, 2003). The milk fat

globule membrane is a combination of protein and lipids, and glycosylated forms, derived from cellular membranes during milk synthesis (Walstra *et al.*, 1999). In milk, the milk fat globule membranes prevent coalescence of fat globules and protect fats from enzyme interaction (e.g. lipolysis). Surface-active proteins can replace disruptions to the membrane so that globules always have an interface between lipid and aqueous phases. The membrane and surfactant proteins can be a barrier to transfer or may have a direct binding influence.

Movement between phases is affected by viscosity, interfacial surface area and binding (Seuvre *et al.*, 2000). Foda *et al.* (1974) speculated that the interface between fat and water phases was important to flavor development. Research shows in several cases that emulsifier layers do not significantly alter partitioning (Landy *et al.*, 1996; Carey *et al.*, 2002; Roberts *et al.*, 2003). Cellular membranes are effective in preventing passage of charged compounds and large molecules, like proteins (Nelson and Cox, 2000). Small hydrophobic compounds experience less resistance to passage through cell membranes and this would likely extend to milk fat globule membranes. In a study where the equilibrium, rather than the kinetics of partitioning is investigated, a disregard of emulsifier layer effects should simplify the study, without a great risk of overlooking important partitioning influencers.

According to Seuvre *et al.* (2000), the effect of β -lactoglobulin binding is dependent on lipid phase volume. Adsorption of proteins to the lipid–aqueous interface causes conformational changes and affects binding sites. As the relative lipid volume rose, the effect of binding from proteins was minimized. Studies by Roberts and Pollien (2000) also found that fat overrides the retention effects of proteins.

13.3.5 Partitioning in milk fat

Partitioning in milk fat may be a key factor in cheese flavor generation and release. In addition to lipid–aqueous partitioning, reversible and irreversible interactions may bind compounds involved in flavor formation (Piraprez *et al.*, 1998). Van der Waal, covalent and electrostatic interactions may influence the apparent partition coefficient in a food (Lee *et al.*, 1995). Movement between phases is affected by physical properties including viscosity, interfacial surface area and binding.

Early observations of partitioning effects were reported in milk fat fractionation studies. Dolby (1970) observed that flavors concentrate in lower melting (liquid) milk fat, while higher melting point fats had a flat flavor. These initial observations are used as evidence to indicate that only liquid fat is involved in flavor retention and release, but this is debatable. Later, van Boekel and Lindsay (1992) studied the partitioning of Cheddar cheese volatiles between milk fat, aqueous and vapor phases. Cheese can be considered as having two liquid phases: a fat phase dispersed in an aqueous phase. For their analysis, they used anhydrous milk fat and a sodium acetate buffer (0.1 M sodium acetate, pH 5.2 and 37.5 g L⁻¹ NaCl) to simulate the aqueous phase of Cheddar cheese. Milk fat

was heated to 40°C to liquefy it entirely. The milk fat was allowed to cool to 25°C before use in headspace analysis. According to van Boekel and Lindsay (1992), crystallization at 25°C decreased the liquid milk fat volume by 10%. They made the assumption that the volatiles would accumulate in the liquid portion only. They also noted that adsorption on proteins is likely to have an impact on volatile partitioning; unfortunately, this aspect was not investigated. van Boekel and Lindsay (1992) report partition coefficients of 3.5 for hydrogen sulfide, 6.13 for methanethiol and 14.25 for dimethyl sulfide, indicating that these compounds partition primarily into the lipid phase. Dimos *et al.* (1996) used partition coefficients from van Boekel and Lindsay (1992) to assist in correlation calculations between headspace volatiles and flavor. According to Dimos *et al.* (1996), improved correlations between headspace volatiles and flavor may be possible if more partition coefficient data were available.

Cornell *et al.* (1970) observed a decrease in $\log K$ between butter oil and water as the temperature was raised from 40° to 80°C. $\log K$ was also less in a milk salt solution (0.6% solids buffered at pH 6.5) than in water. Berg and Rankin (2005) determined $\log P$ values for *n*-chain alkan-1-ols over temperature, comparing a water/octanol system with a water milk fat system. This study showed that for the alcohols studied, temperature had a positive, non-linear influence on $\log P$ values and that the milk fat-containing systems had an approximately one-log decrease in $\log P$ values relative to the octanol standard. The influence of temperature was higher in milk fat systems as a function of the degree of solidification where alcohol motility was hindered by slower diffusion rates and possible entrapment within the solid milk fat material.

Flavor release is an additional topic of current interest in flavor research (Roberts and Taylor, 2000). These studies have shown the impact of fat and aqueous phases on flavor release kinetics in food systems under simulated eating conditions (Chung *et al.*, 2003; Miettinen *et al.*, 2003). Release into headspace considers a third phase (e.g. air), which enables aroma detection. Results of flavor release studies may be used to help define the phase partitioning conditions in cheese during production and aging. Likewise, a better understanding of equilibrium conditions may improve development of flavor release models and implementation of flavor release data into designing foods.

Recent research by Roberts *et al.* (2003) shows that the solid portion of milk fat entraps volatile compounds during solidification, but does not absorb volatiles once in a solid state. Their findings related flavor release of volatiles almost exclusively to the volatile's lipophilicity. Larger fractions of solid fat resulted in greater flavor release, due to less available liquid fat solvent. At 50°C, hydrogenated palm fat and milk fat, both in a completely liquid state, had the same level of flavor release. Adsorption of flavor compounds onto lactose or milk proteins was disregarded and this assumption was validated by an agreement of predictive models based solely on the liquid oil, water and air phase volumes. Differences in lipophilicity of different oils (medium-chain triglycerides versus longer-chain milk fat) did not impact flavor release as long as the fats were in a completely liquid state. Also, surfactants do not affect flavor

release. Dubois *et al.* (1996) found that solid fat impacts volatility of hydrophobic compounds in a comparison of anhydrous milk fat (15% solid at 25°C) to completely liquid tributyrin. Roberts *et al.* (2003) found that two hours of mixing on an orbital shaker was required to reach equilibrium for milk fat (1.36%), water and air phase partitioning trials at 10° to 50°C.

The effect of fat solidification has been shown on the distribution of compounds between phases (Dubois *et al.*, 1996; Roberts *et al.*, 2003). How the measurement $\log K$ relates to this distribution depends on how $\log K$ is defined. The native definition is based solely on partitioning between liquid volumes. However, for comparison, defining $\log K$ with the entire volume of lipid phase may be appropriate. An understanding of the level of solids and the reporting format is necessary to make comparisons and correlations between studies.

Consumer health concerns have created a large market for reduced fat cheeses. Such cheeses have a flavor that is quite different from the full fat version, thus resulting in limited consumer acceptance. Reduction of fat in foods leads to an alteration of the release rates of flavor volatiles, especially for more lipophilic compounds (van Boekel and Lindsay, 1992; Dubois *et al.*, 1996).

13.4 Impact of partitioning on flavor generation in cheese

Milk fat is recognized as a reservoir for fat-soluble flavor compounds (Foda *et al.*, 1974). Milk fat's role in other flavor formation mechanisms is less understood. Lee *et al.* (1995) stated that the physical laws of solubility and partitioning are likely the primary controller of retention and release of compounds in fat containing foods. Individual particles of a food product (e.g. salts, proteins, carbohydrates, as well as their degradation products) may exist dissolved within the lipid or aqueous phase. Particles may also exist at the interface between phases or be bound, entrapped, or adsorbed to other components (Kilara, 1995). Diffusion can be limited by the interactions of the various food components. Surfactants may play a role in substrate availability, as they can solubilize nonpolar compounds into the aqueous phase (Richards *et al.*, 2002).

Metabolism of protein, lipids and carbohydrates produces frank flavor compounds and substrates for additional flavor-forming reactions. The substrates are often hydrophilic in nature, due to their small size and abundant polar groups (e.g. carboxyl, amine and carbonyl). Flavor compounds often arise from reactions that reduce the polarity of the substrate (e.g. decarboxylation, deamination, condensation). The conversion of hydrophilic substrates to hydrophobic flavors exemplifies the importance of the lipid phase during cheese aging. Changes in the phase solubility characteristics or volume likely affect the conversion to important flavor compounds (Table 13.1).

Flavor-forming reactions in cheese occur due to intracellular microbial metabolism as well as extracellular chemical reactions, the latter occurring from the enzyme activities of lysed cells, endogenous milk enzymes and spontaneous chemical reactions. Similar to the effects that phase concentration has on flavor

Table 13.1 Conversion of hydrophilic substrates to hydrophobic flavors¹

Substrate	Log <i>P</i>	Ratio ⁶	Flavor	Log <i>P</i>	Ratio
L-Tryptophan	−1.1 ²	1:13	Skatole	2.6 ³	400:1
L-Phenylalanine	−1.5 ²	1:32	Phenylethanol	1.4 ³	25:1
L-Methionine	−2.0 ²	1:100	Methional	0.21 ⁴	1.6:1
L-Valine	−2.3 ²	1:200	3-Methyl butanal	1.3 ⁴	20:1
Ethanol and	−0.3 ²	1:2	Ethyl butyrate	1.8 ⁵	63:1
Butyric acid	0.79 ²	1:6			

¹ McSweeney and Sousa (2000).² Sangster (1997).³ Leo *et al.* (1971).⁴ Calculated according to Rekker and Mannhold (1992).⁵ Sangster (1989).⁶ Approximate theoretical ratio of concentration of compound in lipid phase to concentration of compound in water.

release and perceived flavor, phase concentration is hypothesized to also impact the extent of flavor substrate conversion reactions (e.g. oxidation, reduction, esterification, hydrolysis). Zeng (1997) utilized this concept in exploring the flavor impact of butyric acid in low fat cheese and how it relates to its dissociation constant and partition coefficient. Zeng's (1997) calculations may have varied if actual milk fat partitioning data had been available; instead these assumptions were based on a paraffin oil partition coefficient.

Ethyl butyrate is an important flavor compound in cheese, but in excess it causes a fruity flavor defect (Bills *et al.*, 1965; Christensen and Reineccius, 1995). Two common products formed during cheese ripening, ethanol and butyric acids, undergo esterification to yield ethyl butyrate. Available substrate, active enzymes and the particular K_{eq} (Equation 13.5) for the reaction conditions limit this reaction. K_{eq} for a reaction is dependent on the concentrations, or more accurately the activities (α), of each reaction participant.

$$K_{eq} = \frac{\alpha_{\text{ethyl butyrate}} \times \alpha_{\text{water}}}{\alpha_{\text{ethanol}} \times \alpha_{\text{butyric acid}}} \quad 13.5$$

Previous research has focused on the activity of enzymes and the concentration of flavor precursors (Ha and Lindsay, 1992; Liu *et al.*, 1998; Fenster *et al.*, 2000). However, solvents have an effect on availability of substrates and the equilibrium position of reactions (Janssen *et al.*, 1993). Christiani and Monnet (2001) differentiate esterases and lipases by their site of activity. Esterases are active in an aqueous environment, as opposed to lipases, which act at lipid interfaces. The location of enzyme activity and substrates will impact the extent of enzymatic conversion. In this example, the product, ethyl butyrate, is less polar and is likely pooled to a greater extent into the milk fat phase, as compared to ethanol and butyric acid (see Table 13.1). This pooling should yield a greater total concentration of ethyl butyrate as opposed to a single phase system. Changes in milk fat content, temperature (solid fat content) and salt would likely

affect how compounds partition, thus changing the activities of each of the reaction participants. Using log P estimations directly, the equilibrium is established at 0.49:1 ratio for ethanol in 1-octanol:water, while butyric acid would partition about an order of magnitude more into the 1-octanol phase (6.17:1). Ethyl butyrate would partition primarily into the 1-octanol phase (66.1:1). These differences in partitioning would alter the availability of reactants and thus the final concentrations of the target products.

Chin and Rosenberg (1997) established relationships between volatile concentration and fat content, temperature and time. However, current knowledge in this field is insufficient to make accurate predictions of the exact effect of changes on partitioning and the resulting effects on flavor formation. Carefully planned studies are needed to establish how the partitioning affects flavor development. Separation of direct partitioning effects from indirect effects from physical changes used to modify partitioning levels poses major complications to the study design. Temperature, salt, pH and fat content will impact substrate availability due to partitioning; however, they will also impact bacterial growth, enzymatic activity and substrate release.

13.5 Future trends

Given the complex, variable nature of cheese as a flavor delivery matrix and the dynamic, equally complex nature of cheese flavor chemistry, the development of sound, usable science to improve flavor performance is a daunting task. The ability of cheese to act as both substrate for and delivery matrix of flavor compounds poses an intriguing challenge. Some areas needing research include the creation of an improved understanding of milk fat as a solvent in liquid and solid (crystalline and amorphous) states and the overall binding and solving properties of individual milk components as well as their properties as a flavor release matrix when present in combination. The dependence of reaction pathways on specific enzymes and cofactors further extends the partitioning question to ask where these required components reside within the cheese matrix relative to their access to target substrates. One may also infer that there is an influence of partitioning on specific enzymatic activity or gene expression mediation mechanisms such as allosteric inhibition. Within living cells, such as those involved in milk fermentation and cheese ripening, there are further deviations from the simple, physical chemistry, entropy-driven gradients of partitioning imposed by such factors as substrate and enzyme compartmentalization within cell organelles and across membrane-bound structures. In summary, from a flavor chemistry perspective, it may be valuable in the future to consider and work to understand cheese as a type of complex 'flavor reactor' where solvents and solutes, reactants and products, enzymes and cofactors may ultimately be manipulated to achieve desirable flavor performance.

13.6 References

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14

Starter culture production and delivery for cheese flavour

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14.1 Introduction

Each cheese of the numerous types can be described broadly in terms of chemical composition (fat, protein, water, salt), but the characteristic properties of each type result from complex interplay of many factors, including milk composition, the manufacturing process, salting, the growth and biochemistry of internal and surface microflora, packaging and maturation conditions. Such factors are addressed in other chapters of this book.

Starter cultures, adjunct cultures and any adventitious non-starter organisms have crucial effects on cheese flavour. Controlling acid production by the starter culture during the cheese make is a key to achieving control over curd pH, moisture and lactose level. These factors in turn have a major influence on the microbial, chemical and biochemical environment of the maturing cheese, with effects on salt uptake, on bacterial growth and survival, and on the activities of coagulant and other enzymes. Controlling the microbial composition of a cheese is also a major step in controlling flavour development in the cheese, and so the strain and species composition of starter and adjunct cultures (and, if possible, other non-starter organisms) should also be controlled.

It is important to identify and characterize cultures with desirable properties if cheese flavour is to be controlled and improved. These cultures must be matched to the production process and to the product flavour specifications (Table 14.1). This chapter will address the technological strategies, options and challenges involved in obtaining and maintaining appropriate starter cultures, characterizing these cultures, preparing them in quantity and delivering them in

Table 14.1 Typical bacterial species in starter and adjunct cultures for some common cheese types¹

Bacterial species	Use ²	Cheese types							Key properties
		Cottage	Brie	Stilton	Gouda	Cheddar	Emmental	Mozzarella	
<i>Lactococcus lactis</i>	S	+	+	+	+	+			Mesophilic starters. Lactic acid production; proteolysis and other pathways producing flavour and aroma precursors
<i>Lactococcus lactis</i> subsp. <i>lactis</i> biovar <i>diacetylactis</i>	S, A	±			+				Similar to other lactococci, but also production of CO ₂ and characteristic aroma compounds from citrate
<i>Leuconostoc</i> species	S, A	±	±		+				Production of CO ₂ and characteristic aroma compounds from citrate
<i>Streptococcus thermophilus</i>	S						+	+	Thermophilic starters. Lactic acid production
<i>Lactobacillus helveticus</i>	S						+	+	Thermophilic starters. Lactic acid production
<i>Lactobacillus delbrueckii</i> subsp. <i>lactis</i>	S						±		Thermophilic starters. Lactic acid production
<i>Propionibacterium</i> species	S, A						+		Production of CO ₂ and characteristic aroma compounds
Other cheese-associated lactobacilli	A					+			Complex flavour and aroma development during cheese maturation

¹ This is not an exhaustive list. Some species are highly typical of the defined cultures for particular cheese types (+) and some variably used (±). Many other bacterial species are used in defined or undefined cultures for cheese making, and yeasts and moulds are also typical of some cheese types.

² S, typical component of defined (DSS) starter culture; A, typical component of defined adjunct culture. Undefined (MSS) cultures contain these and other species.

an active form to the cheese vat at each factory. The production of adjunct cultures follows similar principles (though acid production activity is generally not a concern) but will not be addressed in detail.

14.2 Strategic options

The technology behind starter cultures can be broadly divided into culture collection, culture maintenance, culture characterization, propagation of inoculum cultures, large-scale culture production, delivery to the vat, and on-going technical support (sample testing, data feedback, development of new cultures). Though these steps link into a logical chain of events (Fig. 14.1), they can be separated. Historically, self-sufficient artisan cheese makers carried out propagation, production and delivery within the cheese factory. With greater application of microbiological knowledge and culture preservation technologies, laboratories (attached to the cheese factory or operating independently) became involved in the new science of collecting, maintaining, characterizing and propagating cultures. Specialist laboratories now dominate these activities.

There are two major strategic divisions concerning application of cultures in the modern cheese industry, based on (1) the type of culture used and (2) the method used for delivering the starter culture to the cheese vat. The culture itself can be microbiologically defined or undefined in its composition (Sections 14.3.1 to 14.3.3). The starter culture added to the cheese vat can be grown as a fresh culture at the cheese factory ('bulk starter'; Section 14.8.4), or it can be grown elsewhere by a specialist culture supplier and transported to the cheese factory in a concentrated, preserved 'direct-to-vat' form (Section 14.8.5). The principles of starter culture growth and delivery, and the various available options, are described in the sections that follow.

14.3 Sources of cultures

For most of the history of cheese making, the microbiology of the process has been unknown. Cheese makers relied on habitual process and ritual to create appropriate conditions for lactic souring of milk and subsequent cheese maturation. Scientific analysis of dairy fermentations began in the late nineteenth century, and starter cultures became established as important tools in cheese manufacture. By starting the milk fermentation with a consistent starter culture with defined desirable performance properties, the overall quality, reliability and safety of the cheese are improved. Three different ways to achieve culture consistency were developed and, with technological variations, continue in use today. These are known as natural cultures, undefined (mixed-strain) cultures and defined cultures.

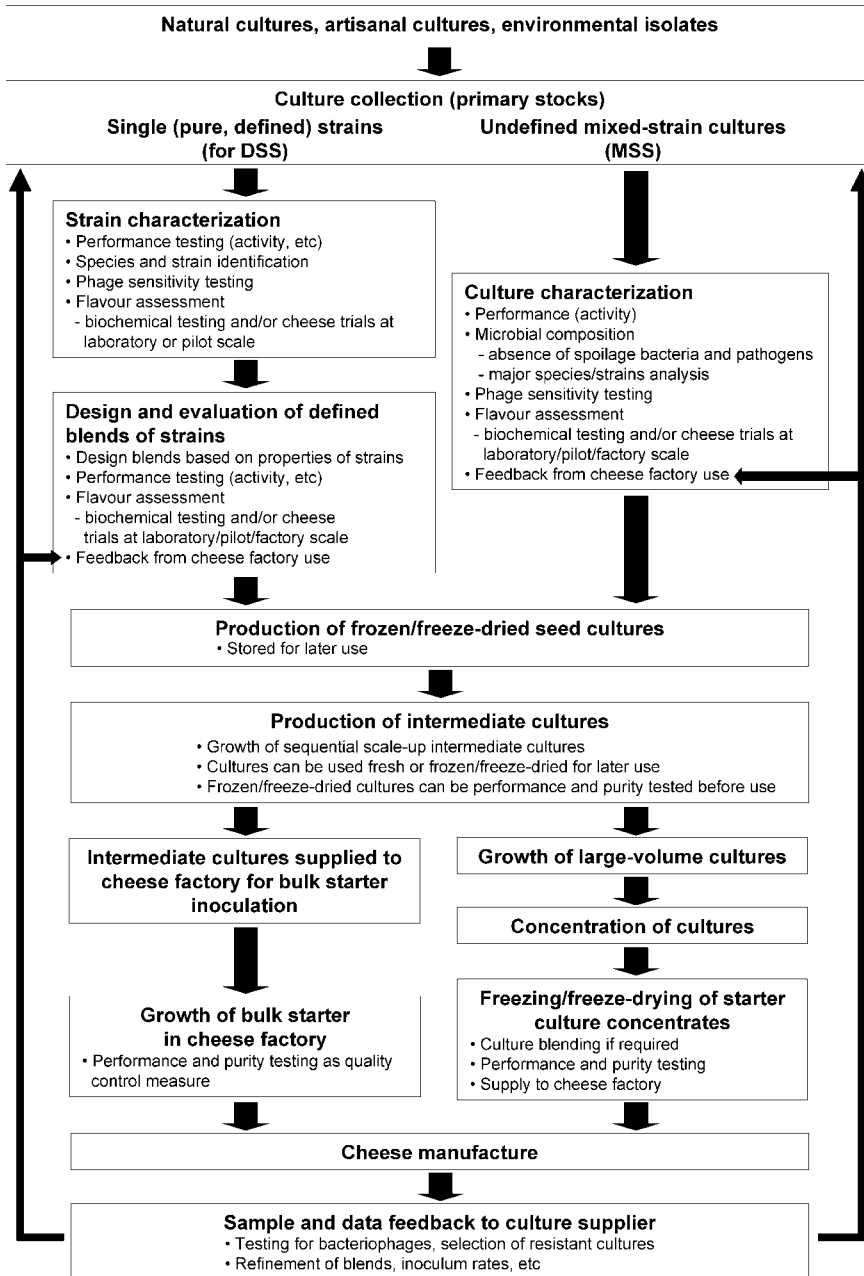


Fig. 14.1 A general scheme for selection, management and growth of MSS and DSS cheese starter cultures. Note the strategic choices of starter composition (top half of figure) and method of delivery to the vat (bottom half of figure).

14.3.1 Natural milk and whey cultures

Various procedures have been used historically to produce 'natural' starter cultures for cheese making (reviewed by Limsowtin *et al.*, 1996). Some of these procedures survive for the production of European regional cheeses.

In the simplest process, natural milk cultures can be prepared by incubating milk at specified temperatures to encourage the growth of appropriate acid-tolerant organisms present in the milk. This process has not survived into industrial-scale cheese manufacture due to variable performance and microbiological hazards.

In the most common process, natural whey cultures are prepared using whey from a recent cheese batch in a selective incubation to produce starter culture for the next batch. The composition of such a culture is unknown at the time of use (though extensive retrospective research studies have been done) and is always changing. With careful process control and quality control testing, this approach remains in use today. It has been adapted to commercial industrial-scale manufacture of traditional cheeses such as Italian water buffalo Mozzarella (Coppola *et al.*, 1988) and Grana (Bottazzi, 1981). More complex procedures (such as the traditional calf stomach in whey method for Swiss cheese starter cultures) give variable results and are widely being replaced by undefined laboratory cultures.

14.3.2 Undefined laboratory cultures

Carefully preserved archive stocks of high quality and consistently performing artisanal starter cultures (e.g., giving good vat performance for pH reduction, good cheese flavour, no defects and free of pathogens) can be used as the basis for a more reproducible culture preparation system. Using basic bacteriological techniques to avoid contamination, cultures can be maintained (frozen, freeze-dried or sequentially subcultured) outside the cheese vat. Initially, cheese makers carried out this work within cheese factories, subsequently it was done by the staff of dairy laboratories, but it is now commonly performed in specialist culture laboratories.

These cultures are maintained as undefined mixtures of different strains and often different species of bacteria. Some cultures also contain yeasts. Improving techniques for bacterial identification (particularly molecular biology techniques; Schleifer *et al.*, 1995) have given culture laboratories greater ability to study the composition of mixed cultures, but the full composition of any such culture is not known. Due to their undefined, mixed nature, they are known by descriptions such as 'undefined cultures' or 'mixed-strain starters' (MSS).

The variability of cultures continually propagated by subculture, especially if exposed to bacteriophages (phage) (see Section 14.5), is illustrated by the work of Stadhouders and Leenders (1984). If subculturing is kept to a minimum and subculture handling follows a prescribed method then the general microbial characteristics remain relatively stable (Stadhouders, 1986), giving more consistent cheese making than either natural starter cultures or starter cultures that

are extensively subcultured. The exact microbial composition of MSS cultures is not known, though some have been well studied (see Stadhouders and Leenders, 1984; Lodics and Steenson, 1990).

14.3.3 Defined cultures

Another approach to achieving reproducibility in starter culture performance is to use single-strain bacterial isolates from natural or undefined cultures as the basis of culture composition. Each strain is initially obtained as a single colony isolate, and is maintained as a 'pure' culture (i.e. containing only a single strain), tasks best performed by a specialist laboratory.

Each strain can be carefully characterized in terms of its growth, biochemistry, genetics (the properties of particular genes, relationships between strains, or complete genomic analysis) and relevant properties such as sensitivity to phage (Section 14.5) or effect on cheese flavour. A culture blend can then be assembled with a mix of strains (typically two to six strains of one or more species) giving the desired cheese making characteristics and flavour development. On the basis of known phage sensitivities, a strain blend can be designed so that no one phage is likely to infect all strains in the culture, making it unlikely to fail in the event of phage infection. These designed multiple-strain cultures are known as defined-strain starter cultures (DSS). They provide rational control over culture composition and the ability to design cultures (blends of strains) with specific properties. Their development is reviewed by Lawrence *et al.* (1978) and Heap (1998).

14.4 Starters, adjuncts and selection of cultures

The many different starter cultures and adjunct cultures have diverse properties that have effects on cheese flavour. These are described at length in other chapters. However, the conventional definitions of a starter culture (primarily responsible for lactic acid production) and an adjunct culture (secondary culture added to influence cheese flavour or texture) are not always easy to apply.

MSS (and their artisanal ancestors) usually contain a complex mix of strains. Only some of these will be fast acid-producing strains, and development of characteristic flavour can be due to other strains in the mix. Defined-strain cultures contain fewer strains and typically give a simpler flavour profile, but adjunct cultures (usually added separately to the cheese vat) can be used to enhance and control flavour development. Cheese makers have used many culture combinations in search of cost-effective flavour customization, including mixtures of defined-strain starter and undefined starter cultures. In this example, both cultures contribute to acid production, but the undefined culture added mainly for its contribution to flavour could be regarded as an adjunct.

Regardless of how cultures are described, they are typically chosen for use on the basis of particular properties (relating to vat performance, flavour potential,

resistance to bacteriophage infection, etc.) known from laboratory testing, pilot-scale cheese making studies or past industrial cheese-making experience. However, a given culture combination can give different results when used in different milk, under different cheese make conditions, or in cheese with different composition. Therefore, any culture combination must be evaluated by the potential user to ensure that it meets required specifications.

Another criterion applied to culture choice is ease of growth and whether a starter culture survives handling and storage. These processes are described for starter cultures in the sections that follow, but the growth of adjunct cultures is not specifically addressed in this chapter. In general terms, many adjunct cultures have similar growth requirements to starter cultures, and can be grown, concentrated and preserved using similar approaches. However, some have atypical nutritional requirements that necessitate different or supplemented growth media. Some adjunct formulations are deliberately attenuated to accelerate enzyme release through cell lysis. A highly concentrated adjunct inoculum is used if rapid development of a specific flavour is required.

14.5 Bacteriophages and strain selection

The cheese maker must always be alert for phage infection of cultures. Phages are viruses that infect bacteria, and infection of starter cultures is a major cause of starter performance variability in cheese making. Industrial consequences of phage infection include changes in strain ratios of MSS or DSS (potentially altering flavour) and slower acid production by the starter culture, often necessitating adjustments to the cheese make. In extreme cases, severely diminished acid production has major effects on cheese compositional properties and (rarely in modern cheese manufacture) can lead to abandonment of production. Precautions to minimize the effects of phage infection on acid production are based on improved factory design and practices (whey containment, control of airflow, general sanitation), phage-free propagation of starter cultures (though this is not possible with some undefined cultures) and better selection of strains used in starter cultures. It should be recognized that, by giving more consistent starter culture performance, these precautions also increase product consistency.

Phage biology (Jarvis *et al.*, 1991; Brüssow, 2001), phage detection (Svensson and Christiansson, 1991) and phage control in the cheese industry (Lawrence, 1978; Accolas *et al.*, 1994; Sturino and Klaenhammer, 2004) are major sciences in their own right, and will not be discussed in detail here.

Infection of starter cultures typically takes place in the cheese vat (i.e. in the relatively exposed conditions of the cheese factory), but infection during culture preparation can also occur if culture handling and hygiene are poor. The ultimate environmental sources of most cheese industry phages are not known, but phages have been shown to enter the factory in association with bacteria in the incoming milk (Heap *et al.*, 1978; Madera *et al.*, 2004). Some undefined cultures

contain phages, causing on-going changes to the strain composition during propagation and in the cheese vat (Stadhouders and Leenders, 1984; Lodics and Steenson, 1990). Whatever its source, once a phage enters a cheese factory and begins to multiply it will spread to any culture that is sensitive to it and is difficult to eradicate.

Phage infection of a culture can cause sufficient cell death that the culture is no longer suitable for use. The strategy for replacement of a phage-infected culture depends on the culture type. MSS must be withdrawn from use and a replacement culture found, similar in cheese making characteristics but different in sensitivity to phage. With defined culture blends, only infected strains within the culture need be replaced. In either case finding an appropriate replacement is not always easy. There are four sources of 'new' cultures:

1. Suppliers typically have an archive collection of pure strains and/or mixed cultures within which suitable replacements might be found.
2. Phage-resistant variants (also known as derivatives or bacteriophage insensitive mutants, BIMs) can often be selected by deliberately exposing cultures to phage infection.
3. Strains carrying genes that give them resistance to one or more species of phage can be used in natural matings (conjugation) to enhance the properties of sensitive strains.
4. Genetic manipulation offers even further improvement of resistance to phage infection, but international consumer opinion has effectively prevented commercial application of this technology.

These approaches are reviewed by Lawrence *et al.* (1978), Limsowtin *et al.* (1996) and Sturino and Klaenhammer (2004).

Whatever the source of a replacement (either a defined replacement strain in a DSS culture or a replacement MSS culture), it must be evaluated to determine whether its properties are appropriate to the cheese maker's needs. In some cases, it might be necessary for the cheese maker to adjust the cheese make to compensate for different performance characteristics. Rotation of cultures (alternate use of two or more DSS or MSS cultures to minimize opportunities for phage multiplication to high levels) has similar problems, unless cultures different in phage sensitivity but very similar in vat performance and flavour characteristics can be found.

Phages in dairy environments are continually changing, and so monitoring of culture performance, testing of whey samples for the presence of phages, rotation design and culture replacement is an on-going process requiring informed technical management and systematic use of cultures.

14.6 Characterization of cultures

Early starter culture technologists could observe how a culture behaved in the cheese vat and what the flavour and other properties of the cheese were like. As

the tasks associated with starter isolation and propagation moved from the hands of the cheese maker into specialist laboratories, more detailed analyses became possible. Laboratory-scale experiments simulating cheese manufacture could be used to assess the behaviour of cultures under various conditions (different cheese make temperatures or cheese compositions, milk from different sources, altered salting levels, etc.) before a culture was used in the cheese factory. For DSS, pure strains could be evaluated on their own and then cultures could be assembled with various strain combinations to assess their performance.

With advances in analytical technologies, it has become possible to study the biochemistry and genetics of starter cultures and to integrate this information into a greater understanding of their properties. Knowing the enzymatic capabilities of pure strains and mixed cultures, and measuring the chemical products of their metabolism, allows researchers to correlate the properties of cultures with desirable and undesirable aspects of cheese flavour. This continues to be a major area of research with increasing impact on culture technology. Genetic studies, particularly of lactococci and lactobacilli, have uncovered the genes underlying many metabolic properties associated with growth in milk and production of specific flavour compounds. Studies of gene sequences and expression (and, more recently, total genome sequences) are gradually revealing more details of the metabolic potential of these bacteria. To date, the greatest practical impacts have been in the following areas: (1) enhancement of diacetyl production; (2) understanding proteolysis, peptidolysis, amino acid metabolism and flavour development; and (3) genetics-based identification of strains, leading to more reliable species classification and strain identification, culture quality control and assessment of relationships between strains. Genetic identification tools have been the primary basis for research examining the strain composition of MSS and for studying the growth and survival of adjunct strains in maturing cheeses.

14.7 Culture requirements

The type and quantity of culture required to make a cheese depend on the manufacturing process used and the flavour characteristics required. The essential issues in culture production and supply are to ensure that the culture is delivered in good quantity and quality. The culture delivered to the cheese vat must be capable of sufficient activity to give the necessary acid production rate, and must be of the desired composition to achieve the development of appropriate flavour notes. The quantity of acidifying culture required varies depending on the rate of acid production required during cheese manufacture. For example, the manufacture of low-moisture, high-acid cheeses may require more than 10 times the amount of culture needed for low-acid, high-moisture cheese manufacture. Major changes to milk composition (such as increased solids content achieved using ultrafiltration techniques) can also alter starter culture quantity requirements.

The way in which the culture responds to the physical environment during cheese making is significant. Changing temperatures and salt addition are stresses that starter strains react to differently, so the appropriate choice of starter culture and production of that starter culture with consistent composition (i.e. consistent strain ratios within the mixed culture) are important contributors to consistent cheese production. Consistency of cheese microflora composition is a key element in controlling flavour development, and so consistency of any adjunct cultures used is also important.

14.8 Starter culture growth and delivery

Whatever their composition, starter cultures are maintained in small quantities by laboratories, in stable frozen and/or freeze-dried (lyophilized) form. On the other hand, the cheese-maker needs large quantities of culture, especially for industrial-scale cheese manufacture. Depending on the cheese type, the make procedure and the activity of the starter culture, this requirement will be in the range of 10 to 200 litres of milk-grown starter culture for every tonne of cheese made. Any technical advances that increase the acid-producing activity of starter cultures will reduce the volume of culture required, thereby reducing the size and cost of the equipment needed to grow, handle and store the cultures. The following sections will describe how commercial quantities of starter cultures are produced.

14.8.1 The starter culture laboratory

Modern starter culture production begins with a reliable source of cultures, carefully preserved, properly identified and characterized, and supplied in a form useful to the cheese maker with guidance on how they should be used and up-to-date information on phages known to infect each culture. In industrial cheese making with tightly defined product specifications, starter culture performance is crucial. The level of technical support required is beyond the abilities of all but the most sophisticated factory laboratories, and so cheese makers increasingly rely on support from specialist laboratories, usually associated with starter culture suppliers.

14.8.2 Culture scale-up

Culture scale-up must be done in a way that minimizes opportunities for strain mutation or changes in mixed culture composition, following the principles that primary stocks should be handled as little as possible and sub-culturing steps should be kept to a minimum. Culture volume is progressively increased from a small seed culture (e.g. 1 ml), through one or more intermediate cultures to a final large-volume culture (thousands of litres), which is to be used as the cheese starter. Unless the culture has special requirements, the initial scale-up steps are

conventionally carried out in milk (usually sterile autoclaved non-fat milk reconstituted from powder). One example scheme, typical of culture handling in industrial cheese factory laboratories during the 1950s to 1970s, is described below:

1. A primary stock vial is used to inoculate many identical culture tubes that can be stored frozen until needed. These act as the seed cultures for later scale-up steps, typically increasing the culture volume 100-fold in each step.
2. A seed culture is incubated when needed (overnight incubation at appropriate temperature) and then sub-cultured by inoculating a larger volume (sometimes called a 'mother' culture).
3. After incubation, this is used to inoculate a larger volume, which in turn is used as inoculum for the final starter culture.
4. After incubation, the starter culture is used as the inoculum for cheese making.

There are many possible variations for the starter culture scale-up scheme, including:

1. The number of steps can be changed by altering seed culture volumes (e.g. 10 ml versus 1 ml) and sub-culture inoculum levels (e.g. 0.1% versus 1% v/v). In some schemes only one intermediate culture is used between seed and final culture. Alternatively, additional steps might be needed to generate sufficient inoculum for a large starter culture vessel. Bulk starter culture volumes of 10,000 litres or more per day are not uncommon in cheese factories. Even larger volumes are typically grown by suppliers of direct-to-vat starter culture concentrates.
2. Seed cultures can be maintained at the factory or supplied by an external laboratory in fresh, frozen or freeze-dried form. Freeze-dried seed cultures were widely used in the days before freezers became readily available.
3. Bulk starter inoculum cultures (frozen or freeze-dried intermediate cultures) can be obtained from an external laboratory.

Culture maintenance and scale-up involve repeated culture handling during sub-culturing. The process is prone to contamination (with bacteria or phages) and provides opportunities for undesirable changes in mixed culture composition unless growth conditions are carefully controlled. Cheese factories and their adjacent laboratories are not the ideal location for this work. Typical cheese factory environments may have significant phage populations (airborne via factory aerosols). Factory laboratories may lack staff with appropriate microbiological skills or facilities for aseptic culture handling under carefully controlled conditions.

One solution to these problems is to remove some or all of the culture scale-up steps to a remote facility specializing in storing and propagating starter cultures. Most cheese made on an industrial scale today makes use of pre-tested inoculum cultures (Section 14.8.3) for growth of bulk starter (14.8.4) or concentrated direct-to-vat cultures (14.8.5).

14.8.3 Pre-tested intermediate (bulk starter inoculum) cultures

Preparation of inoculum cultures by a remote laboratory offers cultures grown under clean, controlled conditions. This removes all culture propagation steps from the cheese factory except for growth of the bulk starter culture, reducing risks of culture contamination with undesirable bacteria or phages. In modern practice, these cultures are typically grown to high culture density in pH-controlled fermenters, so that only a small volume of culture (e.g. 0.01% v/v) is needed as inoculum for the bulk starter culture. Alternatively, high cell densities can be achieved by centrifugally concentrating the cells.

A culture laboratory can easily prepare hundreds of identical inoculum cultures in a single batch, freezing (less commonly, freeze-drying) them for later testing and use. This approach allows thorough quality control testing of purity and performance before the culture is sent to the cheese factory, and the cheese maker can be assured of minimal culture variability within and between culture batches. The same culture production strategy can be adopted within a suitably equipped cheese factory laboratory, although isolating the laboratory from the general environment of the factory can be difficult.

As well as culture reproducibility, this approach offers considerable flexibility in culture choice. With a range of cultures held in the factory freezer (pure strains, defined mixes or undefined cultures) the cheese maker can change culture at short notice to meet product requirements or to respond to phage infection, needing only to decide which culture(s) to use to set the bulk starter culture incubation. Examples of the production and use of bulk starter inoculum cultures are described by Stadhouders *et al.* (1969), Turner *et al.* (1979), Timmons *et al.* (1988), Limsowtin *et al.* (1997) and Fox *et al.* (2000).

14.8.4 Bulk starter culture growth at the cheese factory

In contemporary industrial manufacture of cheeses, usually only larger factories (making at least 30 tonnes of cheese per day) grow bulk starter culture on site. Bulk starter cultures are very cost-effective at this scale, but the financial investment required to install, operate and maintain a clean, reliable bulk starter culture system and the staff training required to manage it are beyond the resources of many small operations. The major issues in bulk starter culture growth are hygiene, consistency of growth medium, culture activity, control of strain ratios and absence of phages. Key features of a bulk starter culture growth system are shown in Fig. 14.2.

The inoculum culture (fresh, frozen or freeze-dried) is added to a larger (bulk) volume of growth medium (see Section 14.8.6). Sterile medium and aseptic inoculation are essential to avoid contamination. A simple inoculation port (with or without local steam or flame sterilization) is sufficient if used in conjunction with a sterile positive-pressure air supply to the bulk starter culture vessel. Special inoculation devices have also been used (e.g. Lewis, 1987; Cogan and Hill, 1993).

This bulk culture is grown at controlled temperature (see Section 14.8.8). When the culture nears the end of its growth (8–18 hours, depending on the

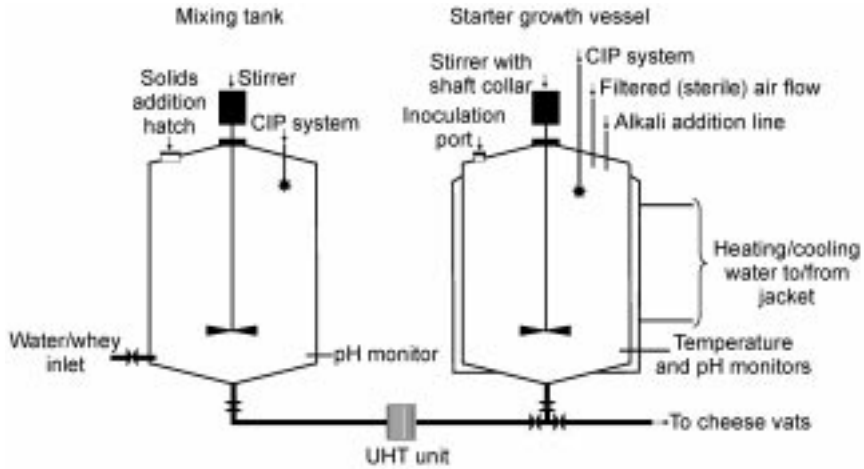


Fig. 14.2 Illustration of features typical of modern bulk starter growth systems. There are many variations on this scheme, for example, (1) heat treatment of the growth medium can be done in the starter growth vessel rather than by using an external heat exchanger, (2) mixing of growth medium components can be done using a special mixing apparatus or in the starter vessel, with heat treatment in the starter vessel, and (3) more rapid cooling of the grown starter can be achieved using an external chilling heat exchanger and a separate holding vessel. If fresh milk is used as growth medium, no mixing is required.

system) but has not yet entered stationary phase or decline, it is chilled as quickly as possible to 4°C or below using a water-jacketed cooling system or, more rapidly, a heat exchanger. The chilled culture remains active and can be used over the next one to two days. Storage for longer is possible if the pH is not too low (the tolerable value is species- and strain-specific) and low temperature is maintained, but some decrease in starter culture activity would be expected. This ability to hold the culture permits limited testing before use if desired (e.g. acid production activity, phage, coliforms or other contaminants).

The absence of pathogens and spoilage organisms in the bulk starter culture relies on having (1) uncontaminated intermediate cultures, (2) clean, sanitized equipment, (3) sterile growth medium, and (4) controlled, filtered airflow. Similar precautions are essential for minimizing phage risks.

Techniques to improve culture growth and acid-producing activity are described in Section 14.8.6. Though these generally help to increase starter culture growth rate and/or cell density, different starter strains inherently grow at different rates depending on such factors as temperature, pH and nutrient availability. Consistent culture composition can, in principle, be achieved through strict adherence to consistent culture growth conditions (at all stages from seed to bulk), and this is the best that can be done with undefined cultures. With defined-strain cultures, tightly specified ratios of culture components in the cheese vat can be achieved by growing each component separately and then mixing them in the desired ratio. With simple culture blends (e.g. a mix of two

strains) this is easily done with two bulk starter culture vessels, but few cheese factories have enough starter culture growth vessels to grow the separate components of more complex culture mixes. For mixed-species cultures (e.g. *Streptococcus thermophilus* with *Lactobacillus helveticus*), the species ratio in the bulk starter culture can be influenced by choice of growth medium, temperature, pH and relative inoculation levels. The observed effects are often strain-specific.

14.8.5 Direct-to-vat cultures

Cheese factories lacking the resources to prepare reliable bulk starter cultures make use of cultures grown off-site, eliminating all culture preparation steps from the factory or its associated laboratory. Culture maintenance and growth are essentially similar to factory bulk starter culture preparation, though usually on a larger scale: one culture production facility provides the starter culture needs of many cheese factories. Storage and transport of such large volumes of culture are not practicable, and so the cultures are concentrated for easier handling, then frozen or freeze-dried for storage. Concentration is achieved using membrane concentration systems and/or by continuous-flow centrifugation (Stadhouders *et al.*, 1969; Stanley, 1998). Cryoprotectants (lactose, sucrose, other sugars, monosodium glutamate, glycerol, etc.) are usually added to reduce damage to starter culture cells during freezing and storage (Kilara *et al.*, 1976).

Concentrated cultures (reviewed by Gilliland, 1985) can be added directly to the cheese vat, and so these cultures are known by various descriptions including direct-to-vat (DTV), direct vat inoculation (DVI), direct vat set (DVS) or direct-set starter cultures. Commercial DTV starter cultures are supplied frozen (requiring storage at -30°C or lower to achieve practical shelf life) or freeze-dried.

In simplest form, cultures are supplied as frozen blocks, now typically supplied in cardboard cartons. Cultures snap-frozen in small pellets have advantages over solid blocks, though the freezing process is more complex. Rapid freezing of pellets (around 0.1 g each) rather than slow freezing of blocks (up to 500 g to 1 kg each) has likely benefits in survival and activity of starter cultures (Keogh, 1970). Culture pellets can easily be blended after freezing, permitting separate growth of the culture components and more extensive testing if required prior to blending. Pellets can be packed in simple plastic bags, permitting cheaper packaging and easier customization of pack size.

Cultures are packaged (ideally under oxygen-free conditions) as specified activity doses suitable for particular vat sizes or production processes, with each manufacturer having its own 'Unit' of culture activity. Small cheese makers typically have to adapt their cheese make to suit the available pack sizes, whereas a culture supplier might be able to supply customized pack sizes specifically for large customers.

Users of bulk starter cultures have fine control over the volume of starter added to each vat, thereby adjusting starter activity for changes in milk

composition, for inhibition due to phage or other non-phage inhibitors, or for different product requirements. Users of DTV starter cultures cannot easily make these adjustments, and so must change make times or temperatures to compensate for these events. However, it is possible to use DTV-style starter cultures in a manner that mimics bulk starter use by dispersing DTV culture in a holding tank and then dispensing the required volume to each vat. This approach is not widely used, as it involves both the equipment cost of holding and dosing systems (as would be used for bulk starter) and the cost of concentrated cultures.

Freeze-dried cultures are typically supplied as a powder in a sealed foil pouch. Dried cultures require less storage space than frozen cultures and transport and storage are simple and relatively inexpensive (shelf life is extended at low temperatures but freezing is not strictly required). For these reasons they are used by many small cheese-makers, especially if they do not have access to reliable freight services. The production process is expensive and some starter strains, notably strains of *Lactococcus lactis* subsp. *cremoris* used in making mild Cheddar-style cheeses, do not survive freeze-drying well (Yang and Sandine, 1979). The alternative technology of spray-drying is a cheaper process but survival is generally even lower, preventing broad commercial application. Freeze-drying results in damage to cells, so that significant activity is lost (adding to the cost of culture production) and surviving cells are slow to reactivate in the cheese vat (Sandine, 1996; To and Etzel, 1997). Therefore, the acid production profile of freeze-dried cultures typically has an extended lag phase and the cheese make should be adapted accordingly. Frozen starter culture concentrates may also suffer this problem.

As with bulk starter cultures (Section 14.8.4), DTV cultures can be defined (14.3.3) or undefined (14.3.2) in their microbial composition. The strategies used to minimize batch-to-batch growth variation of cultures are the same as with bulk cultures – minimal sub-culturing, reproducible controlled growth conditions and, in the case of defined cultures, the option of separate growth of the culture components. Freshly grown cultures of each component can be held chilled while cell count estimates, acid production activity tests and limited purity testing are performed. Defined blended cultures with specified component ratios and activity can then be mixed. Alternatively, components can be frozen or freeze-dried, evaluated and then blended prior to packaging. Starter cultures freeze-dried as a powder or frozen in the form of free-flowing pellets can be handled this way without having to be rehydrated or thawed.

14.8.6 Growth media for industrial growth of starter cultures

Starter (and adjunct) strains generally have complex nutritional requirements, and so they must be grown using media containing various salts, amino acids and vitamins. They grow poorly unless provided with a fermentable sugar to use as an energy source, and must be grown at an appropriate temperature. The requirements differ for each species and, to a lesser extent, for each strain within a species. Historically, milk and whey were the starter culture growth media

most readily available in the dairy industry, and they are the basis of most media in use today.

The lactic acid produced by growing starter cultures causes several problems. Low pH conditions are unfavourable for metabolism, growth and long-term survival, and the accumulation of lactate ions is itself inhibitory. Therefore, pH control and prevention of lactate inhibition are major issues in starter culture production.

Milk-based media

Whole milk or non-fat (skim) milk are the most obvious media to use for starter culture growth. Every cheese factory has milk on hand and it is a rich medium (lactose, vitamins, minerals and high protein content) in which conventional starter cultures can be grown. It has in-built pH buffering capacity, mainly due to casein-associated colloidal calcium phosphate. The only processing step required is a heat treatment to inactivate bacteria and phages, achieved either by heating in the growth vessel to ~90°C for at least 30 min or by passing it through a plate heat exchanger for high-temperature/short time treatment. Temperatures in the range 90–130°C for at least several seconds have been used, with severe treatment more likely to ensure killing of undesirable microorganisms including wild lactic acid bacteria, clostridia and phages.

The rate or extent of starter culture growth in milk is in some cases limited by the proteolytic activity of a culture. Heat sterilization assists growth by causing some denaturation and hydrolysis of caseins, making peptides and amino acids more accessible to the starter culture (Cogan and Hill, 1993). Heating also inactivates some inhibitory agents, notably the lactoperoxidase system (Desmazeaud, 1996). Growth stimulation of many strains can be achieved by adding additional sources of peptides and amino acids, such as whey powder, whey permeate (a by-product of whey protein concentration by ultrafiltration), hydrolysates of casein, whey or vegetable protein, or yeast extract. Yeast extract is also a source of other nutrients that can stimulate starter culture growth. Ingredients used in starter culture growth media are reviewed by Whitehead *et al.* (1993). Commercial blends of growth stimulants are available in powdered form; some are tailored to the mineral and vitamin needs of particular starter culture types. Users should be aware that any change to the growth medium can alter the strain balance of mixed cultures and therefore the flavour profile.

Skim milk reconstituted from spray-dried powder has been widely used, though it is more expensive than fresh milk and requires powder storage and mixing facilities. Unlike fresh milk that varies in composition or in the level of inhibitory agents (e.g. very low level residues of veterinary antibiotic residues are sufficient to slow starter culture growth), milk powder is tested and chosen batches standardized before use. Its nutrient and buffering capacity can be increased by increasing the solids level of the reconstituted milk (e.g. to 14% w/v) (Cogan and Hill, 1993), or it can be fortified with other growth stimulants to increase the cell populations.

Milk was once the standard medium for bulk starter culture growth in cheese factories, and continues to be the dominant medium in countries such as

Australia and New Zealand. Precautions to exclude phages from the bulk starter culture (Fig. 14.2) are an essential part of successful milk-based bulk starter preparation (Lawrence, 1978; Limsowtin *et al.*, 1997).

Milk is not a convenient growth medium for DTV cultures, because of the difficulties in harvesting starter culture cells either by centrifugation or membrane filtration. Milk can be clarified by destabilizing the casein micelles (e.g. by adding trisodium citrate) (Stadhouders *et al.*, 1969). However, the use of a growth medium low in casein or particulate matter (often based on cheese whey) is a simpler option. It has been reported that citrate clarification is not suitable for use with all cultures (Gilliland, 1985).

Whey-based media

Cheese whey is in many ways similar to milk as a growth medium. Its lactose, vitamin and mineral content are similar to the aqueous phase of milk, with the pH dependent exception of an elevated calcium level. Its total protein content is lower, but it contains readily available peptides and amino acids resulting from the combined action of rennet and starter cultures on casein during the cheese make. Whey-based derivatives are also suitable, such as hydrolysed whey (protease treated), whey permeate, deionized whey or whey powder.

Addition of growth stimulants to whey can greatly improve starter culture growth. As with milk-based media, any changes can alter the strain balance of mixed cultures. Growth media can be prepared from fresh 'sweet' whey (e.g. pH ~6.4) or whey powder or using any of a variety of commercially available blends of whey powder and stimulants, mixed and sterilized as for milk-based starter culture media. Whey-based media are suitable for bulk or DTV starter cultures. Formulations low in casein micelles or particulate matter minimize carry-over during centrifugation and reduce fouling of heat exchangers during sterilization and of membranes during concentration of cells.

Whey has relatively poor pH buffering capacity, and so the addition of buffering salts is common (Whitehead *et al.*, 1993). These buffers help to slow the decline in pH that occurs due to lactic acid production by the starter culture, thereby allowing higher bacterial numbers to accumulate and survive, and so producing a starter culture with greater acid-producing activity and which retains activity longer. Many different salts (and blends of salts) have been used and examples are given in Table 14.2. Some are readily soluble (e.g. sodium phosphates), while others are added in particulate form but dissolve as the pH falls during starter culture growth (e.g. trimagnesium phosphate) (Sandine, 1996). Encapsulated buffers designed to dissolve in a pH-sensitive manner have also been used (Whitehead *et al.*, 1993). These added buffering agents are collectively known as 'internal' pH control systems, and can be combined with external pH control (Section 14.8.7) and chilling to achieve greatly improved starter culture growth, performance and retention of activity during storage.

Calcium ions are generally required for phage infection of starter culture, though their precise role remains unclear (Geller *et al.*, 2005). The solution behaviour of associating ions can be exploited to lower the availability of free

Table 14.2 Examples of agents for pH control during starter culture growth¹

Name ¹	Chemical formula(e)	Comments
<i>Internal pH control</i>		
Sodium phosphate	NaH ₂ PO ₄ , Na ₂ HPO ₄	Mono- and di-sodium phosphates mixed to give phosphate buffering at desired pH
Ammonium phosphate	(NH ₄)H ₂ PO ₄ , (NH ₄) ₂ HPO ₄	Mono- and di-ammonium phosphates mixed to give phosphate buffering at desired pH
Trimagnesium phosphate	Mg ₃ (PO ₄) ₂	Insoluble in water at neutral pH but dissolves in dilute acid, providing slow-release phosphate buffering
Magnesium hydroxide	Mg(OH) ₂	Insoluble in water at neutral pH but dissolves in dilute acid, providing slow-release alkali (OH ⁻ raises pH)
Sodium carbonate (soda ash, washing soda)	Na ₂ CO ₃	Generates alkali when dissolved in water. Whitehead <i>et al.</i> (1993) reported use of Na ₂ CO ₃ coated with magnesium stearate. The coating is insoluble in water at neutral pH but dissolves in dilute acid, providing slow release of Na ₂ CO ₃
<i>External pH control</i>		
Ammonia	NH ₃	Ammonia gas dissolves in water to produce alkaline ammonium hydroxide
Ammonium hydroxide (aqua ammonia)	NH ₄ OH	Alkaline product of ammonia dissolved in water
Sodium hydroxide (caustic soda)	NaOH	Dissolves readily in water to give a strongly alkaline solution
Potassium hydroxide (caustic potash)	KOH	Dissolves readily in water to give a strongly alkaline solution

¹ A more extensive list is given by Whitehead *et al.* (1993).

calcium ions by adding phosphate and/or citrate (e.g. as freely soluble sodium salts). This is the basis of the 'phage inhibitory medium' concept (PIM): with less available calcium ions, phage present in the bulk starter culture will not multiply rapidly. Many PIM formulations are used, including phosphated, supplemented fresh whey (Richardson *et al.*, 1977) and commercially available powders typically based on phosphated deionized whey (Anderson *et al.*, 1977).

Though PIM is widely used, in practice phage inhibition is not absolute and some PIM formulations do not support good growth of all starter cultures (Rajagopal *et al.*, 1990; Whitehead *et al.*, 1993). Some phages are less easily inhibited than others (perhaps indicating a lesser or no requirement for calcium ions) and various media differ in their effectiveness against phage replication. Most effective protection against phage infection of the starter culture can be achieved by combining PIM with precautions to exclude phages from the starter culture.

14.8.7 External pH control

A growing cheese starter culture produces lactic acid, which causes the pH of the growth medium to fall. At low pH, growth slows and cell damage and death ensue. In order to grow a culture to high cell density and to retain activity after chilling, the pH must not be permitted to fall beyond the tolerable range. This can be achieved by monitoring the pH of the culture and adding alkali as necessary, either automatically in small increments or as one or two manually added doses (e.g. Richardson *et al.*, 1977; Limsowtin *et al.*, 1980). This is known as 'external' pH control to distinguish it from the 'internal' pH buffering capacity of milk and buffered-whey media.

External pH control can be applied in any medium to growth of inoculum cultures or to bulk or DTV starter culture growth. Sodium hydroxide, potassium hydroxide, ammonium hydroxide and gaseous ammonia have been used successfully for pH adjustment. The bulk starter culture system described in Fig. 14.2 shows the features necessary for external pH control, and Fig. 14.3 illustrates starter culture growth in a pH-controlled system.

The optimum pH and tolerable pH range for rapid growth and maintenance of activity are different for different bacterial species and strains. Externally controlled lactococcal cultures are typically grown at pH ~6. Even if the pH is allowed to fall towards the end of the fermentation, it should not fall below pH 5 (Harvey, 1965); a practical minimum of around pH 5.2 is suggested for best retention of activity (Sandine, 1996). Most *Streptococcus thermophilus* and *Lactobacillus* cultures are more acid-tolerant, and can generally be held at pH 4.5 and 4, respectively (Cogan and Hill, 1993).

Accumulation of high lactate levels in the growth medium results in damage to the culture, and so culture growth cannot be continued indefinitely regardless of the buffering system used. Ideal conditions for any particular strain, MSS or DSS culture must be determined empirically.

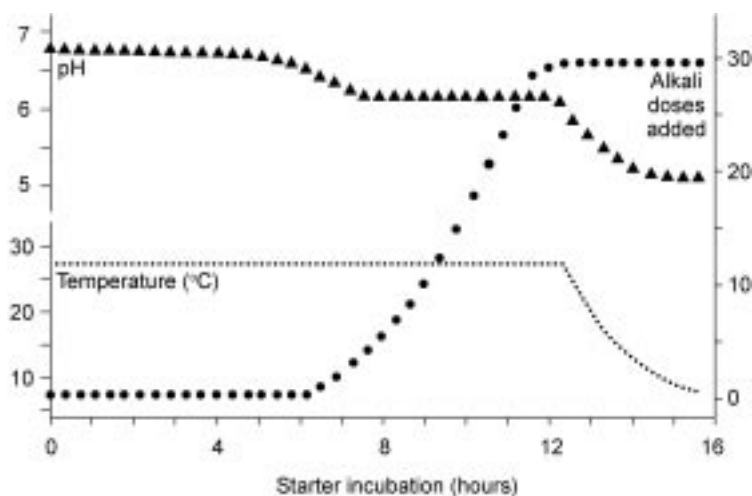


Fig. 14.3 Idealized starter growth with external pH control. The graph illustrates factory bulk starter growth of a lactococcal starter in milk with external pH control (using sodium hydroxide solution as neutralizer). The pH curve shows that (1) acid production during early growth is buffered by the milk, and (2) pH control begins when the desired pH is reached (in this case pH 6.2). In this particular example, pH control ceases and chilling begins when a predetermined amount of alkali (corresponding to the optimal extent of growth for this starter) has been added. Using a starter vessel with a cooling jacket, lactic acid production (indicated by pH change) continues for some time during cooling.

14.8.8 Growth temperatures

The optimum growth temperature of most lactococcal strains is around 30°C. This is seldom used when a combination of strains is being grown because the growth rates of different strains vary, resulting in mixed cultures being dominated by the fastest-growing strains – not necessarily a desirable outcome for cheese flavour development or protection against phage effects. Growth rate differences are also seen at 21°C, a temperature which has been quoted as giving traditional overnight culture growth from a 0.5% to 1% (v/v) inoculum (Cogan and Hill, 1993; Fox *et al.*, 2000). Fast growth (and equipment turnaround) with better strain balance is said to result from growth at 26–27°C (Collins, 1976; Cogan and Hill, 1993), and so these temperatures are often used.

A wide range of temperatures can be used for growing the various thermophilic species used in cheese making; 37–42°C is common, but temperatures in the range 30–46°C have been reported (Hassan and Frank, 2001). When grown as a mixed-species defined culture (e.g. *Streptococcus thermophilus* plus *Lactobacillus helveticus*) or as an undefined culture, higher temperature or lower pH generally favours growth of the *Lactobacillus*.

14.9 Future trends

14.9.1 Resistance to phage infection

Carefully selected starter strains or strain combinations represent significant investments, yet they can quickly be rendered useless by phage infection. The use of physical phage exclusion strategies and/or phage-inhibitory media will remain important ways to minimize phage problems for the foreseeable future. Conventional selection of phage-resistant variants and introduction (via natural conjugation) of transferable phage resistance genes are established techniques for improving the industrial life expectancy of lactococcal starter strains, and these tools will continue to be important as starter strains with specific desirable properties are identified. Other phage resistance strategies based on genetically modified organisms have been demonstrated, but use of modified strains in cheese is currently not acceptable to world public opinion. However, modified strains could see increasing use in specialist fermentations to produce flavour ingredients or other products that can be purified and used without presence of the organism.

The phage resistance of starter strain variants defective in nucleotide metabolism has been studied (e.g. Nilsson and Janzen, 2000). These can grow in suitably supplemented medium, but not in milk. If a cell is infected by phage in the cheese vat, it is unable to support phage replication. The culture is effectively resistant to phage. A limitation in the application of this technology is that these strains do not grow in milk, though they are capable of acid production. The specially grown starter culture inoculum required for cheese making would be abnormally large, and so improved methods of propagation and concentration will be essential to make this approach feasible.

Knowledge of the biology of phage–host interactions is not yet at the stage that would allow targeted alteration of the genes of starter strains that make them susceptible to infection (such as genes that determine cell-surface receptors involved in phage adsorption or DNA injection), except in the case of c2-species phages (Garbutt *et al.*, 1997). This approach could be explored in the search for ‘completely resistant’ starter strains.

14.9.2 Separation of culture functions

In search of controlled acid production and flavour definition, there is a philosophical and practical trend towards separating the two functions, by using an acidifying culture that delivers reliable cheese together with an adjunct culture that directs flavour development (see Chapters 6, 7 and 8). This approach is a response to the realization that combining all desirable properties into one culture is not always easy or possible. It also allows use of the same acidifying culture for making different products (reducing phage risks by minimizing the number of starter strains in use and increasing operator familiarity with starter culture performance characteristics) and gives vat-to-vat flexibility in adjunct culture use.

14.9.3 Compositional changes to enhance culture activity

Lactococcal starter cultures have been used for making Cheddar-style cheeses since the beginnings of industrial culture technology. In recent years there has been increasing use of mixed-species cultures of *Lactococcus lactis* and *Streptococcus thermophilus* (Stokes *et al.*, 2001). This mixture alters the pattern of acid production during the make, with lactococci providing most of the starter culture growth and acid production early in the make and streptococci providing most of the growth and acid made in the later (higher temperature) phases of the make. Overall pH changes are faster (or less volume of starter culture needs to be used, a major issue in DTV culture use) and there is less susceptibility to loss of activity due to phage infection because of the biphasic contribution from the different starter culture components. There can also be flavour effects, with at least one supplier of these cultures not recommending them for cheeses destined for long maturation. Use of suitable adjuncts might overcome this shortcoming.

14.9.4 Methods to improve activity and lower starter culture costs

The production of lactic acid by starter bacteria is an essential function in cheese making, but accumulation of lactic acid is a fundamental obstacle to the cost-effective growth of really high-density cultures. Recent developments in the area of aerobic growth of some lactic acid bacteria might prove useful in increasing starter culture activities (Dywat *et al.*, 2000; Geppel *et al.*, 2001).

Lactic acid bacteria lack the electron transport chain of cytochromes necessary to derive energy from aerobic metabolism. They are generally inhibited under aerobic conditions. Lactococcal cultures grown in the presence of hemin are able to utilize oxygen, achieving increased cell numbers without accumulation of high lactate concentrations. Cultures prepared in this way have two potential uses, either as (1) high-density starter cultures with good storage stability, or as (2) oxygen-scavenging components of starter cultures to remove dissolved oxygen from cheese milk, thereby improving growth and acid production by other strains in the culture. Further development of this concept (including use of a non-animal porphyrin to improve its public acceptability) could lead to significant improvements in the efficiency of starter culture production.

14.10 Sources of further information and advice

Modern cheese makers rely heavily on manufacturers and suppliers for guidance on equipment design and maintenance, bulk starter production and use of bulk or DTV cultures. Manufacturers of stainless steel equipment and ancillary control systems used for milk handling, media mixing, bulk starter culture growth and all phases of the cheese make have valuable specialist skills derived from many aspects of the food industry. Suppliers of bulk starter culture inocula often also supply a range of growth media mixes and supplements.

Starter culture choice should be based on cheese making performance criteria. Culture suppliers are the primary source of information on the expected performance of cultures. The supplier must also be able to provide testing for phages, usually requiring that a cheese whey sample be sent to the supplier on a regular basis. As well as detecting incipient problems, this service is an essential part of the process for selecting future cultures.

Until recent years, there were many government- and industry-funded university departments and dairy research institutes that could provide unbiased guidance on technical issues. Worldwide privatization and deregulation has changed the roles of many institutes, and some have closed. Most are able to undertake defined research and development projects, are involved in genetic and biochemical research (see other chapters of this book), and can provide some level of extension, training or technical advice. Equipment design and culture application are increasingly seen as commercial decisions. Therefore, it is important that cheese manufacturers maintain some in-house technical expertise, to act as an informed interpreter of advice given by equipment and culture suppliers, to assimilate the results of basic research and to actively develop the desired cheese flavour profiles.

The International Dairy Federation (www.fil-idf.org) produces technical bulletins on dairy issues, including starter culture use (e.g. Cogan *et al.*, 1991). Various national dairy technology associations and industry bodies continue to provide a network that can link researchers and consultants with cheese makers. Associations of speciality cheese manufacturers (including traditional manufacturers of cheeses with Protected Designation of Origin) provide valuable regional support for small manufacturers. Major English-language examples serving industrialized cheese manufacture include:

- American Dairy Science Association (www.adsa.org)
- Dairy Industry Association of Australia (www.diaa.asn.au)
- New Zealand Institute of Food Science and Technology (www.nzifst.org.nz)
- The Society of Dairy Technology (www.sdt.org)

Historical and contemporary information on cheese starter cultures can be found in many review articles. Examples include broad reviews prepared by Lewis (1987), Cogan and Hill (1993), Stanley (1998), Fox *et al.* (2000) and Hassan and Frank (2001). These reviews address issues of starter culture composition, taxonomy, biochemistry, genetics, propagation and management as well as phages and other inhibitors. Thunell and Sandine (1985) and Limsowtin *et al.* (1996) target the various types of cultures, propagation strategies and phage control. Parente (2006) provides a rare English analysis of the composition of starter cultures for traditional Italian cheeses. Growth media and production of commercial cheese cultures have been specifically reviewed by Gilliland (1985), Whitehead *et al.* (1993) and Sandine (1996), with related issues for lactic acid bacteria in general addressed by Mäyrä-Mäkinen and Bigret (1993).

14.11 References

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Bacteriocins: changes in cheese flora and flavour

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15.1 Introduction

Bacteriocins are described as peptides produced by some bacteria that kill or inhibit other, sometimes closely related, bacteria (Cotter *et al.*, 2005b). This chapter focuses on how these antimicrobial agents can be used in cheese to improve either the quality or the safety of cheese products. The review will focus on bacteriocins produced by lactic acid bacteria (LAB) given that most starter cultures belong to this category, and also that bacteriocin production is a common feature among this group of bacteria. It is also important to emphasize that addition of LAB bacteriocins to foods in general does not require regulatory approval, given the safe history of use of such cultures in the food industry.

Bacteriocins can play a pivotal role during cheese manufacture and ripening due to their ability to control the flora of cheese. For example, they may play a dominant role in inhibiting the proliferation of non-starter lactic acid bacteria (NSLAB), which are often a source of inconsistency in the final product quality. In addition, they can be used as a tool to promote starter lysis thereby releasing intracellular enzymes into the cheese matrix. Bacteriocins are commonly used as biocontrol agents in cheese for inhibition of pathogenic microorganisms, such as *Listeria monocytogenes*, an organism associated with a number of outbreaks of food-borne illness due to cheese consumption.

15.2 What bacteriocins are and how they work

Food-associated cultures, including starter cultures, produce a plethora of antimicrobial substances including organic acids, diacetyl and reuterin (Holzapfel *et*

al., 1995; Lindgren and Dobrogosz, 1990) and enzymes such as lysins and muramidases, which were classified as Class III bacteriocins by Klaenhammer (1993). In addition, many organisms produce a group of antimicrobial peptides collectively called bacteriocins. These antibacterial peptides are ribosomally synthesized and released into the medium as bioactive peptides with a function that was originally described as 'killing other closely related bacteria with a bactericidal mode of action' (Tagg *et al.*, 1976). Most act on the cytoplasmic membrane of their target organism and dissipate the proton motive force through the formation of pores in the phospholipid bilayer, ultimately resulting in cell death. In addition, or alternatively, bacteriocins can act on the cell wall and inhibit its synthesis (e.g., by binding to lipid II, which is a cell wall precursor). Indeed, the mode of action of nisin involves a dual mechanism and targets both cell wall, through inhibition of its synthesis, and cell membrane, through pore formation. Initially, the N-terminus of nisin binds to lipid II, which blocks peptidoglycan synthesis. Tryptophan fluorescence measurements indicate that lipid II switches the orientation of nisin from parallel to perpendicular with respect to the membrane surface, thus stabilizing nisin polymers into a stable pore structure (Breukink *et al.*, 2003).

Undoubtedly, the best known bacteriocin is nisin, first described in 1928 by Rogers and Whittier as a Lancefield *Streptococcus* group N inhibitory substance (Rogers and Whittier, 1928). It is considered to be the prototype LAB bacteriocin and is produced by some strains of *Lactococcus lactis*. In 1969, nisin was deemed safe for use in food by the Joint Food and Agricultural Organization/World Health Organization Expert Committee on Food Additives. In 1983 it was added to the European food additive list as number E234 and in 1988 it gained Food and Drug Administration (FDA) approval in the USA. To date, nisin is the primary bacteriocin exploited for commercial production and it is approved for use in almost 50 countries to prevent food spoilage and contamination in the food industry. It is worth noting that the success of nisin has stimulated further research targeted towards identifying novel bacteriocins from other LAB for similar applications.

15.3 Bacteriocins of LAB – classification

Bacteriocins of LAB are a heterogeneous group of peptides that were previously classified into five groupings (Klaenhammer, 1993; Nes *et al.*, 1996; Kemperman *et al.*, 2003). The most recent classification scheme proposed by Cotter *et al.* (2005b) contains two classes of bacteriocins. Class I consists of lanthionine-containing bacteriocins, or lantibiotics, which includes both single-peptide (nisin, mersacidin, lactacin 481) and two-peptide (lactacin 3147, cytolysin) lantibiotics. This class contains up to 11 subclasses (Cotter *et al.*, 2005a).

Class II comprise a very large group of non-lanthionine-containing bacteriocins that is further divided into four subclasses; class IIa includes pediocin-like peptides, such as pediocin PA-1(AcH) and leucocin A; class IIb consists of two-

peptide bacteriocins such as lactacin F; class IIc consists of cyclic bacteriocins such as enterocin AS48 and reuterin 6; and class IId consists of non-pediocin single linear peptides such as lactococcin A and divergicin A.

It has been suggested that large heat-labile murein hydrolases such as lysostaphin and enterolysin A (former class III bacteriocins) be moved to a separate designation called 'bacteriolysins', containing non-bacteriocin lytic proteins (Cotter *et al.*, 2005b). It has also been proposed that circular LAB bacteriocins be regarded as class V bacteriocins (Kemperman *et al.*, 2003); however, Cotter *et al.* (2005b) suggest that circular bacteriocins should be included in the non-lanthionine-containing class II category. It is evident that as new bacteriocins continue to emerge, so also does the complexity of classification, and it is without doubt that classification schemes will continue to evolve in the future, with the discovery and our increased understanding of these fascinating antimicrobials.

15.4 Why are bacteriocins used in cheese?

Bacteriocins represent a straightforward approach to engineering an innate immunity into food systems. Since food-associated organisms naturally produce them, at present they do not require legislative approval where their method of incorporation is via a producing culture. However, addition to the food of pure or concentrated bacteriocins still requires governmental approval. Bacteriocins have two main applications in cheese manufacture, both of which involve manipulation of the flora. The first is to improve the quality of cheese products and this is done in two ways – through starter culture lysis or through the inhibition of undesirable and spoilage microorganisms. The second is to improve the safety of the food through inhibition of pathogenic microorganisms.

15.4.1 Bacteriocins for improvements in cheese safety

Listeria monocytogenes is one of the most problematic pathogens in cheese and has been associated with a number of foodborne illness outbreaks associated with cheese consumption. In particular, this pathogen can be a persistent problem in smear cheese varieties such as Tilsit, Limburg, Danbo and Munster (Breer, 1986) and soft cheeses such as cottage cheese. This pathogen survives over a wide pH range, at refrigeration temperatures, and is tolerant to salt concentrations as high as 20% (under ideal conditions of neutral pH and low temperature). Listeriosis, the foodborne illness caused by *Listeria monocytogenes*, accounts for approximately 25% of deaths caused by foodborne pathogens in the US annually (Mead *et al.*, 1999) and was responsible for 71% of food product recalls owing to bacterial contamination in the US between 1993 and 1998 (Wong *et al.*, 2000). It is not surprising, therefore, that over the past two decades attention has focused on identifying bacteriocins capable of inhibiting this organism (Palumbo, 1986). As a result, a number of bacteriocins, classified as Class II *Listeria*-active

bacteriocins, were targeted for protection against this pathogen (Ennahar *et al.*, 2000).

One such bacteriocin, pediocin PA1(AcH), is produced by some strains of *Pediococcus* spp. (Bhunia *et al.*, 1988; Henderson *et al.*, 1992) and *Lactobacillus plantarum* (Ennahar *et al.*, 1996). These and other pediocin-producing starter cultures were incorporated into various cheeses to inhibit the growth of *Listeria monocytogenes*. In one such study, a cell suspension of pediocin-producing *Lactobacillus plantarum* was sprayed on the surface of Munster cheese at the beginning of ripening. While *Listeria monocytogenes* was sometimes detected at very low levels after 7–11 days of ripening, the pathogen did not grow, nor did it survive at the end of ripening (21 days) (Ennahar *et al.*, 1998). Importantly, the ripening process was not adversely affected by the *Lactobacillus plantarum* spray, most probably due to the fact that this bacterial strain commonly exists in Munster cheese (Ennahar *et al.*, 1996). In red smear cheeses, addition of a pediocin PA1(AcH)-producing *L. plantarum* strain led to an almost complete inhibition of *Listeria monocytogenes* when the pathogen was added at 10^2 cfu ml⁻¹ of salt brine solution (Fig. 15.1) (Loessner *et al.*, 2003). This study found that pediocin resistance developed at high frequencies in all *L. monocytogenes* strains tested.

Acquiring resistance is a common feature of Class II bacteriocins (Ennahar *et al.*, 2000; Gravesen *et al.*, 2002). As a result, it is recommended that Class II bacteriocin-producing cultures should not be used over long periods in cheese production (Loessner *et al.*, 2003). In another study, the genetic machinery for pediocin PA1(AcH) production was transferred into a *Lactococcus lactis* ssp. *lactis* strain, which was subsequently used in the manufacture of Cheddar cheese. *Listeria* counts in the cheese decreased from 10^3 cfu ml⁻¹ to 10^2 cfu g⁻¹ within one week and further decreased to 10 cfu g⁻¹ within three months (Buyong *et al.*, 1998). Again, the presence of the bacteriocin-encoding plasmid did not adversely affect the cheese-making quality of the starter culture, as all other cheese-making parameters were similar between control and experimental cheeses.

Another example of a group of Class II bacteriocins that are particularly active against *L. monocytogenes* is the enterocins, which are produced by some cultures of *Enterococcus* spp.

Despite the fact that foods containing enterococci have a long history of safe use, they are not considered as GRAS (generally recognized as safe) organisms. *Enterococcus faecalis* in particular can act as an opportunistic pathogen, causing a variety of infections such as urinary tract infections, bacteremia and infective endocarditis (Moellering, 1992; Jett *et al.*, 1994), and are of immense importance in community-acquired and hospital-acquired (nosocomial) infections (Jett *et al.*, 1994; Simjee and Gill, 1997). One of the contributing factors to their pathogenesis is their evolving resistance to antibiotics. For example, resistance to the antibiotic vancomycin is now widespread among members of the genus, which leaves few options for disease management (Facklam and Sahm, 1995; Klein *et al.*, 1998). However, it is important to emphasize that *Enterococcus*

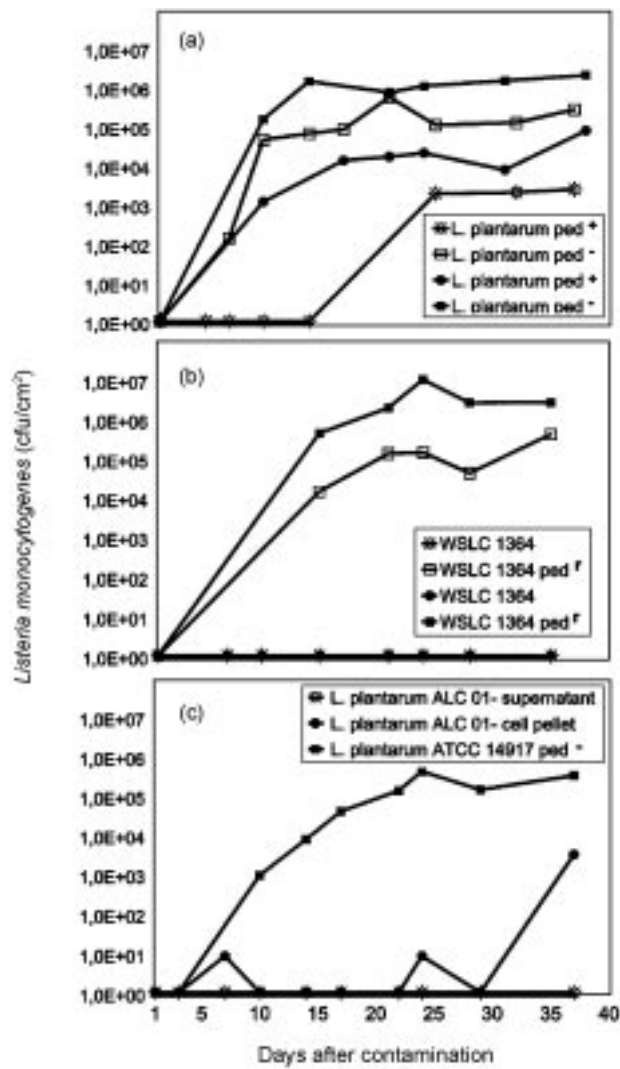


Fig. 15.1 Inhibition of growth of *Listeria monocytogenes* WSCL 1364 by *Lactobacillus plantarum* ALC01. Ripening experiments were performed on soft cheese using a commercial undefined multispecies microbial consortium. (a) *Listeria* cell counts on the cheese surface after contamination at day 1 with 2×10^2 cfu (open symbols) and 4×10^3 cfu (solid symbols) per ml of brine solution. Control cheeses were ripened with the pediocin ACh-negative type strain *L. plantarum* ATCC 14917. (b) Contamination with 10^2 cfu (open symbols) or 10^3 cfu (solid symbols) per ml of brine solution. Control cheeses were ripened with the resistant mutant WSCL 1364R (pedr). (c) *Listeria* contamination with 7×10^2 cfu/ml of brine solution. In this case, cheese ripening was performed with a commercial, defined ripening culture. Either the supernatant or the pellet of the 14 h culture in VisStart TW ALC01 medium was used. Control cheeses were ripened with the addition of a 14 h culture of the pediocin ACh-negative type strain *L. plantarum* ATCC 14917, cultivated in VisStart TW ALC01. Taken from Loessner *et al.* (2003).

faecium strains do have important applications in the dairy industry, particularly in artisanal fermented products. They can be readily isolated from a range of food sources, including raw milk and a variety of cheeses such as artisan cheeses from southern Europe, where they contribute to the development of sensory characteristics during ripening. The production of bacteriocins by a number of *Enterococcus* strains also provides a strong case for their use in foods, but naturally their use with regard to the safety of the food must be carefully addressed before proceeding to their application (Giraffa, 2003). Nunez and co-workers (1997) used an enterocin 4-producing *Enterococcus faecalis* as a starter culture to manufacture Manchego cheese which contained *Listeria monocytogenes* Ohio at an initial level of 10^5 cfu ml⁻¹. Addition of this culture reduced *Listeria* numbers by three orders of magnitude after eight hours and by six orders of magnitude after seven days of ripening. In a similar study, Giraffa *et al.* (1993) used enterocin-producing *Enterococcus* ssp. as starter cultures to manufacture Taleggio cheese which was spiked with *Listeria*. Their study found satisfactory inhibition of the pathogen due to the synergistic activity of bacteriocin production and the pH decrease during cheese-making.

Nisin has a very broad spectrum of inhibition and lactococcal strains producing this bacteriocin are sold commercially as cheese starter cultures. Initially, they were found to be very effective in inhibiting clostridial spoilage in Swiss-type cheeses, where late gas-blowing was a problem (Hirsch *et al.*, 1951; McLintock *et al.*, 1952). While much of this early work demonstrated the potential of nisin for inhibition of pathogenic and spoilage microorganisms, nisin-producing strains can be poor starter cultures in that they have slower acid development rates, limited proteolytic activity and were quite sensitive to bacteriophage infection (Hawley, 1955; Lipinska, 1977). In an effort to overcome some of the problems associated with the use of nisin-producing strains as starter cultures, some investigators have designed multiple-strain starter systems. One such system comprises a nisin-producing strain in combination with a nisin-resistant fast-acid strain (Lipinska, 1977), while another consists of a naturally occurring lactose-fermenting nisin-producing proteinase-positive *L. lactis* strain in combination with a lactose-positive, nisin-positive, proteinase-positive transconjugant. Both these starter systems have adequate acid production for cheese making (Roberts *et al.*, 1992). Using these paired systems for cheese manufacture is effective in controlling *Clostridium sporogenes*, *Staphylococcus aureus* and *Listeria monocytogenes* (Zotolla *et al.*, 1994; Delves-Broughton *et al.*, 1996). Maisnier-Patin and colleagues (1992) demonstrated the potential for using a paired nisin system for the inhibition of *L. monocytogenes* in Camembert cheeses. In this instance, use of the nisin-producing strain resulted in a 3-log reduction in *L. monocytogenes* when compared with the initial level in the cheese milk.

Lacticin 3147 is a broad-spectrum antimicrobial peptide produced by *Lactococcus lactis* DPC3147 (Ryan *et al.*, 1996) and *L. lactis* IFPL105 (Martinez-Cuesta *et al.*, 1997). It is comprised of two structural peptides, LtnA1 and LtnA2, both of which are required for full biological activity of the bacteriocin

(Ryan *et al.*, 1999). The genetic machinery for bacteriocin production and immunity is encoded on a 60.2 kb conjugative plasmid, pMRC01, in *L. lactis* DPC3147, while in *L. lactis* IFPL105 the 46 kb bacteriocin-encoding non-conjugative plasmid is designated pBAC105. The conjugative nature of pMRC01 has been exploited to construct over 30 industrially important lacticin 3147-producing strains (Coakley *et al.*, 1997). Lacticin 3147, like nisin, exhibits a bactericidal effect on a wide variety of Gram-positive species such as *Listeria monocytogenes* and *Clostridium* spp. Indeed, lacticin 3147-producing starters have been evaluated for protection of cottage cheese against *Listeria* (McAuliffe *et al.*, 1999). Using the lacticin 3147-producing culture *L. lactis* DPC4275 as a starter, a 99% reduction in the *Listeria* population was achieved within five days in the cheese as compared with control cheese manufactured with a bacteriocin-negative strain (Fig. 15.2). In another recent study, up to a 1000-fold reduction in the *Listeria* population was observed when a lacticin 3147-producing strain was repeatedly sprayed onto the surface of a smear-ripened cheese (O'Sullivan *et al.*, 2006). Table 15.1 gives a brief overview of instances where bacteriocins have been used to inhibit pathogens other than *Listeria* in cheese.

In each application discussed to this point, the delivery mechanism for the bacteriocin was by means of including the live bacteriocin-producing culture as a component of the starter mix. The introduction of a bacteriocin in this manner has many potential advantages in that it allows for the bacteriocin to be added into the cheese in a simple and homogeneous fashion using the same method that is successful for starter culture addition, and it is the least expensive method of introducing a bacteriocin to the complex, semi-solid matrix of cheese. In addition, direct addition of a bacteriocin-producing strain to a cheese vat precludes the occurrence of local spots of excessive production, since the bacteriocin-producing cells are distributed homogeneously throughout the cheese curd.

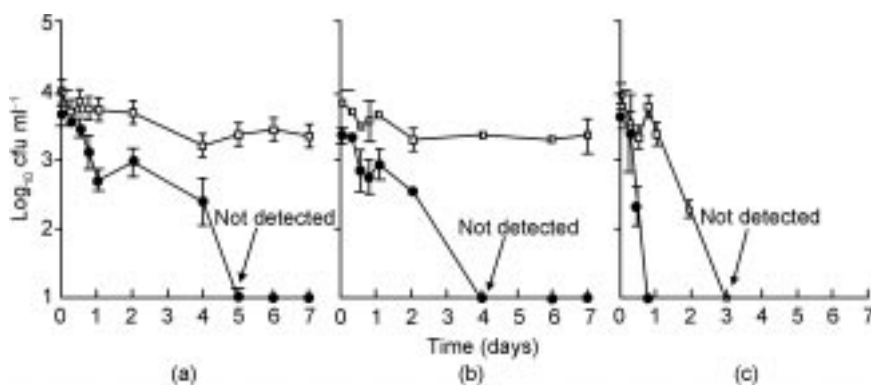


Fig. 15.2 Inhibition of *Listeria monocytogenes* Scott A in cottage cheese produced with a lacticin 3147 starter culture stored at (a) 4°C; (b) 18°C and (c) 30°C. (□), Cheese manufactured with DPC4268, no bacteriocin; (●), cheese manufactured with DPC4275 producing lacticin 3147. Taken from McAuliffe *et al.* (1999).

Table 15.1 Examples of applications of bacteriocin of LAB as bioprotective agents in cheese

Bacteriocin	Application	References
Unnamed	Inhibition of <i>Clostridium tyrobutyricum</i> in small-scale cheese curds by a bacteriocin-producing <i>Streptococcus thermophilus</i>	Mathot <i>et al.</i> (2003)
Enterocin AS-48	Inhibition of <i>Bacillus cereus</i> in non-fat hard cow's cheese by an enterocin-producing <i>Enterococcus faecalis</i> A-48-32	Munoz <i>et al.</i> (2004)
Plantaricin TF711	Bacteriocin produced by <i>Lactobacillus plantarum</i> with activity against <i>Bacillus cereus</i> , <i>Clostridium sporogenes</i> and <i>Staphylococcus aureus</i> for application in goats' milk cheese	Hernandez <i>et al.</i> (2005)
Enterocin L50	Active against <i>Listeria</i> , <i>Staphylococcus</i> , <i>Clostridium</i> and <i>Bacillus</i> , potential application in cheese safety	Achemchem <i>et al.</i> (2005)
Lacticin 3147	Inhibition of <i>Listeria</i> and <i>Bacillus</i> in cottage cheese using a bioactive lacticin 3147 powder	Morgan <i>et al.</i> (2001)

While the addition of bacteriocin-producing organisms with the starter culture to the cheese vat represents the most economical method of introducing bacteriocins to the product, there are however, instances where this is not a viable option. For example, some of the problems associated with using nisin-producing starters for cheese manufacture have already been mentioned above. Indeed, nisin is commonly added to cheese products in a concentrated form, Nisaplin[®] (Danisco, Copenhagen, Denmark), rather than using nisin-producing starters to manufacture the cheese. For example, the addition of Nisaplin to milk used in the manufacture of Ricotta-type cheese effectively inhibited the growth of *L. monocytogenes* for a period of eight weeks or more (Davies *et al.*, 1997). MicroGARD (Danisco) is another commercial powder, produced from a fermentate of *Propionibacterium freudenreichii* and commonly used as a bio-preservative in cottage cheese. It is also a potent inhibitor of Gram-negative bacteria, such as *Pseudomonas*, *Salmonella* and *Yersinia*, as well as some yeasts (al-Zoreky *et al.*, 1991). The antimicrobial action of MicroGard is thought to be associated with the combined effect of bacteriocins with acids, such as propionic acid.

Regardless of the method of administering bacteriocins to a food, especially for the purpose of controlling spoilage and pathogenic microorganisms, a potential problem is the development of bacteriocin resistance among target strains, in a manner similar to the emergence of antibiotic resistance in clinically

important strains. Indeed, a recent study involving strains of *Listeria* revealed that the frequency of development of nisin resistance ranged from 10^{-6} to 10^{-3} depending on the *Listeria* strain tested. Nisin-resistant variants were able to survive and even multiply in milk fermented by a nisin-producing *Lactococcus* (Martinez *et al.*, 2005). *Listeria* readily develops resistance to some Class II bacteriocins (Ennahar *et al.*, 2000; Gravesen *et al.*, 2002) by mechanisms that involve the mannose phosphotransferase system (PTS) permease (Ramnath *et al.*, 2004).

One rational solution to overcoming potential problems associated with bacteriocin resistance is to use combinations of bacteriocin preparations. In this way, the second bacteriocin kills the cells escaping the bactericidal action of the other bacteriocin. Hanlin *et al.* (1993) observed an increased inhibitory activity against several Gram-positive bacteria when a combination of nisin and pediocin PA-1(AcH) are used. Similarly, a combination of nisin and sakacin A had a much stronger inhibitory effect against *L. monocytogenes* than either bacteriocin used alone (Schillinger *et al.*, 1996).

Another approach is the use of live cultures producing multiple bacteriocins *in situ*. Co-production of the bacteriocins enterocin A and pediocin PA-1 (Martinez *et al.*, 2000) and nisin and pediocin PA-1 (Horn *et al.*, 1999) were investigated, but the resulting multiple-producing strains did not demonstrate an improved inhibitory activity when compared with either single-bacteriocin producer alone. O'Sullivan and co-workers (2003b) also described the use of a conjugation strategy to construct a double lantibiotic-producing strain that could be expected to efficiently inhibit spoilage and pathogenic microorganisms. One such strain, *L. lactis* DPC5552pMRC01, was characterized to find that the two bacteriocin-encoding plasmids, pMRC01 (encoding lacticin 3147) and pCBG104 (encoding lacticin 481), were stably maintained with each capable of bacteriocin production. Notably, the double producer was a more effective inhibitor of *Lactobacillus fermentum* and *Listeria monocytogenes* LO28H than strains producing either bacteriocin singly; this could be attributed to the synergistic activity of the bacteriocins, since lacticin 481 alone inhibits *Listeria* only at very high concentrations. This was an interesting finding given that a recent study by Luders and co-workers (2003) reported an antimicrobial synergy between a eukaryotic antimicrobial peptide, pleurocidin, and the LAB bacteriocins, sakacinP, pediocin PA-1 and curvacin A. These bacteriocins alone did not inhibit *Escherichia coli*, but when micromolar concentrations of the LAB bacteriocins were combined with $2 \mu\text{g}$ of pleurocidin ml^{-1} , 100% growth inhibition was obtained.

Combining bacteriocins with other treatments such as high pressure has also been investigated (Hauben *et al.*, 1996; Kalchayanand *et al.*, 1998). Morgan *et al.* (2000) found that co-treatment with high pressure and lacticin 3147 was more inhibitory to *Listeria innocua* and *Staphylococcus* in milk and whey-based growth media when compared to either treatment alone. The combined effect of high-pressure treatment and bacteriocin-producing LAB also inhibited *E. coli* (Rodriguez *et al.*, 2005) and *Staphylococcus aureus* (Arques *et al.*, 2005) in raw

milk cheeses. While high hydrostatic pressure is an expensive technology, in all the above examples its use in combination with bacteriocins allowed for much lower pressures to be used while still achieving a lethal effect, thus presenting a more economical feasible process.

15.4.2 Bacteriocins for control of undesirable populations

Non-starter lactic acid bacteria (NSLAB) are a component of the indigenous microflora of cheese. They are generally composed of lactobacilli and pediococci and grow in the cheese as it matures, to become the dominant flora in cheeses that are ripened for periods over and above four months. Since the starter cultures usually lose viability over this time period, the role of NSLAB in cheese flavour development is unclear but, whether positive or negative, they undoubtedly contribute to an unpredictability associated with Cheddar cheese quality (Fox *et al.*, 1998). Importantly, NSLAB are associated with a number of defects including the development of off-flavours, the formation of calcium lactate crystals and slit formation in cheese (Daeschel *et al.*, 1991; Thomas and Crow, 1983). Control of NSLAB populations is undoubtedly desirable and leads to the development of a more consistent and predictable end product from cheese making.

Bacteriocins may represent an important biological tool to control NSLAB in cheese during ripening. For example, a lacticin 3147-producing *L. lactis* DPC4275 reduces NSLAB populations in full fat cheese by at least 100-fold as compared to the control cheese manufactured with a bacteriocin-negative strain (Ryan *et al.*, 1996). Fenelon *et al.* (1999) found a similar reduction in NSLAB populations in low fat cheese manufactured with *L. lactis* DPC4275 and ripened at elevated temperatures. A lacticin 481-producing culture, *L. lactis* DPC5552, used as a starter culture adjunct for Cheddar cheese manufacture decreased the NSLAB population by 2-log units as compared to the control cheese manufactured without the bacteriocin-producing strain (Fig. 15.3, O'Sullivan *et al.*, 2003a).

Some NSLAB undoubtedly contribute positively to the flavour and quality of cheese and thus it may be undesirable to inhibit them completely. Consequently, a starter culture system was developed that uses lacticin 3147-producing starters in combination with lacticin 3147-tolerant NSLAB. In this case the NSLAB were isolated from well-flavoured cheeses. Specifically, a lacticin 3147-tolerant variant of *Lactobacillus paracasei* ssp. *paracasei* DPC5336 was isolated after repeated exposure to low levels of lacticin 3147. Cheddar cheese was subsequently manufactured using the lacticin 3147-tolerant *Lactobacillus* in conjunction with a lacticin 3147-producing *Lactococcus*. The levels of lacticin 3147 produced in the cheese were sufficient to inhibit NSLAB with the exception of the tolerant *Lactobacillus*, which became the dominant microflora in the cheese (Ryan *et al.*, 2001). A number of important probiotic lactobacilli and bifidobacteria have been made tolerant to lacticin 3147 with the intent of manufacturing cheese that contains probiotic bacteria. This system ultimately

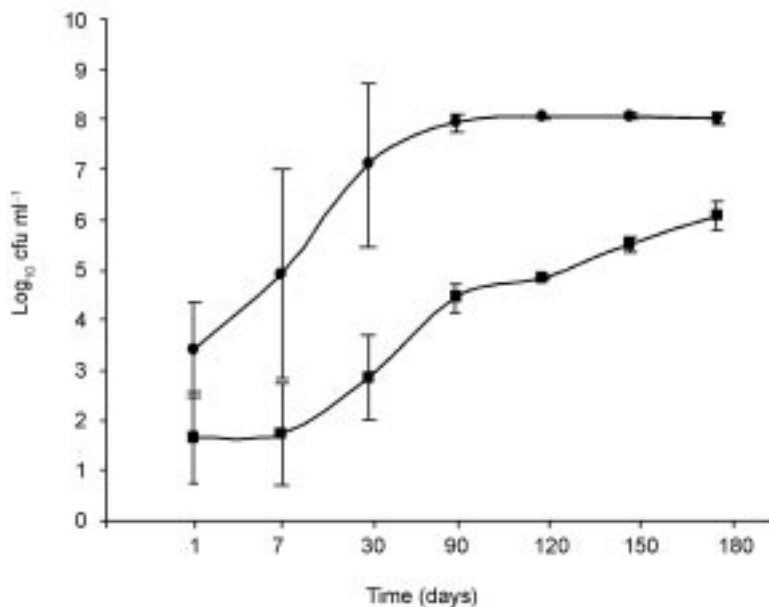


Fig. 15.3 Non-starter (NSLAB) viability counts in cheese manufactured with (●) starter *L. lactis* HP without bacteriocin-producing adjunct and (■) *L. lactis* HP + lacticin 481-producing *L. lactis* 481. Error bars represent the standard deviation of duplicate counts on duplicate trials. Taken from O'Sullivan *et al.* (2003a).

offers cheese manufacturers more control of the microbial populations in cheese and thus the ability to manufacture 'designer cheeses' in terms of a predictable microflora and consistent quality.

15.4.3 Bacteriocins for inducing lysis of starter cells

During cheese ripening the starter culture lyses in a gradual process that occurs from within 24 hours up to one year. As a result of autolysis, intracellular enzymes such as proteinases, and peptidases such as post-proline dipeptidyl aminopeptidase (pep X), are released into the cheese matrix. This results in the hydrolysis of casein into smaller peptides and free amino acids. This process, known as secondary proteolysis, is considered to be an important step in the ripening and flavour development of cheese. In this respect, the peptides formed as a result of proteolysis and the metabolism of amino acids to compounds such as 3-methyl-1-butanal, diacetyl and acetoin contribute to flavour development (Fox and Wallace, 1997).

Thus, there is an important link between the rate of starter cell lysis (autolysis) and cheese flavour development (Chapot-Chartier *et al.*, 1994; Wilkinson *et al.*, 1994). Consequently, there has been a tremendous effort to increase the rate of autolysis, including through the elevation of ripening temperature (Aston *et al.*, 1983; Fedrick *et al.*, 1983; Law, 1986), the use of lytic

bacteriophage (Crow *et al.*, 1995) and the addition of exogenous enzymes (Wilkinson *et al.*, 1992; Fedrick *et al.*, 1986). Bacteriocins also have applications in the acceleration of cheese ripening through a bacterolytic action that results in lysis of the target strain, a process that can occur very rapidly. Initial attempts to use bacteriocins for such purposes involved the use of a lactococcal A, B and M producer, *L. lactis* DPC3286. This 'triple bacteriocin' is plasmid encoded and has a narrow spectrum of inhibition, restricted to other lactococcal strains (Morgan *et al.*, 1995). Cheddar cheese was manufactured using DPC3286 as an adjunct to the cheese-making strain *L. lactis* HP. Lactate dehydrogenase (LDH) was used as an indicator of starter cell lysis over the six-month ripening period. Cheese manufactured with the bacteriocin-producing adjunct exhibited a mean increase in LDH of 62% as compared to a mean increase of 8% for cheese manufactured with a bacteriocin-negative strain. Cheese manufactured with the bacteriocin-producing adjunct also exhibited higher levels of free amino acids and higher sensory scores when graded on the basis of flavour and aroma as well as body and texture. In addition, the graders' comments indicated that cheese made with the bacteriocin-producing adjunct was of better quality than the control cheese (Morgan *et al.*, 1997). However, utilization of this system at pilot scale is sometimes problematic because the strain targeted for lysis is also the acid producer. This may result in increased cheese production times. Indeed, in some instances where bacteriocin-sensitive strains were used, cheese could not be manufactured using this system.

In a further development, a three-strain starter system was designed in order to overcome problems associated with the two-strain system. The three-strain system incorporates the bacteriocin producer as an adjunct with a bacteriocin-sensitive strain – *L. lactis* HP (destined for lysis) – and a bacteriocin-insensitive *Streptococcus* strain that ensured consistent acid production. Cheese manufactured using this strain combination exhibited a 265% increase in LDH above the cheese manufactured with *L. lactis* HP alone (regarded as having 100% LDH release), and a 210% and a 188% increase above cheese manufactured with *L. lactis* HP + *S. thermophilus* DPC1842, and with *L. lactis* HP + *S. thermophilus* DPC1842 + *L. lactis* DPC3289 (Bac–), respectively. Cheese manufactured in the presence of the bacteriocin-producing adjunct had elevated free amino acid levels of 9698 $\mu\text{g ml}^{-1}$ of cheese juice, more than double that observed in cheese manufactured with *L. lactis* HP alone, indicating that significantly more proteolysis occurred in this cheese (Morgan *et al.*, 2002).

Lacticin 3147 was used for increasing starter cell lysis (Martínez-Cuesta *et al.*, 1998, 2001). In this case a two-strain mixture was used for the manufacture of goats' milk cheese and levels of the intracellular enzyme pep X in cheese manufactured with the bacteriocin-producing adjunct were about twice those in the control cheese (Martínez-Cuesta *et al.*, 2001) (Fig. 15.4). In this study, the starter strain contained the genes for lacticin 3147 immunity and as a consequence, it was not inhibited by the bacteriocin producer and acidification was not compromised (Martínez-Cuesta *et al.*, 2001). It is worth noting that the presence of the major autolysin, AcmA, is required in the sensitive strain for

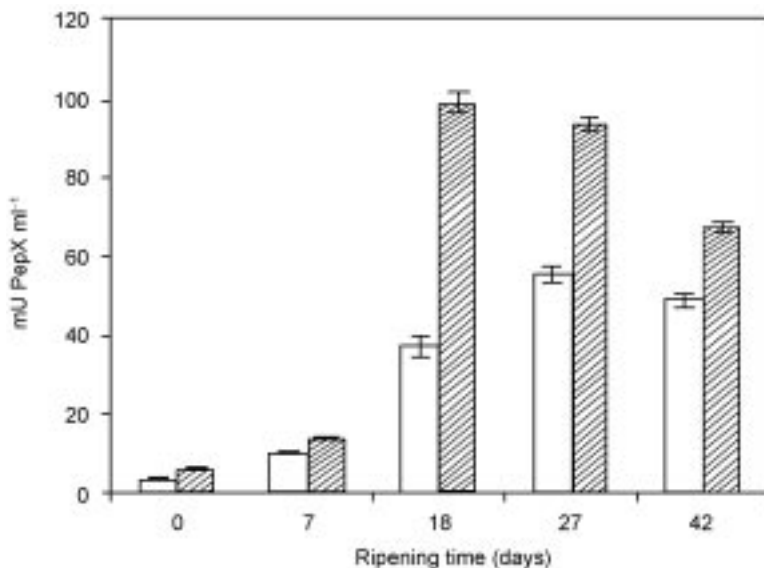


Fig. 15.4 Release of PepX activity, expressed as mU/ml ($\mu\text{mol}/\text{min}/\text{ml} \times 10^3$), in cheeses manufactured with *L. lactis* IFPL359 (A, open bars), or its transconjugant *L. lactis* IFPL3593 (Bac+) (B, striped bars) as starter during ripening. Pep X activity measurements were performed in triplicate for the two independent trials, and the figure shows the standard error ($p < 0.05$). Taken from Martínez-Cuesta *et al.* (2001).

lysis to occur with lacticin 3147, nisin, and lactococcins A, B and M (Martínez-Cuesta *et al.*, 2000).

In another recent study, a bacteriocin-producing *Enterococcus faecalis* strain was used as an adjunct culture for Hispanico cheese manufacture. The resulting cheese demonstrated higher levels of free amino acids, reduced levels of hydrophobic peptides and increased concentrations of diacetyl, acetoin and 3-methyl-1-butanol, and achieved an overall better score for quality and flavour than control cheeses (Oumer *et al.*, 2001).

Another bacteriocin which has also been demonstrated to have an application in flavour development in cheese is lacticin 481, a broad-spectrum bacteriocin produced by a number of lactococcal cultures (Piard *et al.*, 1992; Rince *et al.*, 1994; O'Sullivan *et al.*, 2002). In a recent study, a lacticin 481 producer, *L. lactis* CNRZ481, was used as an adjunct to *L. lactis* HP for Cheddar cheese manufacture and the levels of LDH assessed over the six-month ripening period as a marker of starter lysis. One day post-manufacture the LDH levels in the experimental cheeses were approximately five times greater than in the control. This trend for elevated LDH levels in the experimental cheese continued over the six months of ripening. During this time a considerable quantity of lacticin 481 was being produced in the cheese. The cheese manufactured with the bacteriocin-producing adjunct also had better flavour scores (O'Sullivan *et al.*, 2003a). One advantage of using a lacticin 481 producer in a two-strain system

such as this is that the starter culture continues to produce acid while releasing large amounts of intracellular enzymes. This has obvious advantages over other systems in that a portion of the starter lyses. This is a very interesting and novel phenomenon and may be explained by the gradual death and lysis/permeabilization of some HP cells in the culture, while simultaneously other cells continue to grow and multiply. However, the reason why some cells within a culture population are susceptible to lysis/permeabilization is not known, but it may be related to growth phase and physiology of individual cells. It may also be that the bacteriocin concentration is so low that only a proportion of the sensitive cells have bound sufficient lactacin 481 molecules to cause pore formation, resulting in cell lysis/permeabilization and subsequent release of LDH (O'Sullivan *et al.*, 2002).

Avila *et al.* (2005) demonstrated that plantaricin- or lactacin 481-producing strains in combination with *Lactobacillus helveticus* resulted in the accelerated release of intracellular aminopeptidases.

15.5 Implications for cheese manufacturers

One of the most important advantages to the manufacturer in using bacteriocins in cheese systems is that their use may result in a safer product, particularly with regard to protection against *Listeria*. Bacteriocins can offer an innate antibacterial activity to foods that offers protection from pathogenic and spoilage microorganisms, while also encouraging development of desirable microflora in cheese.

In a cheese manufacturing plant, there is a requirement for acid production to occur in a specific and short time period so as to allow for maximum product yields and minimum losses. In general, starter strains used in the cheese industry serve their purpose and reduce the pH of the cheese milk to 5.2 within approximately five hours. However, overuse of starter cultures as well as sub-standard sanitation can introduce into the cheese manufacturing plant the problem of bacteriophage infection. Infection with bacteriophage (phage) at best compromises acidity development, resulting in cheeses of higher pH, which are associated with defects in flavour, texture and safety. The worst-case scenario is complete vat failure, resulting in substantial economic losses (Coffey and Ross, 2002). The genetic linkage of phage resistance genetic machinery with bacteriocin production and/or immunity determinants on conjugative plasmids in lactococci has proven particularly useful.

Exploiting nisin resistance associated with phage resistance genes assists in selection of transconjugants of the butter starter strain *L. lactis* 425A (Harrington and Hill, 1991) and *L. lactis* LMO230, a common laboratory strain (Gireesh *et al.*, 1992). Using a similar approach we generated over 30 lactococcal transconjugants using immunity to lactacin 3147 for selecting transconjugants containing the phage-resistance plasmid pMRC01 (Coakley *et al.*, 1997). Immunity to lactacin 481 is also used as a selectable marker for the transfer of the phage

resistance plasmid pCBG104, to generate phage-resistant starter strains for cheese making (Mills *et al.*, 2002). It is therefore apparent that the availability of these conjugative systems containing bacteriocin production/immunity as selectable markers provides an effective food-grade approach to starter culture improvement for bacteriocin production, resistance, and phage resistance.

The ripening time for each cheese variety varies. Soft cheeses usually have a shorter ripening time than hard cheese varieties such as Cheddar, which generally take from six months to 2 years. The storage costs incurred by the manufacturer during this time have been estimated to amount to ~\$30 per tonne of cheese per month (US Department of Agriculture report). In Ireland, cheese output has grown considerably in the last few years and is now in the region of 120,000 tonnes per annum. The costs incurred by manufacturers to store this amount of cheese during ripening are in the region of €3,000,000 per month. This is an enormous financial burden and therefore methods to develop a more intense-flavoured cheese in a shorter ripening time represent a distinct economic advantage to the manufacturer. Increasing the rate of starter cell lysis through the use of bacteriocin-producing cultures is therefore a desirable system for manufacturers, in that the earlier release of the intracellular enzymes would, in effect, shorten the ripening time and thus the storage costs, which would result in a cheaper product to the consumer.

15.6 Future trends

The last 20 years have seen a huge increase in the identification of novel LAB bacteriocins from fermented foods with potential food applications. Concomitant with these natural discoveries, genomic advances continue to contribute to the identification of novel bacteriocins by allowing us to understand the regulatory mechanisms involved in bacteriocin production and to exploit such mechanisms for generation of overproducing strains. Indeed, an attractive approach to tailoring the biological activity and spectrum of inhibition of bacteriocins can be achieved using protein-engineering strategies which exploit existing bacteriocin structures as blueprints. Given the 'arsenal' of bacteriocins available and the genetic capabilities of understanding and manipulating their function, the use of bacteriocins in foods is still in its infancy but will only be realized in commercial practice if innovative and imaginative schemes are designed for their use.

One would assume, given the growing concern of consumers towards the use of chemical preservatives in foods, balanced against a requirement for minimally processed, natural, safe foods, that bacteriocins would be increasingly considered natural alternatives to chemical additives for the preservation of foods. However, more than 80 years on from the first description of bacteriocin-mediated inhibition, only three bacteriocin preparations have been approved for use in foods. In 1988, nisin was granted approval for use as a food preservative by the US FDA (Food and Drug Administration), followed some years later by

EU approval as food additive E234. Commercial success followed in that it has been shown that its addition to a range of food products can considerably lengthen shelf-life and nisin is now included in many processed and canned foods. Pediocin PA1(AcH) in the form of ALTA 2431 (Quest) is used commercially for food biopreservation. ALTA 2431 is based on fermentates generated from a pediocin-producing strain of *Pediococcus acidilactici* (Rodriguez *et al.*, 2002) and its use is covered by several European and US patents (Rodriguez *et al.*, 2002; Ennahar *et al.*, 2000). MicroGARD (Danisco) is another commercial powder, produced from a fermentate of *Propionibacterium freudenreichii* that is commonly used as a biopreservative in cottage cheese. Very recently, *Carnobacterium maltaromaticum* strain CB1, an inhibitor of *Listeria monocytogenes* in ready to eat (RTE) meat products, has received FDA approval as a GRAS organism with potential application in the food industry, under conditions set by USDA/FSIS.

It is surprising, not to say disappointing, that other bacteriocins have not followed these initial success stories and some 25 years later we still await the next 'nisin'. Several candidate bacteriocins are under investigation, which show considerable promise, but none have passed the stringent regulatory hurdles required for a novel food additive.

In the short term, it may well be that the types of niche applications described in this chapter, in which a variety of bacteriocins are used as specific biological tools to influence the microbial flora of cheese in a precise and consistent manner, may well represent the best route to exploiting the inhibitory power of bacteriocins in food systems. Given that no regulatory hurdles would preclude their use and that cost implications would be extremely low, bacteriocin-based live culture systems are ready to be implemented by the cheese industry to increase the benefits of higher quality and safety that would accrue to the consumer. Whether these technologies will move from the laboratory bench and pilot plant to the factory floor will depend on the willingness of cheese manufacturers to embrace novel bacteriocin technologies, and perhaps in part on the ability of researchers to convince cheesemakers of their undoubted potential. This will be a significant challenge to both parties, but one that should prove to be worth the effort.

15.7 Sources of further information and advice

15.7.1 Books

- Bacteriocins of Lactic Acid Bacteria*, 1993, edited by Hoover D.G. and Steenson L.R., published by Academic Press, New York.
- Cheese: Chemistry, Physics and Microbiology*, 1993, Second Edition, Volume 1, edited by Fox P.F., published by Chapman and Hall, London.
- Lactic Acid Bacteria: Genetics, Metabolism and Applications*, 2002, edited by Siezen R.J., Kok J., Abbe T. and Schaafsma G., published by Kluwer Academic Publishers, Dordrecht, The Netherlands.

Peptide Antibiotics: Discovery, Modes of Action and Applications, 2002, edited by Dutton C.J., Haxell M.A., McArthur H.A.I. and Wax R.G., published by Marcel Dekker, New York.

15.7.2 Papers

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15.7.3 Websites

- ‘Bacteriocins’, <http://molgen.biol.rug.nl/molgen/research/lactis/bacteriocins.php>
- ‘Teagasc’, <http://www.teagasc.ie/research/reports/dairyproduction/4207/eopr-4207.htm>
- ‘Teagasc’, <http://www.teagasc.ie/research/reports/dairyproduction/4542/eopr-4542.htm>
- ‘US Food and Drug Administration’, <http://vm.cfsan.fda.gov/~mow/intro.html>

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Part III

Monitoring and evaluating cheese flavour

16

Monitoring cheese ripening: new developments

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16.1 Introduction

Many different cheese types and their production processes are described throughout this book. Each of these cheese types can be recognized for its own flavour characteristic that is a result of a unique combination of enzymatic and chemical reactions. The substrates for these reactions are derived from the three main components of milk: (1) carbohydrates (primarily lactose but citrate as well) and primary metabolites (lactate, acetate, ethanol and acetoin); (2) protein (e.g. casein), peptides and amino acids; and (3) fat. During the process of cheese ripening, these components are gradually converted into flavour molecules, at a slow rate because most cheese-ripening processes are carried out at low temperatures of 4–18°C with high acid and salt. The duration of the ripening process ranges from 6 weeks to over 18 months. For many cheese types the exact result of the ripening is unpredictable and can only be established by organoleptic testing and careful attention to the manufacturing process. Especially in the case of large-scale production, this high degree of uncertainty is unacceptable to the consumer and the manufacturer. For this reason there is a growing need for monitoring the exact status of the ripening process and for predicting the exact time needed for completing it. This chapter describes recent developments in the methodology that can be used to analyse and evaluate the ripening process for three distinct cheese types, Gouda cheese, Maasdam cheese and surface-ripened cheese, that each have their own unique ripening process.

16.1.1 Gouda cheese

Gouda cheese is made with a mesophilic starter culture, mainly consisting of *Lactococcus lactis*. Cow milk is usually pasteurized and cooled to 32°C before rennet and the starter culture are added. The milk is coagulated within 30 minutes, the curd is cut and separated from the whey, the whey is washed once to remove excess lactose, and the curd is collected and pressed to reach an initial moisture level of 42%. Subsequently, the pressed curd is immersed in brine for 48 hours, followed by coating with plastic-like substances containing anti-fungal agents such as natamycin. The very young cheese is finally stored at 13°C for ripening for 8 weeks in young Gouda to 3 months with mature Gouda and over 6 months for old Gouda cheese. During this ripening period, the moisture content of the cheese slowly declines and the protein in the cheese is hydrolysed to release peptides and amino acids, which themselves can add to the flavour but mostly serve as precursors for subsequent chemical and biochemical reactions.

16.1.2 Maasdam cheese

Maasdam cheese is very similar to Gouda cheese in its initial manufacture. The major difference is in the starter cultures that are used. In addition to the mesophilic starter culture that is standard for Gouda cheese, propionic acid bacteria and, usually, *Lactobacillus bulgaricus* are added at the start of the process. The curd preparation, the pressing and the brining are all similar, if not identical to Gouda cheese manufacture. A different ripening process is used, with a 'heating period' after three weeks of ripening during which the incubation temperature is raised from 13°C to about 17–18°C. This increase in temperature stimulates the growth and metabolic activity of the propionic acid bacteria. This 'cooking' step lasts about two weeks and during this period a large proportion of the lactic acid is converted to propionic acid, acetic acid and carbon dioxide. The success of this cheese type is determined by the amount of propionic acid and gas that is formed during the cooking.

16.1.3 Surface-ripened cheese

There are many different surface-ripened cheeses produced throughout the world. The best known are the mould cheeses, such as Brie and Camembert, that are coated with a thick layer of the fungus *Penicillium camemberti*. The cheeses that we will describe here do not have fungi on the surface, but instead a mixture of different bacteria and yeasts. Representatives of these cheese types are the less-known Limburger, Munster, Port Salut and the Gouda derivatives, Kernhem and Caractere. After brining, the young cheese is not coated, but instead the cheese surface is treated (wiped) several times with aerobic cultures containing yeasts, *Brevibacterium* and other aerobic microorganisms. The crucial factor in the short two-month ripening process of these cheeses is the succession of microorganisms that finally make up the crust of the cheese. This crust protects the cheese from contamination by undesirable microorganisms and at the same

time is responsible for the generation of distinct flavour compounds that are released into the cheese (Weimer *et al.*, 1999; Rattray and Fox, 1999). The flavour components are generated as a result of both lipolysis and proteolysis.

16.2 Monitoring ripening on the metabolite level

The organoleptic properties of cheese products are, to a large extent, determined by the conversion of milk components through the metabolic processes of the starter culture (Fig. 16.1). These conversions play a primary role in flavour and taste development. For the cheese products described above, many of the key compounds required for the desired flavour have been identified. In recent years insights into the biochemical pathways leading to the production of both desirable and undesirable compounds have been elucidated. Below, an overview is presented of some traditional methods for monitoring cheese ripening and how novel techniques targeting the key cheese flavour compounds or enzyme activities can provide more effective monitoring processes.

16.2.1 Traditional methods to measure proteolysis: new developments

The progress of proteolysis is an important indicator in cheese ripening. Traditionally, this is monitored by the concentration of water-soluble nitrogen (SN) and by monitoring the concentration of nitrogen that is soluble in 12% trichloroacetic acid (AN) (Ardö and Polychroniadou, 1999). The latter fraction contains small peptides with a molecular weight smaller than 1400 Da, all free amino acids and their degradation products. The cadmium–ninhydrin method allows the specific detection of the total fraction of amino acids in cheese extracts (Folkertsma and Fox, 1992).

These methods are suitable for a global estimate of progress in cheese ripening. In many cases the cheese manufacturer focuses on some specific aspects relevant for flavour and taste development such as the accumulation of peptides leading to bitter off-flavours or the production of specific amino acids

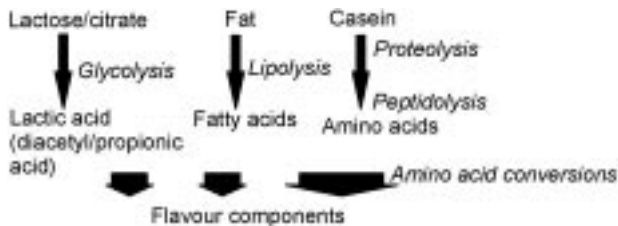


Fig. 16.1 Starter-mediated flavour formation in cheese products. The degradation of the protein part is the major pathway towards the formation of key flavours in most cheeses. In the first stage of ripening milk protein is degraded to peptides and amino acids. In the final phase of ripening free amino acids are converted into volatile flavour components (see also Fig. 16.2).

or flavour compounds. Chromatographic techniques such as HPLC and gas chromatography can be used to separate and identify cheese compounds and simultaneously quantify these using various detection techniques (see Chapter 18 in this book for a review of these methods). Recent developments in mass spectrometry (MS) have revolutionized the methods available for the analysis of the small molecules that are important in cheese flavour (Careri and Mangia, 2003, Cserhati, 2002). The application of HPLC MS-MS allows the rapid identification of peptides in complex mixtures through comparison of fragmentation patterns with reference databases. Moreover, the sensitivity of modern mass spectrometers allows detection of very potent flavour compounds at their odour threshold levels. The development of software for comparative analysis of chromatograms will provide an increasingly powerful tool for recognizing differences in the ripening process in different production runs. Relating these differences to differences in the final product quality should allow the identification of marker compounds or profiles indicative of desired ripening progress.

16.2.2 Flavour and taste components that are characteristic for the ripening process

The progress of cheese ripening can be monitored by determining the progress of metabolite production from primary carbon metabolism through the conversion of (1) lactose and citrate, (2) metabolites derived from lipolysis, and (3) the progress of proteolysis (Fig. 16.1). The relevance of these pathways will be discussed below and several key flavour compounds are listed in Table 16.1. Chapters 3, 4, 5 and 6 of this book describe the exact mechanisms of production for these characteristic compounds.

Gouda cheese

In Gouda cheese, lactose is almost exclusively converted to lactate by the mesophilic starter cultures. This process is finalized within 10 hours after preparation of the cheese curd, and is therefore not a relevant parameter for monitoring of ripening. Lipolysis is very limited in these cheeses and may be relevant with respect to the generation of low levels of C4 to C8 that are subsequently used to produce aldehydes, ketones, esters and thioesters for the production of flavourful cheeses. High concentrations of lipolysis-derived flavour compounds typically indicate growth of spoilage organisms and usually lead to off-flavours in some cheeses, but provide the basis of the strong flavours of other cheeses, such as Italian-style varieties.

Protein degradation is probably the most important route for generation of flavour and taste in Gouda cheese. Traditionally, the progress of proteolysis in cheese ripening is monitored by the increase in SN and AN fractions. The increase in AN correlates to the release of certain peptides and amino acids that contribute to the sweet or broth flavour. Although increase in AN and SN are indicators of the overall progress of cheese ripening, they do not directly correlate to the production of the relevant volatile compounds that are part of the

Table 16.1 Important aroma compounds in Gouda, Maasdam and surface-ripened cheeses

Meta-bolism	Gouda ¹	Maasdam (and Swiss-type) ²	Surface-ripened cheese ³
Sugar	Diacetyl	Propionic acid Diacetyl	
Fat	Butyric acid Butanon Hexanal Pentanal		
Amino acid	3-Methylbutanal 3-Methylbutanol Methanethiol Dimethylsulphide (DMS) 2-methylpropanol Dimethyltrisulphide (DMTS)	Methional 3-Methylbutanal Skatole	3-Methylbutyrate 3-Methylbutanal Methional Methanethiol Dimethyldisulphide
Rest and combined pathways	Ethyl butyrate Limonene	Ethyl butyrate Ethyl hexanoate Ethyl-3-methylbutanoate Phenylethyl acetate	S-methylthioacetate S-methylthiopropionate S-methylthiobutyrate Ethyl-esters (C2-C8)

¹ Engels *et al.* (1997), Neeter and De Jong (1992).² Curioni and Bosset (2002), Preininger and Grosch (1994).³ Leclercq-Perlat *et al.* (2004), Weimer *et al.* (1999).

cheese flavour. Here monitoring the production of specific key flavours or taste compounds using gas chromatography is a much more powerful approach.

Maasdam cheese

Maasdam cheese is produced with mesophilic starter cultures in the same way as Gouda cheese. The applications of additional thermophilic lactic acid bacteria, such as *Lactobacillus delbrueckii*, and propionic acid bacteria have major consequences for the flavour and taste development. As with Gouda cheese, lactose is initially converted to lactate. The thermophilic LAB used in Maasdam manufacture typically have high proteolytic and peptidolytic activities and as a consequence AN ratios tend to increase more rapidly in these cheeses. This contributes to the sweet and *umami* (e.g. glutamic acid) notes in this cheese. When the temperature is raised in these cheeses, the lactic acid is converted to propionic acid and CO₂ by the propionic acid bacteria, which has a major impact on the organoleptic and structural properties. Propionic acid has a distinct sweet and nutty flavour note, whereas the production of CO₂ leads to the characteristic eye formation. The production of proline-specific peptidases by propionic acid bacteria results in elevated levels of proline, which further contributes to the sweet taste of cheeses produced with propionic acid bacteria such as Maasdam and other Swiss-type cheeses. Initiation and progress of the propionic acid

fermentation is a delicate process. Vats that have low activity by the propionic acid bacteria produce what is called a 'weak fermentation' that results in low levels of propionic acid and consequently very little flavour development occurs. Excessive fermentation, especially if the so-called 'late fermentation' uses aspartate as a co-substrate in the fermentation of lactate, results in rapid production of CO₂ and crack formation in the cheese (Bachmann *et al.*, 2002).

Surface-ripened cheese

Surface-ripened cheeses are a very diverse group of cheeses and consequently the flavour and taste compounds indicative for progress of ripening vary for the specific cheeses. However, some general aspects can be recognized. After smearing, outgrowth of yeasts, especially *Debaryomyces hansenii* and *Geotrichum candidum*, leads to the rapid consumption of lactic acid and deacidification of the cheese surface. The yeast flora does not have a major contribution to the smear flavour development. The subsequent outgrowth of aerobic bacteria, especially *Arthrobacter* species, *Brevibacterium linens*, *Corynebacterium ammoniagenes*, and staphylococci, results in the development of the typical sulphury smear aroma. Volatile flavours originating from methionine and cysteine are key components in this aroma. The metabolism of methionine by *B. linens* is of particular relevance and well documented (Ratray and Fox, 1999; Weimer *et al.* 1999). Methanethiol that is produced from methionine by this organism is either oxidized to dimethyldisulphide and dimethyltrisulphide or converted to methylthioesters in collaboration with other microorganisms in the smear flora.

16.2.3 Biochemical or chemical reactions that lead to formation of specific flavour components

The formation of flavours during cheese ripening is a complex and rather slow process involving various chemical and biochemical conversions of milk components (Fig. 16.1). The enzymes involved in the conversion of milk components are predominantly derived from the starter cultures used in the fermentation process. The main organisms in these starters are lactic acid bacteria (LAB), e.g. *Lactococcus lactis*, *Lactobacillus* species, *Streptococcus thermophilus* and *Leuconostoc mesenteroides*. Additional cultures are used, such as *Propionibacterium* in the case of Swiss-type (Maasdam) cheeses, and various aerobic cultures (e.g. *Brevibacterium*, *Arthrobacter*, *Staphylococcus*, *Penicillium*, *Debaromyces*) for surface-ripened cheeses (Bockelmann, 2002; Molimard and Spinnler, 1996).

In the case of the lactose fermentation, the main conversion leads to the formation of lactic acid by the LAB, but a fraction of the intermediate pyruvate can alternatively be converted to various flavour compounds such as diacetyl, acetoin, acetaldehyde, or acetic acid (Fordyce *et al.*, 1984). Moreover, the conversion citrate may further contribute to the production of diacetyl (Hugenholtz *et al.*, 2000). As described above, lactic acid is converted to propionic acid in Maasdam cheese.

Lipolysis results in the formation of free fatty acids, which are potent flavours and precursors of flavour compounds such as methylketones, alcohols and lactones. In Gouda and Maasdam-type cheeses limited lipolysis is a desired as excessive production of fatty acids is considered a defect. LAB contribute relatively little to lipolysis, but additional cultures (e.g. mould) in the case of surface-ripened cheeses (Molimard and Spinnler, 1996) often have high lipolytic activities. Flavours derived from the conversion of fat are particularly important in surface-ripened cheeses and in soft cheeses, such as Camembert and Roquefort.

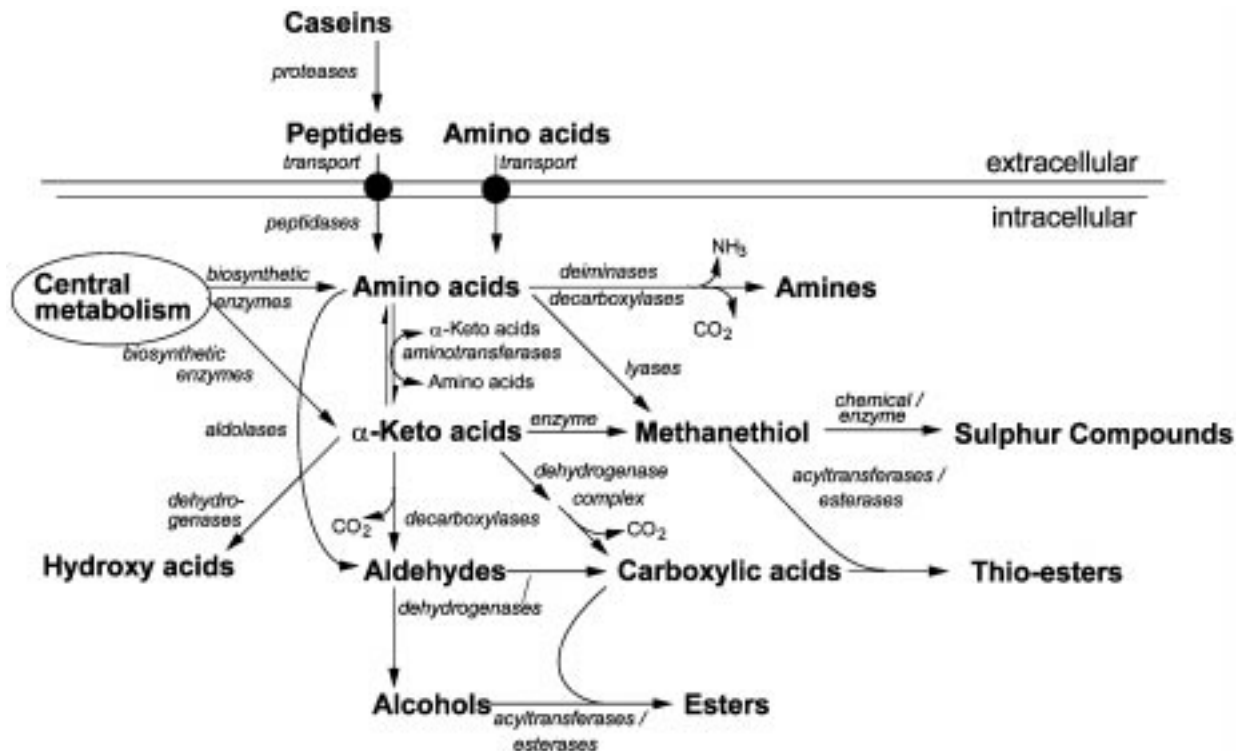
The degradation and conversion of caseins is the most important initial biochemical step for flavour formation in hard-type and semi-hard-type cheeses (Kranenburg *et al.*, 2002). The pathways leading to the production of flavour and taste compounds from milk protein by *L. lactis* are well documented. Degradation of caseins by the activities of rennet enzymes, and the cell-envelope proteinase and peptidases from LAB, yield small peptides and free amino acids that are subsequently utilized for production of other impact compounds. A balance between proteolysis and peptidolysis prevents the formation of bitterness in cheese (Smit *et al.*, 1998). Although it is known that peptides can taste bitter (Lemieux and Simard, 1992) or delicious (Yamasaki and Maekawa, 1978) and that amino acids can taste sweet, bitter, or broth-like (Mulder, 1952), the direct contribution of peptides and amino acids to flavour is probably limited to a basic taste (Engels and Visser, 1996). For specific flavour development, further conversion of amino acids is required to various alcohols, aldehydes, acids, esters and sulphur compounds. Several excellent reviews are available describing various pathways and enzymes leading to the production of key flavour compounds (Smit *et al.*, 2005; Christensen *et al.*, 1999).

16.3 Monitoring ripening on the enzyme level

16.3.1 Description of specific enzymes that play a direct role in the ripening process

The enzymatic pathways for the production of key cheese flavours are extensively studied, highlighting their importance to cheese quality. Most research has focused on the conversions carried out by *Lactococcus lactis* but the majority of the pathways and the enzymes involved are also found in other organisms, such as various lactobacilli. An overview of the pathways involved in the production of flavour compounds from milk protein is presented in Fig. 16.2, and detailed in Chapter 4.

Since the concentrations of free amino acids and peptides are very low in milk, for their growth in milk the starter cultures depend heavily on their proteolytic systems. The degradation of milk proteins (caseins) leads to peptides and free amino acids, which can subsequently be taken up by the cells (Kunji *et al.*, 1996; Christensen *et al.*, 1999). Proteolysis is initiated by a single cell-wall-bound extracellular proteinase (Prt), which can be either chromosomally or



plasmid-encoded. While most dairy LAB strains contain such an extracellular proteinase, several do not and these are mainly dependent on other strains in the starter culture for the production of peptides and amino acids.

Following uptake, the peptides are degraded intracellularly by a variety of peptidases, which have been extensively studied in both lactococci and lactobacilli (reviews by Kunji *et al.*, 1996; Christensen *et al.*, 1999). The peptidases of LAB can be divided into several groups on the basis of their substrate specificity – endopeptidases, aminopeptidases, di- and tri-peptidases, and proline-specific peptidases. The specialized peptidases in LAB for hydrolysis of Pro-containing peptides have been postulated to be important for the degradation of casein-derived peptides, since these are known to have a high proline content.

The balance between the formation of peptides and their subsequent degradation into free amino acids is very important, since accumulation of peptides might lead to a bitter off-flavour in cheese (Stadhouders *et al.*, 1983; Smit *et al.*, 1998). Various bitter-tasting peptides have been identified and especially these peptides should be degraded rapidly in order to prevent bitterness (Stadhouders *et al.*, 1983; Visser *et al.*, 1983; Smit *et al.*, 1998). Specific cultures have been selected with high bitter-tasting-peptide degrading abilities (Smit *et al.*, 1998) and such cultures are nowadays frequently used in the preparation of various types of cheese.

Amino acid catabolism is specifically linked to production of cheese flavour in all cheese types studied to date. The use of amino acids by the starter and flavour adjunct cultures produces a multitude of compounds which presents a huge analytical challenge. The specific chemical methods for analysis of these compounds and the recent elucidation of many of the major biochemical pathways involved in the production of key flavour compounds are reviewed in other chapters. As a result many biochemical assays have been developed that allow the quantitative analysis of key enzyme activities in cell suspensions and cell extracts. Such enzyme activity assays can be effectively applied for screening starter cultures as well as for monitoring the progress of cheese ripening. This section will provide an overview of their application for monitoring cheese ripening, including also newer methods using proteomics and genomics.

16.3.2 *In situ* measurements of these characteristic enzyme activities

The elucidation of the metabolic pathways and the key enzymes involved in the production of cheese flavour compounds has opened new avenues for the development of rational monitoring tools for the cheese ripening process. In order to allow accurate quantitative determination of the activity of enzymes that are released into the cheese matrix, it is necessary to extract the enzymes from the cheese by preparing a cheese extract free of cheese solids and whole cells as described by De Ruyter *et al.* (1997). In this method a cheese sample is diluted five- to ten-fold in 2% sodium citrate buffer and subsequently homogenised for 5 min in a stomacher. Using this procedure, cellular integrity is mostly retained and subsequently cells can be removed from the homogeneous suspension by

centrifugation (10,000 g, 10 min). Enzymes released into the cheese matrix can be quantified by determining the enzyme activity in the supernatant. Crude extracts can be prepared from remaining cell pellets by sonication or bead-beating. These extracts can then be used to quantify the activity of cheese-ripening enzymes retained within the starter cells. Using this general approach, a variety of enzyme activities can be determined by adapting existing enzymatic assays of key ripening enzyme activities. Some examples will be discussed below.

The activities of various enzymes involved in proteolysis, both the extracellular protease as well as peptidases, can be efficiently quantified using commercially available labelled peptide substrates. These typically contain a *para*-nitroanilide group that is released from the substrate upon hydrolysis by the enzyme. The release of *p*-nitroanilide can be quantified online spectrophotometrically (Kunji *et al.*, 1996 and some of its references).

In an elegant study using a peptidase N-overproducing *L. lactis* strain, De Ruyter *et al.* (1997) monitored the release of the peptidase N into the Gouda cheese matrix. The approach that was used included the application of a range of *L. lactis* recombinants producing lytic enzymes as a co-culture thereby inducing *in trans* lysis of PepN-overproducing strains. In cheese produced with the PepN overproducer, the amount of enzyme activity in the curd was approximately 6%, whereas this increased to almost 100% when lysin-producing recombinants were added. In an analogous approach Weimer *et al.* (1997) measured the enzyme activity in low-fat cheese during ripening, as well as the amount of heat-stable enzymes in milk (Koka and Weimer, 2000).

Similarly, the activity of esterase can be efficiently quantified using *para*-nitrophenol-esters. Hydrolysis by esterases results in the release of *para*-nitrophenol that can be detected online using a spectrophotometer (Fernandez *et al.*, 2000). Other methods rely on the use of alpha- and beta-naphthyl derivatives of fatty acids (chromogenic method) or triglycerides (agar-well assay technique and titrimetric test) as substrates for esterases and lipases respectively (Medina *et al.*, 2004). Labelled ester substrates are available with varying fatty acid chain length, allowing quantification of the corresponding esterase activities. Blake *et al.* (1996) used this approach to develop a semi-automated screening method for bacterial enzyme activity for starter cultures and spoilage organisms.

The activities of the lyases involved in releasing the important cheese flavour methanethiol from methionine can be measured analogously using the appropriate buffers and substrates. The C-S lyase activity towards methionine and cystathionine of cystathionine β -lyase and methionine γ -lyase can be analysed by the determination of the free-thiol group formation with DTNB (5,5-dithiobis 2-nitrobenzoic acid) as described by Uren (1987) (see also Fernández *et al.*, 2000, Dias and Weimer, 1998). In a study by Van Hylckama Vlieg *et al.* (2002) CBL was effectively quantified in cheeses manufactured with CBL overproducing recombinants. In cheese produced with a CBL overproducing strain to which co-cultures were added capable of inducing *in trans* lysis up to 41% of total CBL could be extracted from 12-week-old Gouda cheese matrix. The

results showed that elevated CBL activities were associated with a mild flavour improvement.

Finally, a broad range of NAD(H)-dependent enzyme activities can be detected in cheese extracts. These include lactate dehydrogenase and hydroxy-isocaproic acid dehydrogenase, an enzyme involved in depletion of α -keto acids that act as flavour precursors. The activity of these enzymes can be quantified effectively by monitoring the production or depletion of NADH. An example of the quantification of lactate dehydrogenase as a marker for cell lysis is described by de Ruyter *et al.* (1997).

16.4 Monitoring ripening on the bacterial level

16.4.1 Gouda cheese

The bacterial flora, mainly consisting of representatives of the lactic acid bacterium *Lactococcus lactis*, plays a crucial role in the ripening process. The different proteolytic and flavour-generating enzymes, originating from these lactic acid bacteria, have been described above. One factor that has been identified as a rate-limiting step in the ripening process is the release of these (intracellular) enzymes into the cheese matrix. For this to happen, the bacterial cells need to release their content in a process called lysis. It is now generally accepted that the degree of starter lysis is positively correlated with degree of flavour generation, ripening, in the cheese. This would mean that the ripening process could be monitored by monitoring the lysis process of the starter bacteria. For this purpose various methodologies have been described. One popular method is the measurement of specific bacterial enzymes in the cheese matrix that can only be released from lysed lactic acid bacteria, such as mentioned above for lactate dehydrogenase (de Ruyter *et al.*, 1997). Several reports have appeared on measurement of activity of cytoplasmic enzymes such as aminopeptidase N and peptidase XP. The difficulties that are usually encountered in this type of work are that either intact cells also show some enzyme activity (as a result of permeability of the enzyme substrate), that the enzyme activity measurement is severely disturbed by components in the cheese matrix, or that sample preparation before the enzyme activity can be measured leads to lysis of the starter bacteria and is not a good representation of the actual conditions in cheese. Weimer's group has used this tool to screen bacteria for lysis during carbohydrate starvation (Ganesan *et al.*, 2004, 2006; Stuart *et al.*, 1998) to find that a portion of the population of lactococci remain intact and metabolically active.

To circumvent extraction of enzymes, a method was developed that could determine lysis of starter bacteria directly in cheese, without the need for extraction. It is based on fluorescence labelling of intracellular DNA upon permeabilization of cells (Niven and Mulholland, 1997). This methodology, also known as the BacLight Live/Dead staining method (Boulos *et al.*, 1999), was applied on actual slices of Gouda cheese (Bunthof *et al.*, 2001). These slices were

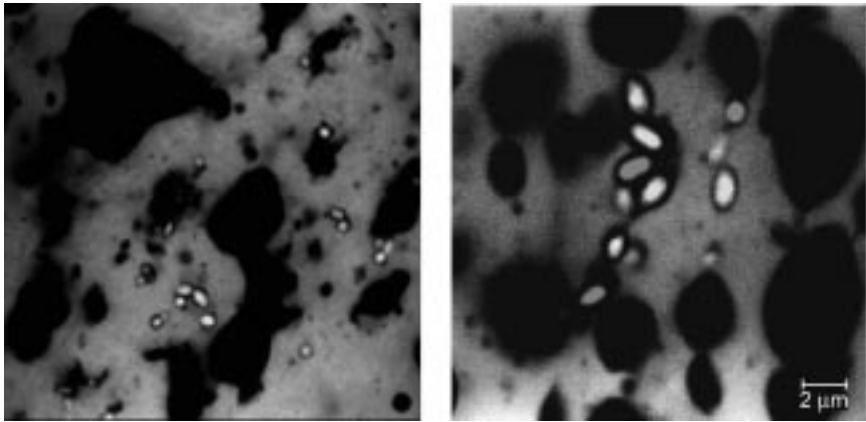


Fig. 16.3 CSLM-images of fluorescent labelled starter bacteria in Gouda cheese. Green (Syto-9) labelled cells are viable and intact, red (propidium iodide) labelled cells are lysing and have become permeable.

treated with two fluorescent labels, the green-fluorescent Syto-9 and red-fluorescent propidium iodide. Syto-9 is lipophilic and will permeate intact bacterial membranes. Inside the cell, the probe will bind to DNA without interfering with viability. In principle, all cells including the dead and/or permeable ones will fluoresce green. When the non-lipophilic and non-membrane-permeable fluorescent dye propidium iodide is added, it replaces the green dye in the dead and permeable cells because of its higher binding affinity for nucleic acids. The final results will show a mixture of green-fluorescent (i.e. intact) cells and red-fluorescent (i.e. membrane permeable and/or dead) cells and the ratio between the two can be determined directly under the microscope. By using a CSLM (Confocal Scanning Laser Microscopy) microscope, which allows zooming-in into relative thick materials, bacterial cells can be observed in the actual cheese matrix (Fig. 16.3) without the need for elaborate extraction techniques. This is a significant advantage in determining the actual integrity of the bacteria during their residence in the cheese, since traditional methods are dependent on a combination of grinding of cheese material and clearing (with citric acid or EDTA) of the casein micelles, all methods that can lead to killing of the cells. Using this technology, a rapid decrease of viable lactococcal cells was observed in Gouda cheese in four weeks of ripening time, replaced by a similar number of dead, but visibly intact, lactococcal cells (Bunthof *et al.*, 2001). Apparently, the cheese matrix leads to maintenance of the cellular structure of dead starter bacteria. The identical staining method was also successfully applied to monitor survival of probiotic bacteria in cheese (Auty *et al.*, 2001).

16.4.2 Maasdam cheese

The crucial process in Maasdam cheese ripening is the proliferation of the *Propionibacterium* population. This population is responsible for production of

propionic acid which renders a specific nutty aroma to the Maasdam cheese, and of carbon dioxide which is responsible for the large eyes that are produced in this cheese type. This cheese is put on the market as relatively young cheese (5–8 weeks) as soon as optimal flavour and gas have been formed. To achieve this, there is growing need for predicting the exact ripening time of the cheese, preferably as early as possible. Traditionally, monitoring of these cheeses has been performed on the basis of gas formation, through mechanical means (knocking on the cheese) or by X-raying the cheese to visualize the eye formation. However, this monitors only the exact moment of the end of the ‘cooking’ period, but is not predictive for how long the ‘cooking’ period should be extended. For accurately predicting when the ripening process is completed, it would be necessary to establish the start of the *Propionibacterium* proliferation during the ‘cooking’ period. A method would need to be developed that would visualize the start of the growth process in these bacteria. A method focusing on transcription of growth-related genes, using whole genome DNA arrays or real-time PCR focusing on transcription of a specific set of genes, could be the method of the future for monitoring the ripening process.

16.4.3 Surface-ripened cheese

A proper ripening process of a surface-ripened cheese, such as Kernhem or Limburg, involves a succession of aerobic microorganisms on the surface of the cheese. Initially, the surface is dominated by different yeasts such as *Geotrichum candidum* and *Debaryomyces hansenii*, as described above. Later in the process, bacteria such as *Arthrobacter*, *Corynebacterium*, *Staphylococcus* and, especially, *Brevibacterium linens* become dominant (Bockelmann, 2002; Molimard and Spinnler, 1996). Especially this last microorganism is responsible for production of the sulphury components that are typical of the aroma of these cheeses. It could be argued that the proliferation of *B. linens* is the determining factor in the ripening process. This would mean that careful enumeration of *B. linens* in the cheese could be used for direct monitoring of the ripening process. Since these microorganisms grow on the cheese surface, it would be relatively easy to sample cheeses without the need to sacrifice them. Applied research is still needed to determine the direct relationship between the *B. linens* cell numbers on the cheese surface and the flavour generation in these cheeses.

16.5 High-throughput tools for monitoring cheese ripening

During cheese ripening, starter bacteria are exposed to a complex environment and challenging conditions that will have a major impact on the cell. Genomics provides excellent prospects for studying such responses in unbiased way. This should provide us with new biomarkers for strain performance that can subsequently be targets for the development of tools for monitoring cheese ripening. Below we describe some examples and possibilities of studying cheese

(ripening) on the transcriptome level, on the proteome level and on the metabolome level.

16.5.1 Global responses at the level of transcription

Several techniques are available for elucidating starter responses at the level of gene activation. Transcriptomics analysis with DNA arrays is the most widely used technique for studying responses at the level of transcription. However, the complex cheese matrix may interfere with the experimental procedures required '(Recombination-based) *in vivo* expression technology', (R)IVET, provides an attractive alternative. It has been developed for the identification of genes that are activated under specific conditions in environments that cannot easily be sampled. RIVET relies on the construction of transcriptional fusions to a promoterless reporter gene encoding a site-specific DNA recombinase. After it is produced, this resolvase functions *in trans* to permanently excise from the bacterial genome a marker gene that is flanked by recombinase recognition sequences. The subsequent deletion of the marker gene serves as an *ex post facto* indicator of increased transcription of the gene fusion, indicating that an activated promoter fragment is located on the genomic DNA fragment located upstream of the resolvase gene. These upstream fragments can be easily recovered from clones that have lost the marker after the screening. RIVET technology may help to identify genes specifically expressed in cheese, thereby increasing our understanding of the cellular response to the cheese matrix and ultimately yielding novel biomarkers for monitoring ripening progress.

Global gene expression using microarrays is now possible for LAB. Use of this approach to define unknown metabolic routes is beginning (Ganesan *et al.*, 2006). This area will expand to provide global analysis of gene expression during the production and ripening once reliable gene chips become available. It has already yielded valuable information about amino acid metabolism in lactococci and brevibacteria. Weimer *et al.* (2004) have developed methods to extract RNA from cheese to monitor the global gene expression during ripening to find that the genes associated with amino acid metabolism are induced. The role of lysis and non-culturable cells can now be explored in detail to assess the respective role for optimizing ripening.

16.5.2 Proteomics and metabolomics of cheese

The determination of all proteins in a ripening cheese is a valuable indication of what can be expected in the cheese during the ripening process. As described above, the lysis of starter bacteria leads to release of intracellular enzymes which subsequently can contribute to the flavour formation in cheese. Only one example of this approach has been published, recently, for Emmental cheese (Gagnaire *et al.*, 2004). Five times during Emmental ripening, at 1, 7, 20, 33 and 76 days, the aqueous phase of the cheese was extracted and eluted on 2D-PAGE, using isoelectric focusing as the one dimension and size separation as the second

dimension. More than 100 spots were detected and these were excised and characterized by MALDI-TOF peptide fingerprinting. A large proportion (i.e. 75 proteins) were identified via protein database searches. A subset of these proteins (i.e. 12 proteins) were milk proteins (from *Bos taurus*), 63 originated from different microorganisms including *Streptococcus thermophilus* (21), *Lactobacillus helveticus* (17) and *Lactobacillus bulgaricus* (7), and only eight proteins originated from *Propionibacterium freudenreichii*. Since this last microorganism plays a similar crucial role in Emmental ripening, one wonders whether the proteomics analysis will help in determining the ripening stage of the cheese if additional extraction methods are developed. Considering that the genome of lactococci contains ~2300 genes, one would expect that this analysis would produce a large number of protein spots on the 2D gel (i.e. >2000). Adding to that the milk proteins and the other bacteria, one would expect >3000 proteins to appear in the analysis.

Metabolomics for cheese flavour

Metabolomics is definitely a high-throughput technique that will be useful in monitoring flavour development and, thus, ripening of cheeses. In principle, the methodology, relying on high-resolution mass spectrometry coupled to chromatographic separation, makes it possible to measure a large number of metabolites in a cheese at a certain time during the ripening. By simultaneously grading the flavour development of the cheeses through organoleptic testing, it should be possible to correlate the presence of a certain set of metabolites with the ripening stage of different cheese types. For some cheeses with relatively simple flavour profiles, such as Maasdam cheese and the surface-ripened cheeses, such correlations can already be drawn as illustrated above for the production of propionic acid and the sulphury compounds. For cheeses with more complicated flavour profiles, sophisticated data processing software programs will need to be developed, to unravel the correlation between metabolite production and ripening stage.

Rapid developments in sensor technology have facilitated the production of devices known as electronic noses that can detect and discriminate the production profiles of volatile compounds (Turner and Magan, 2004). This new technology holds great promise for diagnostic applications in medicine as well as for the assessment of the quality of food products. Recently, a few examples have been published demonstrating its power for determining ripening progress and shelf life of cheese products (Benedetti *et al.*, 2005).

Another interesting new development is the application of Nuclear Magnetic Resonance for monitoring metabolite fluxes in cheese. This has proved to be very useful as it allows one to monitor non-invasively the dynamics of metabolites in cells and food matrices. NMR has been applied to the analysis of carbon fluxes and end-product formation of pure cultures of starter organisms, such as, for instance, the production of succinate from citrate by non-starter lactic acid bacteria or the production of aromatic acids produced from amino acids by *Lactococcus* (Dudley and Steele, 2005; Neves *et al.*, 2005; Ganesan *et*

al., 2006). Other groups have applied NMR to cheese samples allowing discrimination of Emmental cheeses from different geographical regions (Shintu and Caldarelli, 2006). Most NMR studies so far have focused on primary metabolite fluxes. These metabolites typically occur at the high concentrations (mM range) required for NMR analysis. The intrinsic low sensitivity implies that monitoring of many key flavour compounds is not possible using NMR, as these typically occur at ppm or ppb levels (see above).

16.6 Future trends

With the different methods being developed for detailed analysis of the metabolite levels, enzyme levels and lysed or intact starter bacteria in cheese, new ripening markers will be discovered for different cheese types. It will become possible to predict at a much earlier stage the expected quality of a specific batch of cheese production and when the ripening process will be finished, based on a measured concentration of a (set of) metabolite(s) or the measured activity of a flavour-forming enzyme or the extent of lysis of a starter culture in cheese. In the early stage of introduction of these novel monitoring assays, sophisticated laboratories will need to offer their expertise and facilities in support of the cheese industry, but the development of 'labs-on-a-chip', and kits for enzyme assays and for fluorescent labelling of starter bacteria in cheese, will make these monitoring assays available, affordable and easy to use for the cheese manufacturer. This will lead to a much tighter control of the cheese production process, to reduced losses and to huge savings in both time and energy during the production, ripening and storage of the cheeses.

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Defining cheese flavor

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17.1 Introduction

Cheese is a diverse product with a spectrum of flavors and textures which are derived from the starter culture, milk source, age and ripening conditions. Both flavor and texture are crucial to the identity of a particular cheese, and both play a crucial role in consumer acceptance and preference. Flavor and texture are characterized and defined by sensory analysis to better understand these properties and provide superior, specifically tailored products to meet consumer desires.

Sensory analysis is often thought of as a single test or an imprecise analysis method. However, sensory analysis is in reality a powerful toolbox of many different precise tools that when adequately applied, provide cogent results. Perhaps even more important, flavor is a sensory concept and without sensory analysis by human panelists we have no insights into the nature of cheese flavor. The most sensitive instrument provides quantitative information about specific chemicals; however, those chemicals need to be linked to specific sensory attributes to be meaningful for product characterization or to direct flavor development strategies. Sensory tests can be loosely grouped into grading/judging, analytical tests, and affective tests. Within each category, especially the latter two, are multiple types of tests for specific sensory objectives. In general, grading and judging are traditional tests developed by the dairy industry (Bodyfelt *et al.*, 1988). These tests are quality-oriented and subjective and are often not addressed in mainstream sensory analysis textbooks despite their continued use and application by the dairy industry. They are not recommended for research and marketing for numerous reasons discussed elsewhere (Singh *et al.*, 2003; Delahunty and Drake, 2004; Drake, 2004). In contrast, analytical and

affective tests were developed from psychological theories (many dating back to the 1800s) and were designed for the measurement of any human sensory response to external stimuli. Analytical tests are tests that generally use screened or trained panelists. Affective tests measure consumer responses or perceptions and include both quantitative tests as well as qualitative tests. These tools, their general application and purpose, and the psychological tenets for their development are reviewed elsewhere (Lawless and Heymann, 1998; Meilgaard *et al.*, 1999). A review of these tests as they relate to cheese was conducted by Delahunty and Drake (2004), which highlighted and defined the flavor and/or texture of cheese; this provides a platform to understand product inherent variability, stability, changes with time or process, flavor chemistry, and consumer perception. As such, defining flavor is a powerful platform for effective research and competitive marketing.

17.2 The starting point: lexicon development

Defining flavor or texture starts with a defined lexicon. A lexicon is simply a sensory language that documents the sensory properties of a product (Drake and Civile, 2003). As such, a lexicon can encompass flavor, texture, visual, hand-feel, and/or auditory properties of a product. Ideally, a lexicon includes definitions for each descriptor and a food or chemical reference for the descriptor. Many descriptive languages have been identified for cheese, but far fewer have definitions and references for all descriptors (Tables 17.1 and 17.2) (Drake and Civile, 2003; Delahunty and Drake, 2004). Definitions and references ensure clear and precise meanings for descriptors. This enhances taste panelist training, allows calibration and comparison of panels at multiple locations and ultimately provides understanding of flavor chemistry and consumer perception for broad application to cheese flavor.

Development of a lexicon begins with collection of a representative sample set (Drake and Civile, 2003). The lexicon will only be representative of the products it was developed from. Ideally a large and diverse sample set should be collected to promote a broad lexicon. For example, if descriptive sensory analysis was to be applied to differentiate aged Cheddar cheeses made with different adjunct starter cultures, a sensory language might be identified from a limited sample set of 5–10 aged Cheddar cheeses. In contrast, a lexicon for Cheddar cheeses, a language that generally can be used to document flavors in all Cheddar cheeses, might require more than 100 samples from which to identify a robust language (Drake *et al.*, 2001).

Inclusion of previously defined terms from the literature is also needed. There is no need to ‘reinvent the wheel’ and there are numerous descriptors that have been identified, defined and anchored for various cheeses (Table 17.2). Once a sample set is collected and a review of the literature indicates a gap in knowledge, a preliminary language must be identified. Experienced sensory panelists or individuals experienced with the product category are optimal for

Table 17.1 Studies of cheese flavor that have used descriptive sensory analysis

References and cheeses studied	Descriptive vocabularies
Adhikari <i>et al.</i> , 2003	<i>Aroma</i> : Smoky, vinegary, cheddary, buttery, musty, pungent, other (Swiss) <i>Flavor</i> : Smoky, salty, sweet, bitter, acidic, cheddary, sharp, flavor intensity
Low-fat, full-fat and smoked Swiss, Cheddar and Gouda	
Bárcenas <i>et al.</i> , 1999, 2001	<i>Odor</i> : Overall intensity, sharp, milky, brine, rennet, buttery, toasty, smoky, mushroom <i>Flavor</i> : Overall intensity, fruity, butyric, nutty, buttery, acid/yoghurt, sweet, salty, pungent, rennet, smoky
Castellano, Idiazábal, Manchego, Roncal (ewes' milk cheeses), Garrotxa (goats' milk), Tetilla (cows' milk)	
Drake <i>et al.</i> , 2001, 2002, 2003, 2005	<i>Flavor</i> : Cooked, whey, diacetyl, milkfat/lactone, fruity, sulfur/eggy, sulfur/match, free fatty acid, brothy, nutty, catty, cowy/phenolic, age, yeasty, moldy/musty, methyl ketone/bleu, oxidized, waxy/crayon, fecal, bell pepper, rosy/floral, scorched, bitter, salty, sweet, sour, umami, prickle/bite, cultured, creamy, dishcloth, butyric, savory, fermented, maturity
Cheddar, processed cheese	
Heisserer and Chambers IV, 1993	<i>Flavor</i> : Buttery, cooked milk, dairy fat, dairy sour, dairy sweet, animalic, butyric acid, decaying animal, fresh fish, fish oil, goaty, sweaty, waxy, fermented/fruity/winey, nutty, pineapple, sauerkraut, smoky, soy sauce, moldy, mushroom, astringent, biting, pungent, sharp, bitter, salty, sour, sweet
Asiago, Bel Paese, Blue Cheese, Bond-ost, Brisk, Brie, Butter Cheese, Camembert, Cheddar, Chèvre, Colby, Danish Cream Havarti, Edam, Emmentaler, Feta, Fontina, Gorgonzola, Gouda, Gruyère, Jarlsberg, Kreme Käse, Limburger, Manchego, Mozzarella, Monterey Jack, Parmesan, Port Salut, Provolone, Romano, Roquefort, Sap Sago, Stilton, Swiss	
Hough <i>et al.</i> , 1996	<i>Aroma</i> : Total intensity, sweet, sour, lipolysis, milky-creamy <i>Flavor</i> : Total intensity, cheese, salty, sweet, bitter, acid, lipolysis, milky-creamy, tongue-tingling, hot, residual intensity
Reggianito grating cheese	

Lawlor and Delahunty, 2000 Lawlor *et al.*, 2000, 2001, 2002, 2003

Appenzeller, Ambassadeur, Bleu d'Auvergne, Blue Shropshire, Blue Stilton, Cambozola, Cashel Blue, Chaumes, Danish Blue, Dubliner, Emmental, Fontina, Gabriel, Gruyère, Huntsman, Mahón, Old Amsterdam, Raclette, Tête de Moine, Tetilla, Wensleydale

McEwan *et al.*, 1989

Cheddar

Muir and Hunter, 1992a,b,c

Banks *et al.*, 1993

Muir and Banks, 1993

Muir *et al.*, 1995a,b,c,d, 1996, 1997a,b

Cheddar, Farmhouse Cheddar

Murray and Delahunty, 2000a,b,c

Bogue *et al.*, 1999

Fenelon *et al.*, 2000

O'Riordan and Delahunty, 2003

Irish farmhouse and Cheddar cheese

Neilsen and Zannoni, 1998

Hunter and McEwan, 1998

Caerphilly, Cheddar, Comté, Danbo, Edam, Emmental, Fontina, Gouda, Jarlsberg, Parmigiano-Reggiano, Sbrinz, Svenbo

Ordoñez *et al.*, 1998

Idiazábal cheese (ewes' milk cheese)

Odor: Pungent, caramel, mushroom, silage, sweaty/sour, fruity, moldy, cheddar dairy-sweet, sweet, creamy

Flavor: Buttery, caramel, dairy sweet, rancid, mushroom, oily, moldy, nutty, smoky, soapy, silage, processed, sweet, salty, acidic, bitter, pepper, burnt-aftertaste, strength, balanced

Appearance: Color intensity, crumbly, mottling, moldy, softness, openness, shiny

Odor: Strength, creamy/milky, sour, rindy, manure

Flavor: Creamy/milky, strength, sour, manure, salty, acid, smoky, rindy

Odor: Intensity, creamy, sulfur, fruity, nutty, rancid, other

Flavor: Cheddar intensity/overall intensity, creamy/milky, sour/acid, sulfur/eggy, fruity/sweet, nutty, rancid, bitter, cowy, unclean/manurial, salty, other

Aroma: Pungent, caramel, sweaty/sour, sweet, creamy, fruity

Flavor: Pungent, caramel, sweaty, creamy, fruity, buttery, rancid, cheddary, mushroom, moldy, nutty, smoky, soapy, processed, sweet, salty, acidic, bitter, astringent, strength, balanced

Appearance: Color intensity, mottled, uniformity, open, shiny

Smell: Strength/intensity, creamy, yoghurt, fruity/citrus fruit/other fruit/nutty, grass, animal/cowshed, caramel, acid/sour, ammonia, hay/grass

Aroma/taste: Strength/intensity, creamy/yoghurt, grass, fruity/citrus fruit/other fruit /nutty, animal/cowshed, toasted/caramel, sour, pungent, ammonia, sweet, salty, acid, bitter

Odor: Pungent, acid, sweet, characteristic, others

Taste: Pungent, acid, sweet, salty, bitter, characteristic, others

Aftertaste: Pungent, acid, bitter, others, persistent

Appearance: Paste color (internal), eyes (internal), shape (external), rind (external)

Table 17.1 Continued

References and cheeses studied	Descriptive vocabularies
Papademas and Robinson, 2001	<i>Taste and flavor:</i> Salty, bitter, acidity, creamy, milky, minty, intensity <i>Appearance:</i> Color, body
Halloumi	
Piggott and Mowat, 1991	<i>Appearance:</i> White to orange
Delahunty <i>et al.</i> , 1996a, b	<i>Flavor:</i> Milky, buttery, cheesy, moldy, rancid, pungent, sour (aroma), sweet (aroma), salty (taste), sour (taste), bitter (taste), processed, strength, maturity, aftertaste
Jack <i>et al.</i> , 1993	
Cheddar	
Retiveau <i>et al.</i> , 2005	<i>Flavor:</i> Buttery, dairy fat, dairy sour, dairy sweet, cooked milk, must/earthy, moldy, musty/dry, animalic, butyric, goaty, sweaty, aged, fermented, fermented/
French cheeses	fruity, sauerkraut, ashy/sooty, grain, nutty, chemical, green/herbaceous, fruity, floral, astringency, pungency, sharp, biting, bitter, salty, sour, sweet
Roberts and Vickers, 1994	<i>Aroma:</i> Buttery, fatty, fruity, fermented, moldy, nutty, sweaty/sour, pungent, rancid, smokey, spoiled dairy, vinegary
Cheddar	<i>Flavor:</i> Acid, acid bite, astringent, barny, bitter, buttery, cardboard, chemical, fatty, fruity, metallic, milky, moldy, peppery, sweaty/sour, rancid, salty, sharp, smoky, soapy, diacetyl (yoghurt), sweet <i>Aftertaste:</i> Acid, bitter, milky, smoky, fishy, moldy, peppery, soapy, sweaty/sour
Stampanoni, 1994	<i>Flavor:</i> Milky, cooked milk, fatty, buttery, creamy, nutty, butter milk, yoghurt, cottage cheese, caseinate, whey, soapy, fermented, mushroom, earthy, musty, spicy-pungent, blue, ammonia, green-grass, cheese rind, propionic acid, capric acid, butyric acid, fruity, sweaty, animal
Cheese flavors: cheese general, fresh cheese, soft cheese, hard cheese, goat/sheep cheese	
Wendin <i>et al.</i> , 2000	<i>Appearance:</i> Yellow color, granularity, watery, compact <i>Flavor/taste:</i> Sourness, butter, saltiness
Cream cheese	

Source: adapted from Delahunty and Drake, 2004.

Table 17.2 Terms used to describe the flavor of cheese using descriptive analysis methods. Terms in this list were developed, defined and referenced using standard materials by trained panels. Cheeses studied were Cheddar (Murray and Delahunty, 2000b; Drake *et al.*, 2001, 2005a), low-fat, full-fat and smoked Swiss, Cheddar and Gouda (Adhikari *et al.*, 2003), aged natural cheese of many types (Heisserer and Chambers IV, 1993), ewes' milk cheese (Bárcenas *et al.*, 1999), French cheeses (Retiveau *et al.*, 2005) and cheese flavors (Stampanoni, 1994)

Term	Definition	Standard
Acid/ yoghurt, acidic	The taste on the tongue associated with acids (citric, lactic, ...) A sour, tangy, sharp, citrus-like taste. The fundamental taste sensations of which lactic and citric acids are typical	0.35–0.86 g lactic acid per 100 g Ricotta Fermented milk Natural yoghurt Citric acid (0.2% in water)
Age	Flavors indicating age in Cheddar cheese	Aged Cheddar (1 year or older)
Aged	A clear distinct aromatic edge that is sometimes described as sour, astringent, and pungent, frequently seen in aged cheese	Propionic acid
Ammonia	–	Ammonia solution (0.25% in water)
Animal, animalic	The combination of aromatics reminiscent of farm animals and barnyards	4-Methyl-octanoic acid (2% in polyethylene glycol) 1-Phenyl-2-thiourea (5000 mg/kg in PG)
Ashy/sooty	Bark like lingering aromatics associated with a cold campfire	Hickory smoke seasoning salt
Astringent	The complex of drying, puckering, shrinking sensations in the oral cavity causing contraction of the body tissues A mouth-drying and harsh sensation	Alum (0.1% in water) Tea, 6 bags soaked in water for 3 hours Tannic acid (0.05% in water)
Balanced	Mellow, smooth, clean. In equilibrium, well arranged or disposed, with no constituent lacking or in excess	Mild Cheddar
Bell pepper	Aroma associated with freshly cut green peppers	Methoxy pyrazines (5 µg/kg) Freshly cut bell pepper
Biting	The slightly burning, prickling and/or numbness of the tongue and/or mouth surface	Horseradish sauce
Bitter	Fundamental taste sensation of which caffeine or quinine are typical A chemical-like taste	Caffeine (0.02, 0.06 or 0.08% in water) Tonic water, quinine (0.01% in water)
Blue	–	Octan-2-one (1% in PG)
Brine	The combination of aromatics associated with the saturated brine used during traditional ewes' milk cheesemaking	Ewes' milk cheese brine at room temperature

Table 17.2 Continued

Term	Definition	Standard
Brothy	Aromatics associated with boiled meat or vegetable stock soup	Canned potatoes Low-sodium beef broth cubes Methional (20 mg/kg)
Butter milk	—	Pasteurized butter milk
Buttery	Fatty, buttery tasting, of the nature of, or containing butter The aromatics commonly associated with natural, fresh, slightly salted butter Aroma rising from butter at room temperature	Unsalted butter Lightly salted butter Pasteurized cooking butter Diacetyl (1% in PG) Diacetyl in vaseline oil (several concentrations)
Butyric, butyric acid	Sour flavor, similar to baby vomit The aromatics reminiscent of baby vomit; sour and cheesy The volatile compounds associated with butyric acid often described as vomit, sickly, baby burps	Butyric acid, 2500 mg/kg in Vaseline oil = stock solution 2 ml SS + cotton in 60 ml flask Butyric acid (10,000 mg/kg in PG) Butyric acid (1% in PG) Grated Romano cheese
Capric acid	—	Capric acid (pure)
Caramel	The taste and aromatics associated with burnt sugar or syrup; toffee made from sugar that has been melted further	Condensed milk 3-Hydroxy-2-methyl-4-pyrone (2% in PG)
Caseinate	—	Sodium caseinate powder
Catty	Aroma associated with tom-cat urine	2-Mercapto-2-methyl-pentan-4-one (20 mg/kg)
Cheddary	The taste and aromatics associated with typical Cheddar Typical aroma and taste of sharp/mature Cheddar cheese	Processed cheese Mature Cheddar cheese
Cheese rind	—	Cheese rind (Tilsit mild, pasteurized full fat)
Chemical	An aromatic associated with a broad range of compounds, generally known as chemical which may or may not include chlorine, ammonia, aldehydes, etc.	—
Cooked, cooked milk	Aromatics associated with cooked milk The combination of sweet, brown flavor notes and aromatics associated with heated milk The flavors associated with cooked cheese on toast that has cooled to room temperature	Skim milk heated to 85°C for 30 min Evaporated milk UHT milk 3.6% fat, cooked (10 min) Fluid whole milk
Cottage cheese	-	Cottage cheese 25% fat

Table 17.2 Continued

Term	Definition	Standard
Cow/phenolic	Aromas associated with barns and stock trailers, indicative of animal sweat and waste	<i>p</i> -cresol (160 mg/kg), band-aids
Cow	The characteristics associated with the smell of cowsheds and farm animals; may be described as plastic and rubber	
Creamy	Fatty, creamy tasting, of the nature of, or containing cream	Mascarpone cheese γ -decanolactone (0.1% in PG) UHT cream 35% fat Fresh cream
Cultured	The flavors associated with sour cream and cream cheese	Sour cream, cream cheese
Dairy fat	The oily aromatics reminiscent of milk or dairy fat	Whipping cream Unsalted butter 2% fat milk Whole milk ‘Half and half’
Dairy sour	The sour aromatics associated with dairy soured products	Sour cream
Dairy sweet	The sweet aromatics associated with fresh dairy products	Vitamin D milk
Decaying animal	The aromatics reminiscent of decaying animal material	Dimethyl disulfide (bottom notes only) (10,000 mg/kg in PG)
Diacetyl	Aromatics associated with diacetyl	Diacetyl (20 mg/kg)
Dishcloth	The flavors associated with the aroma of an old used dishcloth	
Earthy	–	Geosmin (0.001% in PG)
Fatty	–	Palm kernel fat
Fecal	Aroma associated with complex protein decomposition	Indole, skatole (20 mg/kg)
Fermented	Combination of sour aromatics associated with somewhat fermented/dairy cheesy notes, that may include green vegetation such as sauerkraut, soured hay, or composted grass	Fermented milk, 12% fat Sauerkraut juice Chardonnay wine
Fermented fruity/winey	The combination of aromatics reminiscent of red wine in general; sweet, slightly brown, overripe, and somewhat sour	Burgundy cooking wine vinegar
Flavor intensity	The overall intensity of flavor in the sample, from mild to strong	
Floral	A sweet aromatic associated with flowers	Oil of geranium, citronellol, linalool

Table 17.2 Continued

Term	Definition	Standard
Free-fatty acid	Aromatics associated with short chain fatty acids	Butyric acid (20 mg/kg)
Fresh fish	The aromatics associated with fresh fish	Elodea (an aquatic plant) growing in water
Fruity	The taste and aromatic blend of different fruity identities The aromatics associated with different fruits	Canned fruit salad (in syrup) Trans-2-hexenal (10,000 mg/kg in PG) Canned fruit cocktail juice 'Fruit of the forest' yoghurt Ethyl butyrate (0.1% in PG) Trans-2-hexenal, 300 mg/kg in Vaseline oil = SS, 3 ml SS + cotton in 60 ml flask Fresh pineapple Ethyl hexanoate (20 mg/kg) Dried fruit
Goaty	The aromatics reminiscent of wet animal hair; tends to be pungent, musty and somewhat sour	Hexanoic acid (5,000 mg/kg in PG) Feta cheese
Grain	A general term used to describe the aromatics associated with grains. It is an overall character that is sweet and brown.	All-purpose flour
Green-grass	—	Cis-3-hexenol (1% in PG)
Green/herbaceous	Fresh green slightly sour aromatics associated with green vegetables, newly cut vines, snap peas	Sprig fresh parsley
Maturity	An overall perception summarizing the extent of flavor and texture development in cheese	
Methyl ketone/bleu	Aroma associated with blue-vein cheeses	2-Octanone (40 mg/kg)
Milkfat/lactone	Aromatics associated with milkfat	Fresh coconut meat Heavy cream δ -dodecalactone (40 mg/kg)
Milky	The aromatics commonly associated with ewes' milk raw	Ewes' milk raw Pasteurized milk, 3.6% fat
Moldy, moldy/musty	The combination of tastes and aromatics generally associated with molds; they usually are earthy, dirty, stale, musty, and slightly sour Aromas associated with molds and/or freshly turned soil	2-Ethyl-1-hexanol (10,000 mg/kg in PG) 2-Ethyl-1-hexanol (40 mg/kg) Stilton cheese 2,4,6 Trichloroanisole (1% in PG)

Table 17.2 Continued

Term	Definition	Standard
Mushroom	The taste and aromatics associated with raw mushrooms	Button mushrooms (raw) Brown mushrooms (chopped, raw) 1-Octen-3-ol (0.5% or 1% in PG) 3-Octanol (10,000 mg/kg in PG) 3-Octanol, 5–10 mg/kg in Vaseline oil = SS, 3 ml SS + cotton in 60 ml flask
Musty	Aroma of a damp room or very old book	Cola infusion in ethanol (pure) Damp room Very old book
Musty/ earthy	A slight musty aromatic associated with raw potatoes and damp humus	Raw sliced button mushroom
Musty/dry	Aromatics associated with closed air spaces such as closets and attics (dry)	2,4,6-trimethoxy benzaldehyde
Nutty	The aromatics reminiscent of several dry fruits such as pecans, walnuts and hazelnuts The non-specific nut-like taste and aromatics characteristic of several different nuts, e.g., peanuts, hazelnuts and pecans The nut-like aromatic associated with different nuts	Wheat germ 2 g walnuts + 2 g hazelnuts, minced in 60 ml flask (mixed particulates to be sampled) Mixed crushed nuts 2-Acetyl-pyridine (0.01% in PG) Lightly toasted unsalted nuts Unsalted wheat thins Roasted peanut oil extract Roasted peanuts, ground hazelnuts, ground almonds, 1:1:1 1000-73 nut base by Givaudan-Roure ¹ (10% in PG)
Overall intensity	Strength of the stimuli perceived by the nose Strength of global stimuli originated by the volatiles released during mastication and perceived on the olfactory receptors via the retronasal way	4 g cheese aroma per 100 ml of pasteurized ewes' milk 0.5–3.5 g cheese aroma per 100 g Quark 91549-24 by Givaudan Roure ¹ 91483-24 by Givaudan Roure ¹ 91428-24 by Givaudan Roure ¹ 91125-73 by Givaudan Roure ¹ 10418-71 by Givaudan Roure ¹
Oxidized	Aroma associated with oxidized fat	2,4-Decadienal, 20 mg/kg
Pineapple	The fruity aromatic associated with pineapple	4-Pentenoic acid (10,000 mg/kg in PG) Canned pineapple chunks

Table 17.2 Continued

Term	Definition	Standard
Prickle/bite	Chemical feeling factor of which the sensation of carbonation on the tongue is typical	Soda water
Processed	A bland, shallow and artificial taste. Made by melting, blending and frequently emulsifying other cheeses	Cheese strings
Propionic acid	–	Propionic acid (1% in PG)
Pungent	A physically penetrating sensation in the nasal cavity. Sharp smelling or tasting, irritating Irritative, burnt and/or penetrating sensation in the interior of the mouth	A ratio of 1 part sour cream to 0.68 parts horseradish sauce Danish blue cheese Ammonia (1% in PG) 0.5 g cayenne per 100 ml water, boiled in water for 5 min, 1.5 ml of filtration per 10 g Quark White vinegar
Rancid	The taste and aroma associated with sour milk and oxidized fats. Having the rank unpleasant aroma or taste characteristic of oils and fats when no longer fresh	Cheese stored at 21°C for 4 days Butyric acid (0.1% in PG)
Rennet	The aromatics associated with natural lamb rennet	Natural lamb rennet (33% NaCl)
Rosy/floral	Aroma associated with flowers	2-Phenethylamine, 20 mg/kg
Salty	Fundamental taste sensation of which sodium chloride is typical Fundamental taste sensation elicited by salts Fundamental taste sensation produced by aqueous solutions of several products such as sodium chloride	Sodium chloride (0.25, 0.5, 0.75 or 1% in water) Pecorino Romano sheep cheese 1200 mg NaCl per 100 g Quark
Sauerkraut	The aromatics associated with fermented cabbage	Dimethyl disulfide (top notes only) (10,000 mg/kg in PG) Sauerkraut juice
Savory	A general term used to describe the savory flavors associated with meat broth, roasted meat and Marmite	
Scorched	Aroma associated with extreme heat treatment of milk proteins	Milk heated to 121°C for 25 min
Sharp	The total impression associated with the combination of aromatics that are sour, astringent, and pungent Total impression of penetration into the nasal cavity The perception associated with aged and ripened cheeses, from flat to sharp	Propionic acid (100,000 mg/kg in PG) 5000 mg/kg of propionic acid in Vaseline oil = SS, 2 ml SS + cotton in 60 ml flask

Table 17.2 Continued

Term	Definition	Standard
Smoky	The penetrating, dark brown, acrid aromatic of charred wood Aroma and taste of hickory smoked ham The penetrating smoky taste and aromatics, similar to charred wood. Tainted by exposure to smoke Perception of any kind of smoke odor (hickory, apple, cherry, mesquite or artificial flavoring)	Oil of cade Hickory smoked ham Applewood cheese Guaiacol (0.5% in PG) Guaiacol in Vaseline oil (several concentrations) Liquid smoke flavoring. 401 + cotton in 60 ml flask
Soapy	A detergent-like taste and smell. Similar to when a food is tainted with a cleansing agent	Lauric acid (pure) Mellow processed Cheddar
Sour	Fundamental taste sensation elicited by acids Fundamental taste sensation of which lactic and citric acids are typical	Citric acid (0.08% in water) Lactic acid (0.05 and 0.085% in water)
Soy sauce	The aromatics that are reminiscent of soy sauce; sour, slightly brown and pungent	Soy sauce
Spicy/ pungent	—	Valerian acid (1% in PG)
Strength	The overall intensity of aroma and flavor, the degree of mildness and maturity	English blue Stilton cheese
Sulfur	Aromatics associated with sulfurous compounds	Boiled mashed egg, H ₂ S bubbled through water, struck match
Sweaty	The aromatics associated reminiscent of perspiration-generated foot odor; sour, stale, slightly cheesy and found in unwashed gym socks and shoes	Isovaleric acid (10,000 mg/kg in PG) Isovaleric acid (0.1% in PG) Isobutyric acid (5% in PG) Cheese stored at 30°C for 3 hours
Sweet	Fundamental taste sensation of which sucrose is typical Fundamental taste sensation elicited by sugars Fundamental taste sensation produced by aqueous solutions of several products such as sucrose or fructose	Sucrose (1, 3, 4, or 5% in water) Condensed milk 1.2 g sucrose per 100 g Quark
Toasty	The combination of sweet aromatics produced after food toasting or cooking	Cooked condensed milk Ciclotene (several concentrations in water)
Umami	Chemical feeling factor elicited by certain peptides and nucleotides	Monosodium glutamate (1% in water)

Table 17.2 Continued

Term	Definition	Standard
Vinegary	Aroma described as acidic, fermented and sweaty by the panelists	Combination of acetic, butyric and propionic acids
Waxy, waxy/crayon	The sweet aromatic that is associated with waxed paper or wax candles Aromatics associated with medium chain fatty acids	Decanoic acid (pure) Capric acid, lauric acid or decanoic acid (100 mg/ml)
Whey	Aromatics associated with Cheddar cheese whey	Fresh Cheddar whey Whey powder
Yeasty	Aromatics associated with fermenting yeast	Raw yeast dough Yeast in 3% warm sucrose water
Yoghurt	–	Yoghurt, 3.2% fat

¹Codes refer to commercially available flavour mixtures that can be provided by Givaudan Roure. Source: adapted from Delahunty and Drake, 2004.

this process, but it can also be part of the descriptive panel training process. Once the preliminary language is generated, definitions and references should be identified for terms. Consultation with flavorists or flavor chemists leads to lexicon terms or references that are not food-specific. In many cases, both food and chemical references are ideal as individuals may best associate with different references and the use of multiple references ensures that the descriptor concept is readily identifiable. Definitions are quite useful but may not always provide a frame of reference for all panelists or accurately or fully describe the concept. For instance, the descriptor ‘rancid/free fatty acid’ in a Cheddar cheese lexicon is defined as the aromatic compounds associated with short chain free fatty acids (Drake *et al.*, 2001). For many panelists unfamiliar with the word or concept, the definition does not necessarily provide a frame of reference. However, when provided with butyric acid (chemical reference) or Feta cheese (food reference), a concept and common point of reference becomes readily grasped by all panelists. Chemical references or ‘anchors’ may require additional research for identification (Drake and Civille, 2003; Singh *et al.*, 2003).

Following identification of the lexicon, sensory panelist training is ready to commence. This phase is critical for maximizing the standardized descriptors for the entire group, which leads to more precise and useful data to describe the product flavor profile. Standard descriptive sensory panel training involves discussion of multiple product samples. Throughout the course of product evaluation and discussion, lexicon terms and definitions are clarified. Redundant terms may be identified and combined or eliminated. Statistical analysis of sensory data may also enhance identification of similar or redundant descriptors (Drake *et al.*, 2001; Drake and Civille, 2003).

It is important to note that lexicons are not finite and that they may change with time as additional products are evaluated and new terms identified. Drake *et al.* (2001) identified a lexicon for Cheddar cheese flavor with 26 flavor attributes. The term mothball/grassy was later added to this lexicon when a large number of international and Farmstead Cheddar cheeses were evaluated (Drake *et al.*, 2005). Further, lexicons by their very nature are mobile and fluid or flexible. Drake *et al.* (2002) demonstrated that an anchored cheese lexicon could be readily applied to sensory panels at multiple locations. Lexicons are flexible, like sensory tests in general, in that the lexicon can be readily adapted to specific objectives. The full array of descriptors can be used for a product set or fewer, simpler descriptors may be used. The ability to use a simpler, less complex language is useful when a specific project objective does not require a more advanced language. Less panel training is required and thus, less time and money is needed. A language of 28 attributes was identified for characterization of Cheddar cheese flavor (Drake *et al.*, 2001). However, a simpler language of 15 attributes readily and precisely differentiates most US Cheddar cheeses made from pasteurized milk (Drake *et al.*, 2001) (Table 17.3). Nine terms adapted from this language can differentiate fresh Chèvre-style goat cheeses (Carunchia-Whetstine *et al.*, 2003) (Table 17.4).

Lexicon descriptors can also be subdivided for more precise information. The Cheddar flavor attribute sulfur can be subdivided into total sulfur, match sulfur, and catty sulfur (Drake *et al.*, 2003), and the attribute brothy can be further subdivided into rosey/floral, beefy/brothy, chicken, and dirty/garbage (Carunchia-Whetstine *et al.*, 2005). Descriptor subdivisions often require extensive panelist training so that panelists consistently recognize flavor nuances between closely related samples. As such, application of the extended lexicon may not be feasible or desirable unless project objectives specifically require it.

17.3 Building a foundation: how the lexicon provides the platform

Once complete, the lexicon is a platform to build and enhance product understanding. It is a tool that delves further into flavor, rather than a finite end product, that allows the flavor to be directed to change specific aspects. One of the primary applications of a defined sensory language is to increase understanding of product flavor and flavor variability. Inherent product variations, processing effects, and storage effects can be effectively compared from a sensory perspective. Understanding flavor variability aids in defining effectively market strategies to develop specific products and an effective approach to control flavor development. Descriptive sensory analysis has been widely used to determine the effect of starter and adjunct cultures on Cheddar cheese flavor (Muir *et al.*, 1996; Drake *et al.*, 1996, 1997; Broadbent *et al.*, 2002, 2004; Banks *et al.*, 1993). The impact of other processing variables such as different milk

Table 17.3 Basic sensory language for Cheddar cheese

Term	Definition	References
Cooked	Aromatics associated with cooked milk	Skim milk heated to 85°C for 30 min
Whey	Aromatics associated with Cheddar cheese whey	Fresh Cheddar whey
Diacetyl	Aromatics associated with diacetyl	Diacetyl (2,3-butanedione)
Lactone	Aromatics associated with milkfat	Fresh coconut meat, heavy cream, δ -dodecalactone
Sulfur	Aromatics associated with sulfurous compounds	Boiled mashed egg, struck match, hydrogen sulfide bubbled through water
Brothy	Aromatics associated with boiled meat or vegetable stock	Knorr beef broth cubes, Knorr vegetable broth cubes, Wyler's low-sodium beef broth cubes, canned potatoes
Free fatty acid	Aromatics associated with short chain fatty acids	Butanoic acid
Fruity	Aromatics associated with different fruits	Fresh pineapple, canned pineapple juice
Nutty/malty	The nut-like aromatic associated with different nuts	Lightly toasted unsalted nuts, 2/3-methyl butanal
Catty	Aromatics associated with tom-cat urine	2-Mercapto-2 methyl-pentan-4-one, 20 ppm
Sweet	Fundamental taste sensation elicited by sugars	Sucrose (5% in water)
Salty	Fundamental taste sensation elicited by salts	Sodium chloride (0.5% in water)
Sour	Fundamental taste sensation elicited by acids	Citric acid (0.08% in water)
Bitter	Fundamental taste sensation elicited by caffeine, quinine	Caffeine (0.08% in water)
Umami	Fundamental meaty taste elicited by monosodium glutamate (msg)	Monosodium glutamate (1% in water)

Source: adapted from Drake *et al.*, 2001.

sources, heat treatment and/or flavoring can also be compared (Retiveau *et al.*, 2005; Rehman *et al.*, 2003a, 2003b, 2003c).

Parameters evaluated can be as diverse as regional differences in Cheddar cheese to possible flavor gradations across a 291 kg industrial block of Cheddar cheese. A defined lexicon is the key to define flavor differences or variation among cheeses. Carunchia-Whetstine *et al.* (2006) used a defined cheese sensory language to document the effect of fat removal from fully aged blocks of Cheddar cheese. Removal of 50% of the fat from aged Cheddar cheese

Table 17.4 Goat cheese sensory lexicon and references adapted from the basic Cheddar cheese sensory language

Term	Definition	References
Cooked/ milky	Aromatics associated with cooked milk	Skim milk heated to 85°C for 30 min
Whey	Aromatics associated with Cheddar cheese whey	Fresh Cheddar whey
Diacetyl	Aromatics associated with diacetyl	Diacetyl
Milkfat/ lactone	Aromatics associated with milkfat	Fresh coconut meat, heavy cream, δ -dodecalactone
Waxy/ animal	Waxy/crayon-like aromatic primarily associated with cheeses (and other dairy products) made from goat or sheep milk	4-Methyl octanoic acid and 4-ethyl octanoic acid, 100 ppb of each in MeOH in a sniffing jar
Brothy	Aromatics associated with boiled meat or vegetable stock	Knorr beef broth cubes, Knorr vegetable broth cubes, canned potatoes
Sweet	Fundamental taste sensation elicited by sugars	Sucrose (5% in water)
Salty	Fundamental taste sensation elicited by salts	Sodium chloride (0.5% in water)
Sour	Fundamental taste sensation elicited by acids	Citric acid (0.08% in water)

Source: adapted from Carunchia-Whetstone *et al.*, 2003.

slightly impacts the sensory perception of many specific cheese flavors and the removed fat, itself, displayed very little flavor (Table 17.5). Such detailed information on the specific sensory perception of cheese flavor is required in order to determine the effect of processing changes.

17.4 Flavor chemistry linkages

Identification of organic compounds that contribute to flavor is an important area of research and is covered elsewhere in this book. Generally, most compounds that contribute to flavor are volatile and extensive instrumental analysis is required to extract, separate, and identify these components. Care must be taken to select the appropriate volatile extraction technique as no technique recovers all volatile components equally. Further, only a small percentage of the volatile components in a food are odor-active (Friedrich and Acree, 1998; Singh *et al.*, 2003). Relative amounts of compounds in foods are not necessarily a measure of their sensory impact due to different thresholds and the effects of the food matrix. Long lists of volatile components extracted from cheeses and identified using gas chromatography coupled to a mass spectrometer (GC-MS)

Table 17.5 Application of a defined sensory language to document the effect of fat removal on specific sensory properties of Cheddar cheese¹

Attribute	39 months full fat	39 months full fat reformed	39 months cheese fat	39 months reduced fat
Cooked ²	2.41 ^{ab}	2.6 ^a	ND ³	2.16 ^b
Whey	ND	ND	ND	ND
Diacetyl	ND	ND	ND	ND
Milkfat/lactone	2.59 ^a	2.48 ^a	ND	1.74 ^b
Fruity	1.17 ^b	0.70 ^c	1.76 ^a	ND
Sulfur	2.11 ^a	1.97 ^a	0.50 ^b	1.78 ^b
Free fatty acid	ND	ND	ND	ND
Brothy	3.05 ^b	2.85 ^b	1.29 ^c	2.93 ^b
Rosy/floral	ND	ND	ND	ND
Nutty	1.31 ^{cd}	0.88 ^d	1.48 ^c	1.38 ^c
Catty	0.80 ^a	ND	0.90 ^a	ND
Sour	3.25 ^{abc}	3.00 ^c	ND	3.13 ^{bc}
Sweet	2.54 ^b	2.63 ^b	ND	2.60 ^b
Salty	4.06 ^{cd}	3.93 ^d	ND	4.65 ^{ab}
Bitter	ND	ND	ND	ND
Umami	2.72 ^a	2.56 ^{ab}	ND	2.31 ^{ab}

¹ Intensities are scored on the 15-point universal intensity Spectrum™ scale where 0 = none and 15 = very high (Meilgaard *et al.*, 1999; Drake *et al.*, 2001).

² Means in a row followed by different letters are different ($P < 0.05$).

³ ND = no data.

Source: Carunchia-Whetstine *et al.*, 2006 (with permission).

are useful in their own right, but this information tells us very little about flavor unless sensory analysis with a defined sensory language has been conducted to fully characterize the sensory perception of the cheese.

GC-sniffing or gas chromatography-olfactometry (GC-O) is a sensory technique used to identify compounds that are aroma-active in a food or cheese extract. The GC effluent is split between the instrumental detector and a sniffing port where an individual records the aroma character and its retention time and intensity. Several approaches can be used for GC-O (Van Ruth, 2001), but the primary goal is to identify and characterize the dominant volatile components in a food from the total number of volatile compounds (not all of which contribute to flavor). As such, the technique can be used to identify all of the volatile components in a food or screen for compounds that might be responsible for specific desirable or undesirable flavors. For products such as cheese, many of the volatile compounds that contribute to flavor are also found in other dairy products while other compounds are unique to the cheese and its microflora, processing and ripening conditions (Table 17.6). Again, this information is useful and one step of several to link instrumental analysis to sensory analysis. Individual aroma character is not necessarily indicative of the flavor the compound causes in the food product (Avsar *et al.*, 2004; Drake *et al.*, 2005b). Concentration, sensory threshold, the presence of other compounds, and the

Table 17.6 Aroma-active components in various dairy products determined by gas chromatography–olfactometry (GC–O) of solvent extracts

No.	Compound	Phase ¹	Odor ²	Compound was present in samples ³									RI ⁴		Method of identification ⁵
				Fresh SMP	Stored SMP	Fresh WMP	Stored WMP	WPC 80	WPI	Young Cheddar cheese	Aged Cheddar cheese	Chevre style goat cheese	DB-5	DB-WAX	
1	acetic acid	Ac	vinegar	+	+	+	+	+	+	+	+	+	685	1424	RI, odor, MS
2	2,3-butanedione	N/B	buttery	+	+	+	+	+	+	+	+	+	680	955	RI, odor, MS
3	2/3-methyl butanal	N/B	malty/chocolate	-	-	-	+	-	-	-	+	-	686	925	RI, odor, MS
4	ethyl butanoate	N/B	fruity/solvent	-	+	-	+	-	-	-	+	-	730	1000	RI, odor, MS
5	3-hydroxy-2-butanone	N/B	buttery	-	-	-	-	+	-	-	+	+	730		RI, odor
6	methyl-2-butanoate	N/B	butterscotch	-	-	-	-	+	-	-	-	+	787	1006	RI, odor
7	dimethyl disulfide	N/B	garlic/rubbery	-	-	-	-	+	-	-	-	-	777	1071	RI, odor
8	2-methyl thiophene	N/B	plastic	+	+	+	-	+	+	-	+	+	800	1026	RI, odor
9	hexanal	N/B	green grass	+	+	+	+	+	+	-	+	+	803	1051	RI, odor, MS
10	unknown	N/B	skunk	-	+	+	+	+	-	-	+	-	827	1067	odor
11	unknown	N/B	ammonia	+	+	+	+	+	-	-	-	+	835	1140	odor
12	o-xylene	N/B	geranium	-	-	-	-	-	-	-	-	+	837	1140	RI, odor
13	1-hexen-3-one	N/B	cooked/vegetable	-	-	-	-	-	-	-	-	+		1153	RI, odor
14	butanoic acid	Ac	cheesy/rancid	+	+	+	+	+	+	+	+	+	840	1650	RI, odor, MS
15	unknown	N/B	fruity/solvent	-	+	-	+	+	+	-	+	-	856	1072	RI, odor
16	2-methyl-3-furanthiol	N/B	mushroom/beefy	-	+	-	+	+	-	+	+	+	883	1218	RI, odor
17	propionic acid	Ac	Swiss cheese	-	-	+	-	-	-	-	-	-	883	1495	RI, odor, MS
18	Z-4-heptenal	N/B	fatty/fishy	+	+	+	+	+	+	-	+	+	905	1220	RI, odor
19	methional	N/B	potato	+	+	+	+	+	+	+	+	+	915	1433	RI, odor, MS
20	2/3-methyl butanoic acid	Ac	sweaty/dried apricots	+	+	+	+	-	-	+	+	-	925	1527	RI, odor, MS
21	pentanoic acid	Ac	sweaty	+	+	+	+	+	+	+	+	+	930	1043	RI, odor, MS
22	heptanal	N/B	mushroom/fatty	-	-	-	-	+	+	-	-	+	926	1254	RI, odor, MS

Table 17.6 Continued

No.	Compound	Phase ¹	Odor ²	Compound was present in samples ³									RI ⁴		Method of identification ⁵
				Fresh SMP	Stored SMP	Fresh WMP	Stored WMP	WPC 80	WPI	Young Cheddar cheese	Aged Cheddar cheese	Chevre style goat cheese	DB-5	DB-WAX	
23	methyl hexanoate	N/B	citrus/fruity	-	-	-	-	-	-	-	+	-	938	1118	RI, odor
24	2-acetyl-1-pyrroline ⁵	N/B	popcorn	+	+	+	+	+	+	-	+	+	939	1317	RI, odor
25	dimethyl trisulfide	N/B	cabbage	-	+	-	-	+	+	-	+	-	960	1362	RI, odor
26	2-pentanol	N/B	minty	-	-	-	-	-	-	-	-	+	959	1120	RI, odor
27	1-octen-3-one	N/B	mushroom	+	+	+	+	+	+	+	+	+	980	1285	RI, odor
28	ethyl hexanoate	N/B	fruity	-	-	-	-	-	-	-	+	-	996	1221	RI, odor, MS
29	(Z)-1,5-octadien-3-one	N/B	geranium	+	+	-	+	+	+	-	-	+	997	1312	RI, odor
30	(E,E)-2,4-heptadienal	N/B	fatty	+	+	-	+	-	-	-	-	-	1001	1345	RI, odor
31	octanal	N/B	citrus/green	+	+	+	+	+	+	+	+	+	1005	1275	RI, odor, MS
32	hexanoic acid	Ac	sweaty	+	+	+	+	+	+	+	+	+	1019	1850	RI, odor, MS
33	2-acetyl thiazole	N/B	nutty	+	+	+	+	+	-	-	+	+	1043	1588	RI, odor
34	heptanoic acid	Ac	sweaty	-	-	-	+	-	-	-	-	+		1890	RI, odor, MS
35	phenylacetaldehyde	N/B	rosy	+	+	+	+	+	+	+	+	-	1044	1619	RI, odor, MS
36	unknown	N/B	coconut	-	-	-	+	-	-	-	-	-	1060		odor
37	(E)-2-octenal	N/B	citrus/fatty	+	+	-	+	+	+	-	-	+	1070	1345	RI, odor, MS
38	<i>p</i> -cresol	N/B	cowy/phenolic	-	+	-	+	-	+	-	+	-	1088	2123	RI, odor
39	2,5-dimethyl-4-hydroxy-3-(2H) furanone (Furaneol TM)	Ac	burnt sugar	+	+	-	-	+	+	-	+	-	1072	2047	RI, odor
40	2-isobutyl-3-methoxypyrazine	N/B	bell pepper /burnt	+	+	-	+	+	+	+	+	+	1082	1403	RI, odor
41	2-methoxy phenol (guaiacol)	N/B	smoky	+	+	+	+	+	+	+	+	+	1095	1464	RI, odor
42	nonanal	N/B	fatty/citrus	+	+	+	+	+	+	+	+	+	1098	1385	RI, odor, MS
43	unknown	N/B	rosy	-	-	-	-	-	+	-	-	-	1101		odor
44	unknown	N/B	metallic/beefy	-	-	-	-	-	-	+	+	-	1105	1305	odor
45	2-acetyl-2-thiazoline	N/B	popcorn	-	+	+	+	-	+	+	+	+	1106	1763	RI, odor
46	hydroxymethyl-pyrone (maltol)	Ac	burnt sugar	-	+	+	-	-	-	-	-	+	1113	1459	RI, odor

47	(E,E)-2,4-octadienal	N/B	fatty	-	-	+	+	+	+	+	+	-	1846		odor
48	3-hydroxy-4,5-dimethyl-2(5H)-furanone (sotolon)	Ac	maple/spicy	+	+	+	+	+	+	+	+	+	1120	2210	RI, odor
49	(Z)-2-nonenal	N/B	fatty/green	-	-	+	+	-	-	+	+	-	1126		odor
50	2-ethyl-4-hydroxy-5-methyl-3(2H)-furanone (homofuraneol)	Ac	brunt sugar	+	-	+	+	-	-	-	+	+	1142	2058	RI, odor
51	2-phenethanol	N/B	rosy	+	+	+	+	+	+	+	+	+	1150	1873	RI, odor
52	(E,Z)-2,6-nondienal	N/B	cucumber	+	+	-	-	+	+	+	+	+	1160	1555	RI, odor
53	(E)-2-nonenal	N/B	cucumber/ old books	+	+	+	+	+	+	+	+	+	1168	1582	RI, odor, MS
54	2-me-3-methyldithio-furane	N/B	cooked/ rubbery	+	+	-	+	+	+	-	-	+	1179	1520	RI, odor
55	o-cresol	N/B	plastic/ phenolic	-	-	-	+	-	+	-	-	-	1188		RI, odor
56	butyl hexanoate	N/B	floral/citrus	+	+	-	+	+	+	+	+	-	1203	1536	RI, odor
57	(E,E)-2,4-nonadienal	N/B	fatty	+	+	+	+	+	+	+	+	+	1217	1609	RI, odor
58	decanal	N/B	fatty	-	+	+	+	+	+	+	+	+	1223	1483	RI, odor, MS
59	benzothiazole	N/B	plastic/rubber	-	+	+	+	-	-	-	-	+	1250	1572	RI, odor, MS
60	phenylethyl acetate	N/B	rosy	+	+	-	+	+	+	+	+	+	1255	1820	RI, odor
61	phenyl acetic acid	Ac	honey	-	+	-	+	+	+	+	+	+	1260	1602	RI, odor
62	indole	N/B	musty/fecal	-	-	-	-	-	-	-	-	+	1263	1796	RI, odor
63	unknown	N/B	cilantro	-	-	+	+	+	+	+	+	+	1268		odor
64	(E)-2-decenal	N/B	fatty	+	+	+	-	-	-	-	-	+	1276	1585	RI, odor
65	unknown	N/B	oatmeal	-	-	-	-	+	+	+	+	+	1280		odor
66	̑-octalactone	N/B	coconut	+	+	+	+	+	+	+	+	-	1287	1924	RI, odor, MS
67	octanoic acid	Ac	sweaty/waxy	+	+	+	+	+	+	-	-	+	1307	2343	RI, odor, MS
68	undecanal	N/B	medicinal/ fatty	+	+	-	+	+	+	+	+	+	1305		RI, odor
69	̒-butyrolactone	N/B	sweet	-	-	-	-	+	-	-	-	-	1314	1653	RI, odor
70	(E,Z)-2,4-decadienal	N/B	fatty	+	+	-	-	-	+	-	-	-	1316	1621	RI, odor
71	o-aminoacetophenone	N/B	grape/tortilla	+	+	+	+	+	+	+	+	+	1320	2223	RI, odor
72	(E,E)-2,4-decadienal	N/B	fatty/ oxidized	+	+	+	+	+	+	+	+	+	1330	1710	RI, odor, MS
73	4-methyl octanoic acid	Ac	waxy/animal	+	+	-	+	+	+	-	+	+	1359	2173	RI, odor
74	̒-nonalactone	N/B	coconut	+	+	-	+	+	+	+	+	+	1360	2011	RI, odor

Table 17.6 Continued

No.	Compound	Phase ¹	Odor ²	Compound was present in samples ³									RI ⁴		Method of identification ⁵
				Fresh SMP	Stored SMP	Fresh WMP	Stored WMP	WPC 80	WPI	Young Cheddar cheese	Aged Cheddar cheese	Chevre style goat cheese	DB-5	DB-WAX	
75	5-ethyl-(3H)-furan-2-one	N/B	sweet/tobacco	-	-	-	-	+	+	-	-	+	1380	1694	RI, odor
76	β -damascenone	N/B	floral/fatty	-	+	-	+	-	+	-	-	+	1387	1765	RI, odor, MS
77	dodecanal	N/B	citrus/fatty	+	+	-	-	+	+	-	-	-	1400	1762	RI, odor, MS
78	unknown	N/B	paper/metallic	+	+	-	+	+	+	+	+	-	1425	1938	odor
79	3-methyl indole (skatole)	N/B	fecal/mothball	+	+	+	+	+	+	+	+	+	1425	1885	RI, odor
80	3-methoxy-4-hydroxy-benzaldehyde (vanillin)	Ac	vanilla	+	+	-	+	+	+	-	+	+	1435	1892	RI, odor
81	4-ethyl-octanoic acid	Ac	waxy/animal	-	-	-	-	-	-	-	-	+	1438	2216	RI, odor
82	unknown	N/B	peach	-	-	-	-	+	+	-	-	+	1443		odor
83	unknown	Ac	waxy	-	-	-	-	+	+	-	-	-	1454	2216	RI, odor
84	δ -decalactone	N/B	coconut	+	+	+	+	+	+	+	+	+	1481	1990	RI, odor, MS
85	γ -decalactone	N/B	peach	+	+	+	+	+	+	+	+	+	1508	2103	RI, odor
86	γ -octalactone	N/B	coconut	+	+	+	+	+	-	-	+	+	1547		RI, odor, MS
87	tridecanol	N/B	wet dog/musty	-	-	-	-	+	+	-	-	-	1593	1952	RI, odor, MS
88	6-(Z)-dodecen- γ -lactone	N/B	soapy/sweet	+	+	-	+	+	+	+	+	-	1630	1972	RI, odor
89	γ -dodecalactone	N/B	coconut	+	+	+	-	-	-	-	-	-	1664	2399	RI, odor, MS
90	δ -dodecalactone	N/B	peach	+	+	+	+	+	+	+	+	+	1675		RI, odor, MS
91	unknown	N/B	plastic/soap	-	-	-	-	+	+	-	-	-	1789		odor
92	nonanoic acid	Ac	sweaty/waxy	+	+	-	-	+	+	+	+	+		2072	RI, odor
93	decanoic acid	Ac	sweaty/waxy	-	-	-	-	-	-	-	+	+		2286	RI, odor, MS

¹ Ac = acidic fraction of solvent extract, N/B = neutral basic fraction of solvent extract (Carunchia-Whetstine *et al.*, 2006).

² Odor description at the GC-sniffing port.

³ Compound was detected in the samples by GC/O.

⁴ Retention indices were calculated from GC/O data.

⁵ Compounds were identified by comparison with the authentic standards on the following criteria: retention index (RI) on DB-WAX and DB-5MS columns, odor property at the GC-sniffing port, and mass spectra in the electron impact mode. Positive identifications indicate that mass spectral data was compared with authentic standards.

components of the food matrix play a crucial role, which makes sensory analysis an absolute necessity in flavor chemistry investigations.

When characterizing volatile components of a particular cheese, rather than a specific flavor, in a model system analysis with key compounds can be used with a model subtraction ($N - 1$) and model addition ($N + 1$) approach with trained sensory panelists and a defined sensory language. This method provides further confirmation that the compounds do represent the flavor of the product, not simply food-associated descriptions. This approach has proven to be very robust to identify the key volatile compounds responsible for the flavor of many foods including strawberry, raspberry, rye bread, citrus fruits and milk powder (Roberts and Acree, 1996; Kirchoff and Schieberle, 2001, 2002; Buettner and Schieberle, 2001a, 2001b; Schieberle and Hofmann, 1997; Karagül-Yüceer *et al.*, 2004) but is less successful with the complex flavor of cheeses, particularly Cheddar (Preininger *et al.*, 1996; Warmke *et al.*, 1996; Dacremont and Vickers, 1994; House and Acree, 2001; Yang and Vickers, 2004).

Despite the limitations of instrumental analysis in terms of its linkage to sensory perception in regards to total cheese flavor, the use of a defined sensory language for cheese flavor provides the key to establish links between specific flavors and volatile components (Singh *et al.*, 2003; Drake *et al.*, 2007). The general approach to linking sensory and analytical data is comprised of three basic steps: (1) selection of products with the desired or target flavors using descriptive sensory analysis; (2) instrumental volatile analysis; and (3) confirmation of volatile compounds using quantitation, threshold analysis, and descriptive sensory analysis of model systems. When both instrumental and sensory analysis techniques are used optimally, the results are mutually beneficial. Table 17.7 provides a list of Cheddar cheese flavors that are linked to volatile components

Table 17.7 Cheddar cheese flavors that have been linked to specific volatile components through exhaustive instrumental and sensory analyses. A defined sensory language for Cheddar cheese flavor was used throughout

Sensory flavor	Volatile compounds responsible	Reference
Nutty	Strecker aldehydes	Avsar <i>et al.</i> , 2004
Rosey/floral	Phenylacetaldehyde, phenyl acetic acid	Carunchia-Whetstine <i>et al.</i> , 2005
Beefy/brothy	Methional, furaneol, 2-methyl-3-furanthiol	Cadwallader <i>et al.</i> , 2006
Cow/barny	<i>p</i> -Cresol	Suriyaphan <i>et al.</i> , 2001
Earthy/bell pepper	2-Propyl-3-methoxy pyrazine, 2-isobutyl-3-methoxy pyrazine	Suriyaphan <i>et al.</i> , 2001
Waxy/crayon	4-Methyl and 4-ethyl octanoic acids	Carunchia-Whetstine <i>et al.</i> , 2003
Bitter taste	Peptide β -casein fragment 193-209 (β -CN f193-209)	Singh <i>et al.</i> , 2005

using this approach. Once a link has been established using both sensory and instrumental techniques, additional research in cheese biochemistry can be conducted to determine methods of control over flavor generation.

17.5 Understanding the consumer

Understanding consumer perception of cheese flavor is crucial for effective marketing and product development. Affective sensory tests, which measure consumer responses, can provide information on consumer likes and dislikes. Internal preference mapping (e.g., principal component analysis of consumer data) is used to visualize consumer responses for product-specific differences. However, they are not quantitative for specific flavor profiles or consumer responses. Differences documented by trained panelists are not necessarily relative to or indicative of consumer likes and dislikes. Consumers may use terms that are ambiguous, have multiple meanings, are associated with 'good' or 'bad' or are combinations of several more specific terms in the lexicon. For example, if consumers like or dislike a cheese, we do not know specifically why unless flavor profiles of these products are determined with a trained sensory panel using a defined lexicon.

Rarely are consumer perceptions straightforward to associate those data with specific lexicon terms. An examination of hedonic responses from consumers and descriptive data may clearly indicate why specific products are liked or disliked. Carunchia-Whetstone *et al.* (2006) conducted descriptive analysis using a defined sensory language using aged Cheddar cheese with the fat removed after aging (Table 17.5). Trained sensory panel flavor profiles indicated that the flavor of the full and reduced fat cheeses was not different and that the cheese fat contributed very little flavor to the end product. These cheeses were subsequently presented to 75 consumers (Fig. 17.1). Consumers agreed with the trained panelists in that flavor intensity of the cheeses was not different ($p < 0.05$). However, scores for consumer-liking preferences were significantly different ($p < 0.05$) based on texture between the three cheeses tested. Additionally, written comments provided by 45/75 consumers indicated a 'strange' or 'different' texture in the cheeses with the fat removed. A defined cheese flavor language with trained panel profiling in conjunction with consumer testing indicated that texture, not flavor, was the driving force in product dislike.

Often, defining consumer desires requires additional work beyond descriptive profiling. Combining descriptive and hedonic data requires a statistical technique called external preference mapping. Using this method allows consumer market segments to be linked with specific target flavor profiles, thereby clearly defining consumer likes and dislikes.

Lawlor and Delahunty (2000) determined consumer preferences for 10 speciality Irish cheeses using external preference mapping. Diverse flavor differences were observed among the different cheese varieties. Seven distinct

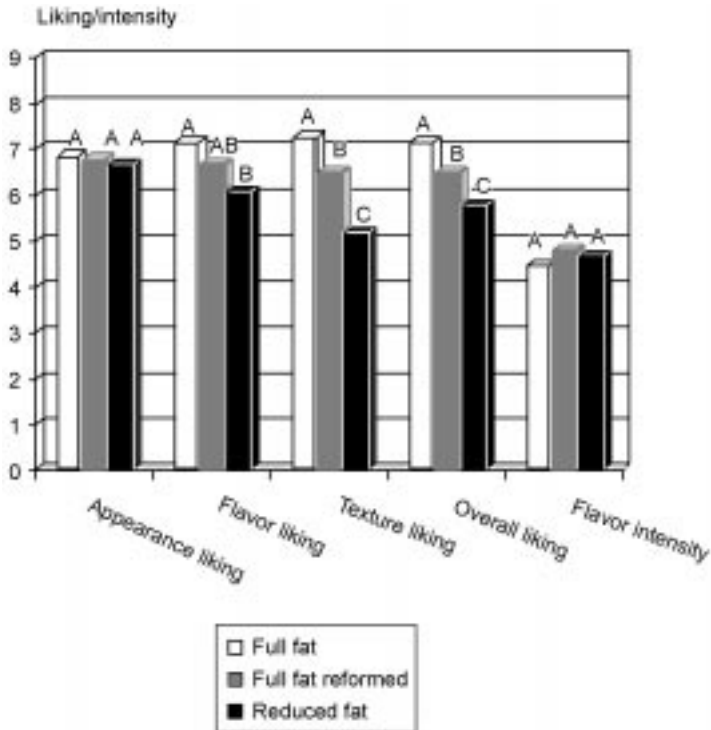


Fig. 17.1 Consumer evaluation of full fat, full fat reformulated, and reduced fat cheeses ($n = 75$). A 9-point hedonic scale was used where 1 = dislike extremely and 9 = like extremely for appearance liking, flavor liking, texture liking, and overall acceptability. A 7-point scale was used where 1 = not intense and 7 = very intense for flavor intensity. Different letters indicated significant differences for all attributes ($P < 0.05$). Cheese flavor was profiled using a defined sensory language and a trained panel (Table 17.5). Taken from Carunchia-Whetstine *et al.* (2006).

consumer clusters were identified and related to their respective cheese sensory profiles. Murray and Delahunty (2000c) conducted preference mapping with factory and farmstead Cheddar-type cheeses. Again, a wide variety was observed in descriptive flavor profiles of cheeses and in distinct consumer preference clusters. Young *et al.* (2004) conducted preference mapping with seven Cheddar cheeses produced in the United States. Trained panelists documented precise flavor profiles of each cheese using a defined lexicon. A wide variability in cheese preferences within one specific type of cheese, Cheddar cheese, was documented. The concept of 'Cheddar cheese' flavor varied widely among American consumers. Six distinct consumer clusters were identified (Fig. 17.2) and the number of consumers in these clusters differed between two geographic locations within the United States. This precise information about consumer preferences would not be possible without a defined sensory language.

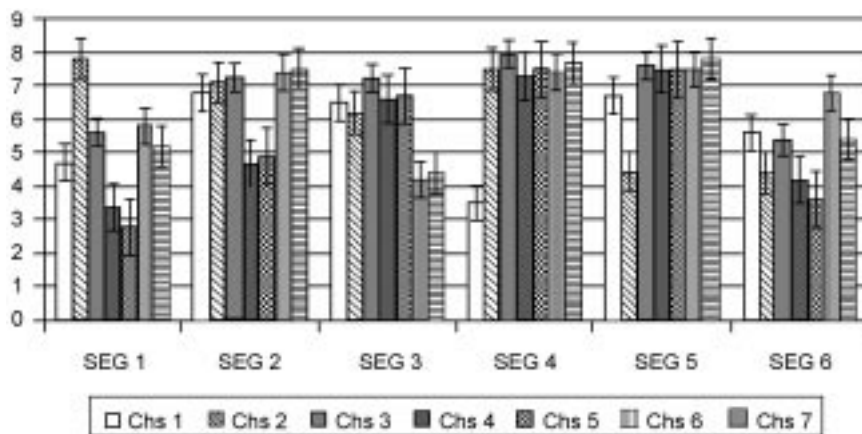


Fig. 17.2 External preference map of combined consumer data ($n = 240$) with descriptive analysis results for seven different Cheddar cheeses. Six consumer segments are identified. Adapted from Young *et al.* (2004).

17.6 A global perspective

Recent work has emphasized the need for a uniform language for international communication and marketing. Using one lexicon, Drake *et al.* (2002) conducted ring trials with Cheddar cheese at three locations in the United States to demonstrate that trained panels using the same language produce similar results. While overall differences were consistent between the locations, subtle differences between locations were noted and attributed to differences in panel training and panelist leadership. Application and calibration of panels at multiple locations is a challenge within a single country.

These hurdles are pronounced when different cultures or languages are involved. Hirst *et al.* (1994) and Risvik *et al.* (1992) used descriptive analysis with hard cheeses at two different international sites to evaluate cross-cultural differences in sensory evaluation of cheese. They noted cross-cultural differences and similarities. Consistent sensory evaluation of hard cheese in the European Union (EU) was addressed. Ring trials at seven different sites across the EU were conducted and a core sensory language was developed (Hunter and McEwan, 1998; Nielsen and Zannoni, 1998). Drake *et al.* (2005) conducted ring trials with Cheddar cheese in the United States, New Zealand, and Ireland. Each location had its own defined anchored sensory language and a trained panel experienced with the Cheddar cheese lexicon. Cheeses were differentiated in a similar manner by panels at each location. Some similar descriptors were identified between the three lexicons, but for the most part, attributes were applied to cheese differently even when the written descriptor appeared identical. In some cases, the actual references for the descriptor were quite distinct, providing further evidence of the importance of references in addition to descriptor definitions. In other cases, the descriptors were simply applied in a different manner by the panels at the

different locations. These examples highlight that cultural differences and experiences play a role in cheese flavor lexicons.

Trained sensory panels using lexicons produce identical product differentiation on sensory profiling of products. Future work with ring trials, consumers in different countries, and discussions with panels and panel leaders in different countries can aid in the characterization of cultural differences in language and language application and provide further clarification and progress towards a single global language. Development of a global defined sensory language is a distinct possibility and future research efforts should address this global marketing and communication need.

17.7 Future trends

Defined sensory languages for cheese are crucial tools for marketing and research. Vital information on product flavor, flavor variability, and flavor stability is obtained. Further, links to chemical components and understanding of consumer concepts can be established. Finally, their use can enhance global communication. Defining cheese flavor using descriptive sensory analysis is the key to a comprehensive understanding of cheese flavor.

17.8 Sources of further information and advice

Fortunately, there are several reputable and peer-reviewed journals that regularly publish the latest findings on cheese flavor research. These journals include dairy product-specific journals such as the *Journal of Dairy Science* and the *International Dairy Journal* as well as journals that are not specific to dairy foods such as the *Journal of Sensory Studies*, the *Journal of Food Science and Agricultural and Food Chemistry*. There are also several scientific conferences and meetings that are major outlets for current cheese flavor research. These conferences are sponsored by organizations such as the International Dairy Federation (www.fil-idf.org), the American Dairy Science Association (www.adsa.org) and the International Food Technologists (www.ift.org). More detailed information can be found by viewing their specific websites.

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18

Measuring cheese flavor

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18.1 Introduction

Improvement of cheese flavor is of great economic importance since flavor is a major determinant of consumer choice and acceptance, and because development of cheese flavor during ripening is a slow and expensive process. Flavor formation in cheese is a complex process that involves numerous microbiological and biochemical reactions including glycolysis, lipolysis and proteolysis. These processes lead to the formation of numerous compounds that provide aroma (odor) and flavor (aromatics and taste) properties to cheese products.

Most often it is the aroma components that are the predominant determinants of the characteristic flavor of cheese products. It is for this reason that most cheese flavor research has focused on the analysis of the volatile aroma components, since an understanding of the aroma chemistry leads to better control of the manufacturing and aging processes to suppress formation of undesirable aroma compounds and enhance the formation of desirable ones.

The first and often most critical step in aroma analysis is the isolation of the volatile compounds from the nonvolatile matrix components. Following isolation, instrumental methods of analysis, such as gas chromatography (GC), are employed to separate, identify and quantify the various volatile components of the isolate. In some cases, combined sensory–instrumental methods, such as GC–olfactometry (GCO), are used to indicate important contributors to the characteristic aroma of the product. This chapter focuses on procedures commonly used for the isolation and extraction of volatile compounds and the analytical methodology used for determining the volatile aroma profiles of cheese products.

18.2 Isolation of volatile components

The analysis of volatile compounds in cheese products is a complicated process due to the presence of only minute amounts of volatile solutes in a highly complex nonvolatile matrix. Furthermore, cheese volatile compounds exist as various chemical classes, such as acids, ketones, aldehydes, alcohols, etc., and are subject to further chemical breakdown due to oxidation and thermal decomposition. Because of this the volatile isolation procedure is usually the most critical step in the analysis. The isolation of volatile constituents may be accomplished by considering the differences in chemical properties between the volatile compounds and non-volatile cheese matrix components. Generally, this means taking advantage of the higher volatility and/or the relative non-polar nature of the volatile components. Methods that depend on the volatile nature of the aroma components include headspace and distillation techniques. Meanwhile, solvent extraction and adsorption methods rely on the relative nonpolar nature of the volatile compounds to isolate them from the product matrix. Often it is useful to take advantage of both volatility and polarity differences by combining two processes in the isolation step, such as combined headspace–adsorption and extraction–distillation methods.

When considering a volatile isolation procedure it needs to be understood that there is no single ‘perfect’ method and all volatile isolation techniques will impart some degree of sampling bias into the resulting GC volatile profile (Etiévant, 1996; Reineccius, 2006). For greatest accuracy the method chosen should maintain sample integrity, minimize loss of labile (sensitive) aroma compounds, and isolate all volatile compounds to the same degree. Most often it is best to use two or more complementary isolation methods that are based on different separation criteria. In this way, the sampling bias of each method is accounted for in the final result. Methods most commonly employed in the analysis of volatile constituents of cheese products are discussed here.

18.2.1 Headspace methods

All aroma compounds possess some degree of volatility. Headspace isolation techniques take advantage of this property by monitoring the gaseous headspace above a liquid or solid product in a sealed container. The methods are therefore limited to compounds that partition into the gas phase. Headspace methodologies, including static headspace, dynamic headspace and purge-and-trap, have been reviewed (Hinshaw, 1990; Cole and Woolfenden, 1992; Wampler, 2002). Some unique advantages of headspace methods are that they provide some indication of the composition of the volatiles above the food (headspace aroma composition), they are nondestructive (mild conditions), and minimal sample preparation is required.

Static headspace analysis

Static headspace analysis (SHA) is in principle the simplest among the headspace analysis techniques. A unique attribute of SHA is that it provides

some indication of the aroma composition encountered when one directly smells a food product. In SHA the food product (liquid or solid) is contained in a closed vessel (typically a vial is used), and the volatile components are allowed to come into equilibrium between the sample matrix and the surrounding headspace. An aliquot of the headspace is then withdrawn and injected into a GC for analysis. SHA results are influenced by numerous parameters, such as temperature, sample and container size, ratio of sample to headspace volume, nature of the sample matrix, addition of matrix modifiers (salting-out) and whether or not the sample is stirred or agitated during equilibration. Advantages of SHA include simple sample preparation, elimination of reagents (no solvent peak during GC analysis) and low risk of artifacts; however, SHA is generally limited to products that contain appreciable amounts of highly volatile 'headspace' components. SHA has been used to only a limited extent for cheese volatile analysis due to two main limitations: (1) the method lacks sufficient sensitivity, since most cheeses do not contain appreciable levels of highly volatile constituents; and (2) the matrix of cheese is very complex, making it difficult to accurately standardize the method. SHA may be a good choice for targeted analyses of some highly volatile components of cheese, such as acetaldehyde, hydrogen sulfide, methanethiol, dimethylsulfide and low molecular weight Strecker aldehydes (e.g. methylpropanal, 2-methylbutanal and 3-methylbutanal). SHA has been used for the analysis of total headspace volatiles (Fernández-García, 1996) and short-chain free fatty acids (Tungjaroenchai *et al.*, 2004) in cheese. In recent years, SHA combined with solid-phase microextraction (H-SPME, discussed on page 404) has become a popular method for isolation and analysis of cheese volatiles.

Dynamic headspace analysis

The efficiency and sensitivity of headspace analysis is greatly improved by use of an intermediate trapping or adsorption step to enrich the volatile constituents prior to GC analysis. This technique is commonly referred to as dynamic headspace analysis (DHA) or purge-and-trap analysis and is a useful method for samples that contain low levels of headspace volatile components. In DHA, an inert gas (nitrogen or helium) is swept over or through a thermostatted sample (contained in a closed vessel) for a period of time sufficiently long enough to 'extract' most of the volatile constituents. During this process the volatiles are enriched by trapping onto adsorbent materials (porous polymers or charcoal) or by cryogenic focusing. Adsorbent trapping is most commonly used, since it avoids the trapping of water vapor that adversely affects the cryogenic injector or GC column performance. Immediately after collection, trapped volatiles are transferred, most commonly by thermal desorption, to the GC for subsequent analysis. In order to improve GC performance, it is common practice to cryo-focus the thermally desorbed volatiles in the GC inlet prior to analysis. Applications of adsorbent trapping-thermal desorption techniques in flavor analysis are discussed in greater detail elsewhere (Hartman *et al.*, 1993; Butrym, 1999; Wampler, 2002). DHA has similar advantages to SHA in that there is only

minimal sample preparation required, no reagents are used (which means there is no solvent peak during GC analysis) and there is a low risk of artifact formation from sample decomposition. A major limitation is that DHA is not an efficient method for analysis of semi-volatile compounds. Due to its ease of use, high sample throughput and relatively low cost per unit analysis, DHA has been commonly used for the analysis of volatile components of cheese products (Dunn and Lindsay, 1985; Barbieri *et al.*, 1994; Thierry *et al.*, 1999, 2004; Larráyoz *et al.*, 2001; Rychlik and Bossett, 2001a; Valero *et al.*, 2001; Qian and Reineccius, 2002a, 2003a, c; Boscaini *et al.*, 2003; Avsar *et al.*, 2004).

Headspace–solid phase microextraction

During the past decade headspace solid phase microextraction (H-SPME) has become one of the most commonly used methods for the isolation of volatile compounds from foods. H-SPME is a rapid, solventless technique based on the partitioning of the volatile components between the sample headspace and a polymer-coated fiber. For analysis, the adsorbed volatiles are thermally desorbed in the heated inlet of the GC. The application of H-SPME in the analysis of volatiles in foods has been reviewed and critically evaluated (Roberts *et al.*, 2000; Harmon, 2002; Marsili, 2002b; Werkhoff *et al.*, 2002).

Several adsorbent phases and film thicknesses are available, allowing for selectivity and specificity in H-SPME. All the parameters of SHA should be considered in H-SPME, as well as nature of the fiber coating and exposure (i.e. extraction) time. It is very important that sampling temperature does not result in any sample decomposition. Roberts *et al.* (2000) recommends the use of short extraction times (1–5 min) for highly volatile compounds and longer extraction times (5–30 min) for semi-volatile compounds. Transfer of the volatiles from the fiber to the GC is generally accomplished by hot splitless injection, which may result in loss of thermally labile volatile compounds.

Dufour *et al.* (2001) demonstrated the potential of H-SPME and GCO for the analysis of Cheddar cheese aroma. The fiber coating used profoundly influenced the aroma profiles obtained. Of five coatings evaluated, polydimethylsiloxane-divinylbenzene (PDMS-DVB) and Carboxen-PDMS fibers gave the highest recovery of aroma-active compounds. Despite the inherent sampling bias introduced by the fiber coating, H-SPME has in recent years been used extensively for the analysis of cheese volatiles (Pérès *et al.*, 2001; Lecanu *et al.*, 2002; Mortensen *et al.*, 2002; Pinho *et al.*, 2002, 2003; Kim *et al.*, 2003; Lee *et al.*, 2003; Chiofalo *et al.*, 2004; Frank *et al.*, 2004; Tavaría *et al.*, 2004; Verzera *et al.*, 2004; Burbank and Qian, 2005; Mondello *et al.*, 2005). Ease of use and ability to automate the analysis are possible reasons for the increased popularity of H-SPME.

18.2.2 Extraction–distillation methods

The combination of distillation with solvent extraction has been used for over four decades for the isolation of volatile compounds from foods (Chaintreau,

2001; Parliment, 2002). The traditional method of simultaneous distillation–solvent extraction (SDE) has, in recent years, been replaced by milder methods (e.g. direct solvent extraction followed by a high vacuum distillation as a ‘clean-up’ step) that avoid the formation of thermally generated artifacts due to sample (i.e. thermal) decomposition and minimize the loss of thermally labile volatile components.

Direct solvent extraction

Most volatile organic compounds in foods are considerably less polar than the bulk, mostly aqueous, food matrix. Most cheese products are an exception to this rule in that they are low moisture, high fat and high protein products. Despite this difficult matrix it has become common practice to use direct solvent extraction to isolate the volatile components from cheese products. The main concern with this approach is that the resulting solvent extracts contain appreciable amounts of nonpolar and nonvolatile lipids and minor amounts of other nonvolatile material. Therefore, these extracts cannot be injected directly into a GC without taking some precautionary steps. One such approach is to use an additional clean-up step (discussed on page 406) to isolate the volatile components in the solvent extract away from the nonvolatile material prior to GC analysis.

Direct solvent extraction is an effective means of isolating a broad range of volatile constituents from cheese products. It is especially suitable for extracting semi-volatile constituents (e.g. lactones, free fatty acids, phenolics, etc.) that cannot be effectively analyzed using headspace methods. Repeated extractions are generally necessary to minimize some of the bias induced by the inherent selectivity of the solvent chosen for the extraction, i.e., some aroma compounds are efficiently extracted while others are only poorly extracted. A good general solvent is diethyl ether, because it has good selectivity toward most aroma compounds, it has a relatively low density that enables ease of recovery, and it has a low boiling point so that it is readily removed by evaporation without significant losses of the extracted volatile substances.

An important consideration in solvent extraction is solvent purity. This includes water if it is used in the analysis or in the preparation of other reagents. One must use the highest purity solvents available, which often means the use of an in-house purification (distillation) process just prior to use of the solvent. Furthermore, it is prudent to run a blank to account for any impurities or artifacts introduced by the solvent or other reagents used in the analysis.

Numerous studies employ direct solvent extraction for the recovery of cheese volatiles. For hard and semi-hard cheeses, the product is typically frozen, grated or ground followed by extraction with the solvent (Preininger and Grosch, 1994; Kubickova and Grosch, 1997; Milo and Reineccius, 1997; Suriyaphan *et al.*, 2001; Zehentbauer and Reineccius, 2002; Qian and Reineccius, 2002b, 2003c; Avsar *et al.*, 2004; Carunchia-Whetstine *et al.*, 2005; 2006). For soft and some semi-hard cheeses, an aqueous extract is sometimes prepared first and then this extract is extracted repeatedly with the solvent to isolate the volatile constituents (Moio *et al.*, 1993). Once a solvent extract is prepared, it is usually subjected to

a high-vacuum distillation clean-up step, separated into neutral/basic and acidic fractions and subsequently concentrated by removing the bulk of the extraction solvent prior to GC analysis. These steps are discussed below.

High-vacuum distillation

High-vacuum distillation is the method of choice for sample clean-up of volatile extracts prepared by direct solvent extraction. This step is especially important if the aroma extract of the sample is analyzed by on-column GC or another injection technique where the nonvolatile material will either interfere with the injection or lead to formation of thermally generated artifacts when a heated GC inlet is used. A highly efficient solvent assisted flavor evaporation (SAFE) distillation system was developed for high-vacuum distillation of either liquid products or solvent extracts (Engel *et al.*, 1999). This technique, or slightly modified versions, is used in preparation of aroma extracts from cheese products (Carunchia-Whetstine *et al.*, 2005, 2006; Cadwallader *et al.*, 2006; Schlichtherle-Cerny *et al.*, 2006). SAFE is effective for the extraction of most volatiles from solvent extracts of cheese, including difficult polar and semi-volatile constituents (Werkhoff *et al.*, 2002).

Class fractionation and concentration of extract

Aroma extracts prepared by direct solvent extraction with high-vacuum distillation often contain hundreds of volatile constituents of varying polarities and containing different functional groups. Various methods, such as adsorption column chromatography and preparative GC, are useful to simplify the analysis by fractionation of the extract prior to GC analysis. One of the simplest and commonest methods is the use of acid/base chemistry to fractionate the extract into its acidic, basic and neutral components. The advantage of performing this pre-fractionation step is that the GC chromatograms are easier to interpret since they have fewer peaks. Furthermore, one does not need to compromise as much on GC column selection since the optimum column may be used for each separate fraction. It is generally not beneficial to fractionate the basic compounds from the neutral ones since most cheeses contain only a small number of basic volatile compounds (e.g. *o*-aminoacetophenone, indole and skatole). Exceptions to this are Parmigiano Reggiano (Qian and Reineccius, 2003a, b, c) and Gruyère (Rychlik and Bosset, 2001a, b) cheeses which contain pyrazines among their characteristic aroma components.

The general approach for fractionation of aroma extracts is to back-extract the acidic components from the solvent extract using aqueous bicarbonate or other suitable aqueous base (Carunchia-Whetstine *et al.*, 2005). The solvent extract retains the neutral/basic components, while the aqueous base is acidified and subjected to solvent extraction to yield the acidic fraction.

Prior to GC analysis, it is usually necessary to enrich the concentration of the volatile analytes in the aroma extract. Most often this is accomplished by distillation or by slow evaporation of the solvent under a gentle stream of inert gas (e.g. nitrogen). A major drawback to this concentration step is that some volatile constituents will be lost (evaporated) along with the extraction solvent.

Thus direct solvent extraction is generally not suitable for the analysis of highly volatile components. Complementary methods such as headspace analyses should also be conducted to evaluate the potential importance of highly volatile compounds.

18.2.3 Miscellaneous methods

In addition to the methods described above, there are some new and emerging methods introduced in the past few years that have not yet been widely applied to the study of cheese products. These include stir bar sorptive extraction (SBSE; Baltussen *et al.*, 1999), solid-phase dynamic extraction (SPDE; Bicchi *et al.*, 2004) and single droplet microextraction (SDME; Wood *et al.*, 2004). The main attraction of these methods is that they are suitable for use with multifunctional GC autosamplers.

18.3 Instrumental considerations

Tandem gas chromatography–mass spectrometry (GC–MS) is the method of choice for the analysis of volatile food components. The pre-eminence of GC–MS is due to the fact that high-resolution GC provides the highest overall efficiency and performance of all separation methods. Meanwhile, mass spectrometry is one of the most powerful techniques for identification of unknown compounds and is readily operated in tandem with GC.

18.3.1 Gas chromatography

A modern GC involves the use of high-resolution open tubular columns with bonded phases that are capable of separating hundreds of volatile constituents in a single run. GC is a mature methodology and the theory and general application will not be discussed here, except for two critical parameters that profoundly influence the results of GC analysis, injection technique and analytical column stationary phase.

Injection method

The injection inlet is used to introduce the volatile compounds, typically 1–3 μL of an aroma extract or 1–25 mL of headspace vapor, into the GC column. Programmable temperature vaporizer (PTV) and on-column injectors represent the best options among the many available GC injectors. The PTV inlet has particular appeal, since it allows for cryogenic focusing and ramped heated (programmable) injections in either split or splitless modes among other possible configurations. Cool on-column injection is the method of choice for analysis of aroma extracts prepared by direct solvent extraction–high vacuum distillation since it avoids thermal degradation and all of the components of the extract are completely introduced into the GC column (i.e. there is no injector discrimination).

Stationary phase

In general, only two types of GC phases are required for the identification and quantitation of the volatile compounds of cheese products. Polarity of the stationary phase is the most important parameter and should be matched with the polarity of the analytes as closely as possible. For identification purposes it is best to determine retention indices on two columns for each analyte of interest. Polyethylene glycol phases, such as a Carbowax (e.g. DBWAX) or free fatty acid phase (e.g. FFAP), are suitable for the analysis of most polar compounds. A 95% dimethyl–5% diphenylpolysiloxane phase (e.g. DB5) is commonly used for the analysis of nonpolar compounds. Some compounds are incompatible with certain phases (e.g. short-chain free fatty acids do not chromatograph well on non-polar phases). In such cases it may be necessary to use an intermediate polarity phase, such as an 86% dimethyl–14% cyanopropylphenylpolysiloxane (e.g. DB-1701). A detailed discussion of the importance of the GC stationary phase in aroma research is reviewed by Blank (2002).

Gas chromatography–mass spectrometry

Many detectors are available for GC. The flame ionization detector (FID) is the most commonly used for routine GC; however, for the analysis of highly complex volatile mixtures a mass spectrometric (MS) detector is preferred since it provides both qualitative and quantitative information. The main advantage of GC–MS is that it allows for mass spectral library matching for identification of unknown chromatographic peaks. The total ion chromatogram generated by full scan GC–MS can also be used for routine peak quantitation.

For analysis of trace constituents, selected ion monitoring (SIM) mass spectrometry and ‘mass chromatography’ are often used. In SIM, only selected ions representative of a specific compound or class of compounds are recorded during GC–MS analysis. The technique provides extremely high sensitivity for analysis of known constituents but does not provide any useful information for the identification of unknown compounds, since full mass spectra are not recorded. Mass chromatography can be considered as retrospective SIM. With this method complete mass spectra are recorded throughout the GC–MS run rather than just selected ions. The data analysis software is used to re-plot only specific ions from full spectra data, with the aim of resolving co-eluted peaks. This issue is more or less resolved with the availability of recent models of MS detectors which can perform simultaneous true scan and SIM. One such example is the Agilent 5975MSD.

Gas chromatography–olfactometry

Since the early 1960s most aroma studies considered that all measurable volatile constituents (i.e. those compounds appearing as peaks during GC–MS analysis) impact the aroma of a food product. In the past two decades, however, the research community realized that among the numerous volatile constituents found in foods relatively few actually contribute to the aroma of a particular food. Various approaches are taken to identify these key odorants. These include the calculation of odor-activity values (OAVs), use of GC–olfactometry (GCO) and sensory analysis of aroma models (Grosch, 1993, 1994, 2001).

As mentioned earlier, high-resolution GC is capable of separating and detecting hundreds of volatile compounds in a single run. However, it is likely that only a small number of these compounds actually contribute to the aroma of the product. The aroma-active components in a volatile isolate can be determined by combining GC with olfactometry. In GCO, the analytes are first separated by the GC and then delivered, generally in parallel with an electronic detector, to an olfactometer (sniff port) where they are mixed with humidified air. Human panelists continuously evaluate (nasally) the air emitted from the olfactometer and record their perceptions, such as odor intensity and odor description of the detected compounds. GCO is reviewed by Acree (1993) and Blank (2002). Common methods based on GCO include aroma extract dilution analysis (AEDA) (Grosch, 1993, 1994), CharmAnalysis (Acree, 1993), Osme (McDaniel *et al.*, 1990), nasal impact frequency (NIF – Pollien *et al.*, 1997; SNIF – Chaintreau, 2002) and post peak intensity scaling (Avsar *et al.*, 2004). These methods differ mainly in how the GCO data are recorded and analyzed. All GCO methods are considered as screening methods since they do not provide an absolute measure of odor potency. Instead, GCO data are used to indicate odor-active volatiles for subsequent sensory studies using recombination and model studies. Among the various GCO methods available, the dilution techniques are most often used in the analysis of cheese aroma. The two most common types of GCO dilution methods are discussed.

- **Aroma extract dilution analysis.** AEDA relies on the GCO analysis of a serially diluted series of an aroma extract. Each odorant is assigned a flavor dilution (FD) factor based on the highest dilution at which it was last detected by GCO. AEDA is considered a screening method since FD-factors are relative values and do not correct for volatile losses during extraction and workup procedures. AEDA is used for aroma characterization of various cheeses (Kubickova and Grosch, 1997, 1998a; Milo and Reineccius, 1997; Rychlik and Bosset, 2001a; Suriyaphan *et al.*, 2001; Qian and Reineccius, 2003c; Avsar *et al.*, 2004; Carunchia-Whetstine *et al.*, 2005, 2006).
- **GCO-headspace dilution analysis.** GCO-headspace techniques were developed based on the dilution concept of AEDA. Two common methods are GCO of decreasing static headspace volumes (GCO-H) and dynamic headspace dilution analysis (DHDA). In these methods dilutions are made by decreasing the headspace gas volume in GCO-H or by decreasing the purge gas volume in the case of DHDA. GCO-headspace dilution methods are applied as a complementary method to AEDA to assess the potential importance of highly volatile components (e.g. acetaldehyde, hydrogen sulfide, methanethiol, dimethylsulfide and low molecular weight Strecker aldehydes) in the aroma of cheese (Kubickova and Grosch, 1997; Milo and Reineccius, 1997; Rychlik and Bosset, 2001a; Qian *et al.*, 2002; Zehentbauer and Reineccius, 2002; Qian and Reineccius, 2003a,c; Avsar *et al.*, 2004).

Compound identification

In GC, positive compound identification is achieved by matching the retention indices (RIs), determined on at least two different column phases, and electron-

impact (EI) mass spectrum of an unknown compound against those of an authentic reference compound analyzed under identical conditions. The RI is a measure of the relative retention of a compound compared with a series of *n*-alkane reference standards on a specific GC phase (Van den Dool and Kratz, 1963). RI values are of particular value since they are compared against published literature values or computer databases. When GCO is conducted, the odor property of the unknown compound is also compared with that of the reference compound. Furthermore, in the case when an unknown compound is below MS detection limits, the combination of RI and odor property by GCO is often used as the sole basis for compound's identification (generally considered a tentative identification).

Quantitative analysis

The ideal quantitative analysis procedure should have high precision and accuracy. Internal standard methodology is the preferred method for routine analysis of volatile compounds in cheese products. In the internal standard method, known amounts of surrogate internal standards that mimic the analytes of interest as much as possible are added to the sample matrix prior to performing the volatile isolation procedure. The internal standards compensate for variations in extraction procedures, injection volume and detector drift. Internal standards should possess chemical, spectral and chromatographic properties that are similar to the compounds being analyzed. They should be readily available, stable, inexpensive, of high (or known) purity, and must not interfere with the analytes during GC. For highest accuracy it is important to determine the relative recoveries and GC response factors for the internal standards relative to the target analytes. These recovery factors are based on GC–MS (full scan, SIM, or mass chromatography) or use of a selective detector (e.g. FPD for selective analysis of sulfur-containing compounds).

The perfect internal standard is an isotopic analog of the analyte of interest. This method is called isotope dilution analysis (IDA) and involves the use of stable isotopes (deuterium or carbon-13 labeled) as internal standards. GC–MS analysis is required since the isotopic internal standard co-elutes with the analyte but is resolvable by MS. The method is self-correcting for recovery, losses during sample workup and chromatography, since the labeled and unlabeled compounds have essentially the same physical and chemical properties. The main disadvantage of IDA is the cost associated with the synthesis of the isotopic analogs. IDA has been used in the analysis of cheese aroma (Preininger *et al.*, 1996; Kubickova and Grosch, 1998b; Rychlik and Bosset, 2001b).

18.4 Linking of sensory and analytical data

To thoroughly understand the flavor of a food product the chemical data used to characterize its aroma profile should be related to its sensory characteristics. This is done using a variety of techniques that are reviewed in this section.

18.4.1 Thresholds and odor-activity values

The odor detection threshold and odor-activity value (OAV) are important when considering the potential contribution of a volatile compound to the aroma of a food product. Most potent odorants have low odor detection thresholds, which means the odor of these compounds can be detected when they are present at low concentrations. An OAV, sometimes called odor unit or odor value, is used to estimate odor potency in terms of the ratio of the concentration of a volatile compound to its odor detection threshold. To assure accuracy of the OAVs, concentrations must be accurately known and thresholds must be determined in a medium that is similar to the food product being studied. Another important limitation of the use of OAVs to rank odorants in foods is sometimes one or more potent odorants are present at levels below the instrument detection limits, which prevents their inclusion in the ranking analysis. Nonetheless, OAVs are useful in the evaluation or ranking of potent odorants in cheese products (Preininger and Grosch, 1994; Qian and Reineccius, 2002b, 2003b).

18.4.2 Sensory analysis of model systems

Many aroma researchers use sensory analysis of model mixtures to validate results of their detailed chemical analyses. Two effective ways of accomplishing this, omission studies and dose–response analysis, are discussed below.

Omission studies

In omission studies a synthetic mixture of odorants is prepared in an appropriate medium that closely resembles the matrix of the food being studied (Grosch, 2001). Typically, the mixture is initially compared to the original food or a simple extract by sensory evaluation (difference testing or descriptive analysis). In the case when a close match is attained, omission studies are conducted to evaluate the sensory impact of omitting one or more of the odorants of the mixture. Generally, one odorant at a time is omitted ($n - 1$ studies) and the resulting model mixtures are compared by difference testing or descriptive analysis against the complete mixture. The results give some indication of which odorants actually contribute the most to the aroma of the food product and provide insight into potential interactions, such as mixture suppression, among odorants in the mixture. These types of studies have been conducted with some success with Emmental (Preininger *et al.*, 1996), Gruyère (Rychlik and Bosset, 2001b) and Camembert (Kubickova and Grosch, 1998b) cheeses, but had only limited success in the case of Cheddar cheese (Dacremont and Vickers, 1994; Wang and Reineccius, 1998; House and Acree, 2001).

Dose–response analysis

Often the matrix of a food product, such as cheese, is too complex to allow for adequate complete model mixture studies. In these cases, dose–response sensory analysis is applied to study the sensory impact of a single odorant or mixed odorants in a complex food matrix. In this method a sensory descriptive panel

rates the intensity of a specific aroma term as a function of the increase of an odorant that is spiked into the food matrix. Generally, the panel is unaware of the treatment and scores the product using a sensory lexicon of terms. An increase in the perceived intensity of the term in question provides compelling evidence that the odorant is, at least in part, responsible for this aroma attribute. This approach is particularly appealing for difficult matrices such as cheese products. Suriyaphan *et al.* (2001) applied dose–response sensory studies to demonstrate that *p*-cresol and 2-isopropyl-3-methoxypyrazine are responsible for cowy/barny and earthy/bell pepper notes, respectively, in British Farmhouse Cheddar cheese. The same method was later used to demonstrate compounds responsible for nutty (Avsar *et al.*, 2004), rosy/floral (Carunchia-Whetstone *et al.*, 2005) and beefy/brothy (Cadwallader *et al.*, 2006) flavors in Cheddar cheese.

18.5 Future trends

Flavor will continue to be an important element of cheese research. Of particular importance is our understanding of how the variables in cheese making, such as use of accelerated ripening, genetically modified starter, adjunct cultures, etc., impact the flavor chemistry of cheese products. Linking of sensory attributes to the underlying flavor chemistry is another critical area, especially of how flavor chemistry relates to consumer acceptance. The objective of this chapter was to present the state-of-the-art in methods used for the analysis of cheese aroma components. All of these methods discussed here have a place in cheese flavor research. Detailed aroma characterization and identification of key odorants in cheese is accomplished by use of difficult and labor intensive methods based on GCO and sensory analysis of model systems. On the other hand, relatively simple and easy to use methods, such as SHA, DHA and H-SPME, can provide useful, although somewhat limited, information about the volatile components of cheese products.

18.6 Sources of further information and advice

The reader is encouraged to consult texts that provide exhaustive reviews and critical evaluations of new and emerging methods for measuring volatile compounds in foods (Marsili, 2002b; Reineccius, 2006).

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Part IV

Improving the flavour of different types of cheese: case studies

Hard Italian cheeses: Parmigiano-Reggiano and Grana Padano

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19.1 Introduction

In the fertile river valleys and plains of Italy, cheese has been made for nearly two millennia with production on a commercial scale developed as early as the eighteenth century. Today, Italy has the second widest range of cheese varieties, many of which are internationally famous. To make the diverse cheese varieties, Italians use milk from cow, sheep, goat and buffalo as well as the mixtures of these milks. In addition to the type of milk utilized for the manufacture of cheese, geographical environment and manufacturing artisanship play important roles in the distinctive aromas, flavors, and textural qualities of the cheeses. Hard Italian-style cheeses are made at high temperatures under unique conditions and often aged for a long time, which give particular sensorial qualities that are characteristic of these types of cheeses. This chapter will discuss the numerous volatile compounds identified in these cheeses, in particular Parmigiano-Reggiano and Grana Padano, and their contribution to the overall perceived aroma during the enjoyment of their consumption.

19.1.1 Classification of Italian-style cheeses

Classification of Italian cheeses can be difficult since cheeses produced in discrete regions, or during a particular season, have different names even though the cheeses are made with similar production techniques and have comparable sensory attributes. The classification given to a particular cheese is based on many aspects including the fat content and consistency of the finished product, or according to the preparation and production technique of the cheese. Often cheeses are classified by their moisture content; for example, most aged Grana-

style cheeses have low moisture content (around 30%) due to moisture loss during extended length of ripening (Battistotti and Corradini, 1993). Additionally, some varieties of cheese are consumed at various stages of ripening and can be called 'table cheese' at 2–4 months of aging but 'grating cheese' after 10 months; therefore, length of ripening also lends itself to the classification of cheese.

'Grana', or grainy textured, Italian-style cheeses are produced using high temperatures to cook the curd along with extended lengths of aging to develop their distinctive flavors. These types of cheeses include Grana Padano, Pecorino Romano, and the well-known Parmigiano-Reggiano. Today, the latter two cheeses are produced internationally under the common names of Romano and Parmesan, respectively; however, the characteristic flavor and aroma are clearly different from the cheeses made in Italy with the same name (Battistotti and Corradini, 1993). Romano is traditionally produced with sheep's milk; however, outside Italy, it is most often made with cow's milk. Grana cheeses are most commonly served as a grating cheese for topping dishes; Parmigiano-Reggiano is the most famed member of this family.

The term 'Parmesan' is often used synonymously with Parmigiano-Reggiano; however, this interchangeability is dreadfully incorrect as there are very specific distinctions between these two cheeses. In order to call a hard, high-temperature cheese 'Parmigiano-Reggiano', it must have been produced in the Po valley of Italy, which includes the small areas of Parma and Reggio Emilia, where most of this cheese is produced, and also the towns of Modena, Bologna, and Mantova (Mantua). The exclusive naming is due to its Protected Designation of Origin (PDO), which is essentially a trademark identifying the geographic area of the product and thus preventing outside manufacturers from using the same identification when selling similar products. In 1996, the European Commission issued a list of foodstuffs and products that cannot be reproduced 'in close resemblance and called by the same name as the original'. Thirty of these foodstuffs are cheeses that can only be produced in particular regions of Italy, including Parmigiano-Reggiano. The making of Parmigiano-Reggiano uses established production processes that have remained relatively unchanged for over 800 years.

19.1.2 Production of Parmesan cheese

Parmesan-type cheese is made from pasteurized and clarified skim milk that is based on gravity separation. For traditional Parmigiano-Reggiano, raw milk is used. The milk is placed in shallow vats to stand overnight whereby natural creaming occurs. In the morning, the milk is partially skimmed and a limited amount of acidity develops. This milk is then combined with fresh whole milk procured in the morning. The fat content of the morning milk is adjusted (Reinbold, 1963) by use of a clarifying separator prior to combination with the evening milk, where the final fat percentage for the combined milk is approximately 2.5% (Battistotti and Corradini, 1993). This is done so that the

proper casein:fat ratio is maintained in the final product. Another difference for traditional Parmigiano-Reggiano cheese versus common Parmesan is the source of the acidifying bacteria. In the manufacturing of traditional Parmigiano-Reggiano cheese, fermented whey incubated at room temperature from the previous day's production is used. The bacterial composition of this natural whey culture is complex; the main organisms include the thermophilic lactic acid bacteria (LAB) of *Lactobacillus helveticus*, *Lactobacillus delbrueckii* ssp. *lactis*, and *Lactobacillus delbrueckii* ssp. *bulgaricus*. For common Parmesan, LAB starter cultures such as *L. bulgaricus* or *Streptococcus thermophilus* are often used to provide the source of acidification. Because the milk is cooked at high temperatures during production, the starter cultures are naturally selected to be thermophilic bacteria in order to survive.

After addition of culture, the milk is brought to the renneting temperature (30–35°C). Rennet, a natural enzyme derived from the lining of cow stomach, is added to coagulate the milk within 15 minutes. Following coagulation, or curd formation, the mass is cut into small pieces no larger than wheat kernels using a wire implement. The curds are slowly heated with periodic gentle stirring until the temperature reaches 52–55°C, usually within 10–12 minutes. The rate of cooking is important because if it is too fast, excessive dehydration occurs at the curd surface, leading to the formation of a skin around the curd particle. This is known as 'case hardening' (Fox *et al.*, 2000), which slows or stops syneresis, resulting in a high moisture content in the final cheese, an undesirable condition for Parmesan. It is also important that the curds be continuously stirred during cooking so that the pieces do not stick together, another factor that would have a negative effect on syneresis and the final cheese moisture.

Once cooked, the curds are wrapped in cheesecloth, strained, and placed into large molds. Because acidification occurs mainly after molding (Fox *et al.*, 2000), light pressure is applied to the molds to encourage whey expulsion, a process that takes 12–24 hours (Battistotti *et al.*, 1983). Afterwards, the pressed curd mass is salted by immersion in brine at room temperature for 20–23 days and placed in a cool (16–18°C), ventilated room with 85% relative humidity to mature (Battistotti *et al.*, 1983; Reinbold, 1963). During ripening, the cheeses are turned at regular intervals and the rinds are cleaned often. Parmesan can have color or coating added to the rind, so it will more closely resemble the color of traditional Parmigiano-Reggiano, which has a natural pale straw or gold color when mature. The minimum length of ripening for Parmesan manufactured in the United States is 10 months (21 CFR 133.165, April 2004 edition, US Code of Federal Regulations); however, traditional Parmigiano-Reggiano is often aged for two years or more.

19.1.3 Differences in production of other Grana cheeses

The techniques used for making other Grana cheeses are very similar to those used for Parmigiano-Reggiano with some subtle differences. For example, Grana Padano is made from raw cow's milk obtained during a single day's

milking, and is partially skimmed after 6–8 hours of natural creaming; it does not stand overnight as with Parmigiano-Reggiano (Coppola *et al.*, 2000). Since this milk is not mixed further with whole milk, Grana Padano is generally less fatty than Parmigiano-Reggiano. Additionally, the feed for Grana Padano has fewer restrictions than that for Parmigiano-Reggiano. Under strict regulations, the milk used for Parmigiano-Reggiano can only come from cows that eat hay gathered from local meadows. On the other hand, the cows used for Grana Padano cheese can eat mixtures of grasses and corn stalks, which are forbidden for Parmigiano-Reggiano cheese. Grana Padano cheese is usually aged for 14–16 months.

Romano cheeses can be made with various types of milk; the terms Pecorino, Caprino, and Vacchino indicate that the cheese is made with sheep, goat or cow's milk, respectively. During cooking of the curds, the final temperature for Romano is not as high as that for Parmigiano-Reggiano, rather only 45–48°C. After pressing Romano cheese is brine- or dry-salted and aged for eight months. Bagozzo and Grana Lodigiano are also members of the Grana family of Italian-style cheeses; however, they are not as well known, with distribution limited to the areas where they are produced. In the case of Grana Lodigiano, the milk is more thoroughly skimmed after standing overnight. Another extra-hard, granular textured cheese is Sbrinz, which originated from Switzerland but is now produced in parts of Italy; it can be considered as the Swiss version of Parmesan. Sbrinz cheese uses full-fat cow's milk, the curds are cooked at high temperature (54–56°C) and the cheese can be aged up to three years (Fox *et al.*, 2000; Battistotti *et al.*, 1983).

19.1.4 Sensory descriptions

Grana cheeses have a grainy, crumbly texture that melts easily in the mouth. The taste of well-aged Italian Parmigiano-Reggiano is rich, fruity, nutty and slightly sweet while Parmesan produced outside Italy has less intense flavor and aroma, most likely due to use of pasteurized milk along with shorter lengths of aging (Battistotti *et al.*, 1983). An important factor that contributes to the taste of the cheese is the diet of the cows from which the milk is procured. If corn stalks are part of their feed, then the cheese will generally be whiter in color and taste milkier compared to cheese from hay-fed cows, which is more yellow and can have hay-like overtones in the flavor (Phillips, 2005). Similarly, the season also plays a role in the overall flavor; in the spring and summer months, the cows graze on fresh grass, resulting in a richer flavored cheese.

The precise flavor and aroma of aged Grana cheeses depends on many factors; however, this family of cheeses is one where the chemical composition is quite similar but the amounts of certain flavor compounds vary. Therefore, for a particular hard Italian-style grating cheese, it is the relative concentration of each compound that plays the largest role in its overall aroma. An in-depth look at the chemical composition of the most well-known cheese of the Grana family, Parmigiano-Reggiano, will be discussed to provide insight about the aroma

compounds making major contributions to the delectable flavor and aroma of Parmesan cheese.

19.2 Aroma analysis

The volatile compounds that comprise aged Italian-style cheeses have been studied extensively (Frank *et al.*, 2004; Boscaini *et al.*, 2003; Lee *et al.*, 2003; Moio and Addeo, 1998; Barbieri *et al.*, 1994; Careri *et al.*, 1994; Engels and Visser, 1994; Virgili *et al.*, 1994; Ha and Lindsay, 1991b, 1990; Meinhart and Schreier, 1986; Woo and Lindsay, 1984; Lindsay, 1983; Manning and Moore, 1979; Dumont *et al.*, 1974). Using gas chromatography as a means of separation and mass spectrometry for identification, the volatile profiles of Parmesan and other hard Italian-style cheeses are found to contain numerous classes of compounds. However, many of these studies focus mainly on the most abundant compounds without considering that, even though a compound may be present at a high concentration, it may have less influence on the overall aroma than some compounds at lower amounts (Qian and Reineccius, 2002a). This is because the impact that a particular volatile compound has on the overall aroma depends on its sensory threshold, which is the minimum concentration at which the aroma of the compound can be correctly identified. Hence, volatile compounds that have low sensory thresholds need not be present at high concentrations in order to be perceived. In order to understand the aroma profile of Parmesan cheese, it is necessary to study the aroma impact compounds in the cheese.

Successful aroma analysis depends highly upon the technique that is used to isolate the aroma compounds. Unfortunately, there is no single universal method that can be used to extract all aroma compounds. Various extraction techniques have been attempted to isolate aroma compounds from Parmesan cheese, including simultaneous distillation–extraction, dynamic headspace, ion exchange chromatography, solvent extraction with high vacuum distillation, and solid-phase microextraction (SPME) (Frank *et al.*, 2004; Boscaini *et al.*, 2003; Lee *et al.*, 2003; Qian and Reineccius, 2003a, c, 2002a; Moio and Addeo, 1998; Barbieri *et al.*, 1994; Careri *et al.*, 1994; Virgili *et al.*, 1994; Ha and Lindsay, 1991b; Meinhart and Schreier, 1986). These methods are commonly used for sample preparation of many food matrices; each has individual pros and cons, which will not be discussed here. By utilizing these techniques, the volatile aroma compounds of Parmesan and similar Grana cheeses have been analyzed, and a wide variety of chemical classes have been positively identified, including acids, esters, ketones, aldehydes, alcohols, lactones, phenols, and sulfur- and nitrogen-containing compounds. Chapter 18 describes the analytical methods for the compounds important to the flavor of cheese. This section describes the analysis and olfactory methods associated with the compounds of Italian-style cheeses.

Gas chromatography/olfactometry (GC/O) is widely used to identify important aroma compounds in the food. The aroma is typically extracted from the

food matrix prior to GC/O analysis. However, the type of food matrix, such as water- or oil-based, from which the compounds are extracted, will have a major impact on how the odor qualities of the compounds are perceived. This is because the composition of the matrix directly affects the vapor pressure of the volatile compounds. Most volatile aroma compounds are hydrophobic in nature, so they prefer to partition into the lipid phase (Harrison *et al.*, 1997) where the release of aroma compounds proceeds at a lower rate than in an aqueous phase (Piraprez *et al.*, 1998; De Roos, 1997). These reduced rates can be attributed to higher van der Waals interactions and higher resistances to mass transfer in fats and oil than in water. Because the lipid content of Grana cheeses is quite high, many of the volatile aroma compounds tend to stay within the cheese rather than being released to the headspace, hence lowering the perceived aroma intensity (Boscaini *et al.*, 2003).

To take into account the impact of the matrix, an odor activity value (OAV) can be calculated by dividing the concentration of an aroma compound in the sample by its sensory detection threshold in the same matrix (Patton and Josephson, 1957). This approach is based on the theory that in order for an aroma to be perceived, its concentration in the matrix must exceed the sensory threshold in that matrix, i.e. when a compound's $OAV > 1$, that compound will probably contribute in part to the overall aroma. Hence, the calculation of OAV is yet another approach to ascertain the influence of an aroma compound on overall flavor and aroma as it directly relates to the matrix in question, thus it can be used to further narrow down the important aroma compounds in a particular sample. While the calculation itself is very simple, accurate quantification of aroma compounds is not easy due to extremely low concentrations of many aroma compounds. At the same time, the determination of sensory thresholds is extremely tedious because values reported in the literature from similar matrices often vary by several orders of magnitude, if not more, which can make the OAV technique less attractive.

19.3 Aroma compounds of Parmesan and related Italian-style cheeses

The aroma profile of Parmesan cheese is quite complex and there is no single aroma compound responsible for the characteristic 'Parmesan' aroma. A sensory study (Virgili *et al.*, 1994), in which product acceptance of Parmigiano-Reggiano was tested, found that trained panelists agreed to the terms 'fragrant' (as related to ester compounds) and 'buttery', to a lesser extent, as good descriptors for the pleasant aroma of Parmigiano-Reggiano. It is possible that the recombination of many aroma compounds discussed below provides the full rich aroma character of Parmesan cheese. The following sections will take a closer look into each chemical class and their contribution to the overall aroma and flavor of Parmesan cheese.

19.3.1 Acidic compounds: free fatty acids

Free fatty acids (FFAs) are important to many varieties of cheese, particularly Italian-style cheeses. In addition to their contribution to cheese aroma in general, FFAs are also precursors to many other important aroma compounds including aldehydes, methyl ketones, esters and lactones. The production of FFAs is reviewed in Chapter 5. This section discusses the FFAs specifically important to hard Italian-style cheeses.

Butanoic, hexanoic, and octanoic acids are often described as rancid, sweaty (body odor), goat-like, and generally unpleasant, but these fatty acids also have additional aroma descriptors such as cheesy and sharp (Table 19.1). These even-numbered short-chain fatty acids (C4 to C8) are highly desirable in cheese in order to impart certain characteristics to the overall flavor and aroma. On the other hand, FFAs with medium chain lengths, like decanoic (C10) and dodecanoic (C12) acids, are not as desirable due to their soapy and waxy aromas. FFAs with longer chain lengths, such as C14 to C20, are virtually odorless, therefore do not contribute to the overall aroma of Parmesan. Calculation of the OAV for FFAs in Parmesan cheeses (Qian and Reineccius, 2002a) demonstrates that the FFAs of chain length C2 through C8 have high OAV values, so they are important in aroma contribution of Parmesan cheeses (Tables 19.2 and 19.3).

Besides the straight-chain FFAs, some branched-chain FFAs also appear to be significant to the overall flavor quality of Parmesan cheese (Ha and Lindsay, 1991b; Woo *et al.*, 1984; Woo and Lindsay, 1984). Branched-chain FFAs are minor components of ruminant lipids (DePooter *et al.*, 1981; Duncan and

Table 19.1 Aroma attributes and sensory thresholds of the volatile free fatty acids found in Parmesan cheese

Compound	Aroma attributes	Threshold (ppm)	Media
Acetic acid	vinegar, sour, pungent	22–100	water
		0.12–7	oil
Propanoic acid	sour, pungent	20–40	water
Butanoic acid	rancid, cheesy, sharp	0.3–6.8	water
		0.14–3	oil
2-Methylpropanoic acid	cheesy, rancid, caramel	0.05–8.1	water
Pentanoic acid	cheesy, sour, meaty, sweaty	1.1–6.5	water
2-Methylbutanoic acid	cheesy, rancid, sour, sweaty	0.07	water
		0.02	oil
Hexanoic acid	cheesy, goaty, sharp	0.29–27	water
		2.5–10	oil
Heptanoic acid	cheesy, goaty, rancid	0.28–10.4	water
Octanoic acid	cheesy, sweaty	3–19	water
		10–350	oil
Nonanoic acid	fatty, green	2.4–8.8	water
Decanoic acid	soapy, waxy	1.4–10	water
		5–200	oil
Dodecanoic acid	soapy, metallic	2.2–16	water
		700	oil

Table 19.2 Free fatty acid concentrations (ppm, mg/kg) in various brands of hard Italian-style cheeses

Cheese ¹	Origin	Straight chain				Branched chain	
		Butanoic C4	Hexanoic C6	Octanoic C8	Decanoic C10	2-Methyl butanoic	3-Methyl butanoic
1 Reggiano A	Italy	397	249	146	374	— ²	—
2 Reggiano B	Italy	231	166	76	87	0.6	2.3
3 Parmesan	— ³	502	174	98	223	—	—
4 Grana Padano	Italy	346	181	116	246	—	—
5 Italian	Italy	678	363	234	355	—	—
6 Sbrinz	Italy	110	56	35	92	—	—
7 American A	USA	95	50	50	87	—	—
8 American B	USA	432	183	111	231	—	—
9 American C	USA	1890	447	219	410	—	—
10 American D	USA	417	421	45	98	1.2	2.1
11 Romano	—	1756	843	328	942	—	—
12 Romano	USA	6585	3385	1781	4600	18	31
13 Romano	Italy	1055	1489	620	808	40	40

¹ Lines 1, 4–9 from Qian (2002b); lines 2, 10, 12, 13 from Ha and Lindsay (1991b); lines 3, 11 from Lindsay (1983).

² Not reported.

³ Not specified.

Garton, 1978; El-Shazly, 1952a) and are naturally present in milk as free fatty acids or bound as glycerides. The bound branched-chain FFAs can be released from glycerides during cheese ripening; however, unlike the normal straight-chain fatty acids, branched-chain fatty acids are poorly hydrolyzed by most lipases (Ha and Lindsay, 1993). It has been reported that the α -carbon atom of the branched-chain fatty acid ester resists hydrolysis by pancreatic lipase (Bergstrom *et al.*, 1954) and it has been demonstrated that a methyl group at either the α - or β -position of fatty acid esters inhibits lipase activity (Sonnet and Baillargeon, 1991; Borgstrom *et al.*, 1957; Tryding, 1957). Thus, their hydrolysis is much slower than the straight-chain fatty acids. Degradation of some amino acids such as leucine, isoleucine, and valine also results in certain branched-chain fatty acids (El-Shazly, 1952a, b).

Most branched-chain FFAs have similar aroma characteristics as linear FFAs, such as cheesy, sweaty, and rancid; however, branched-chain FFAs often have lower sensory thresholds than their linear equivalents, partially due to higher vapor pressures (Brennand *et al.*, 1989; Heath and Reineccius, 1986). For that reason, they can make subtle contributions to the aroma of Parmesan cheese even when present at very low concentrations. Branched-chain FFAs have been found in the acidic fraction of Parmigiano-Reggiano cheese (Ha and Lindsay, 1991b; Meinhart and Schreier, 1986), where 2-methylpropanoic, 2-methylbutanoic, 3-methylbutanoic, 2-ethylbutanoic, 2-methylhexanoic, 2-ethylhexanoic and 2-ethyloctanoic acids are positively identified. Quantitative analysis demonstrates

Table 19.3 Calculated odor activity values (OAV)^{1,2} in various brands of hard Italian-style cheeses

	Brand	Butanoic acid	Hexanoic acid	Octanoic acid	Decanoic acid	2-Methyl butanoic acid
1	Reggiano A	253	40	0.8	3.6	—
2	Reggiano B	147	27	0.4	0.8	30
3	Parmesan	320	28	0.5	2.2	—
4	Grana Padano	220	29	0.6	2.4	—
5	Italian	432	58	1.3	3.5	—
6	Sbrinz	70	9	0.2	0.9	—
7	American A	61	8	0.3	0.8	—
8	American B	275	29	0.6	2.3	—
9	American C	1204	72	1.2	4.0	—
10	American D	266	67	0.3	1.0	0.6
11	Romano	1118	135	1.8	9.2	—
12	Romano	4194	542	9.9	44.9	900
13	Romano	672	238	3.4	7.9	2000

¹ OAV = concentration/threshold; higher OAV signifies greater importance to aroma.

² Concentrations from Table 19.2; threshold value is average of reported range from Table 19.1.

that only 2-methylbutanoic acid and 3-methylbutanoic acids make contributions to the overall aroma and flavor (Ha and Lindsay, 1991a), and the cheesy aromas are mainly contributed by the linear short-chain fatty acids (C4, C6, and C8) due to the high level of lipolysis occurring in Parmesan cheese (Qian and Reineccius, 2002b).

Because the presence of most FFAs in cheese is the result of enzymatic degradation of lipids during the aging process, FFAs are related to the length of cheese aging (Woo and Lindsay, 1984). Different Italian-style cheeses contain varying concentrations of fatty acids due to differences in the milk source (type and fat level), the addition of particular bacterial strains and/or enzymes, and also different ripening temperatures and lengths of aging. Extensive lipolysis occurs in many hard Italian-style cheese varieties, including Parmesan, Romano and Grana Padano. The concentrations of FFAs are also subject to seasonal variations. An investigation (Barbieri *et al.*, 1994) into this matter found that Parmesan cheeses manufactured in the winter and summer months had higher levels of butanoic acid than did those cheeses produced in the spring and autumn months. Other studies (Qian and Reineccius, 2002b; Ha and Lindsay, 1991b) find that the location in which the cheese is made can also affect the FFA content, presumably due to the microflora of the local production facility.

Parmigiano-Reggiano cheeses have a much higher proportion of free short-chain fatty acids than that in the milk fat. This is due primarily to the rennet paste used in the manufacturing of these cheeses (Fox *et al.*, 2000). Rennet paste contains pregastric esterase (PGE), and PGE is highly specific for the release of short-chain fatty acids from the sn3 position of triglycerides. Therefore, the concentrations of short-chain FFAs become elevated in hard Italian-style

cheeses as the cheese ages, which lends to their distinctive flavors. Many Italian-style hard cheeses use PGE enzyme to enhance flavor.

Although Italian-style hard cheeses all have very high lipolysis, Parmigiano-Reggiano contains relatively lower levels of total volatile FFAs as compared to Pecorino Romano cheese (Fox *et al.*, 2000; Ha and Lindsay, 1991b; Woo *et al.*, 1984). It is suggested that the lower concentration of FFAs in Parmigiano-Reggiano cheese may be due to (a) re-esterification of FFAs to glycerol, (b) esterase-mediated interesterification between short- and long-chain FFAs, or (c) esterification of FFAs with alcohols due to decreased moisture content during aging (Ha and Lindsay, 1991b). In general, Romano cheese has a stronger 'sharp' (or *piccante*) aroma than Parmigiano-Reggiano, which has a comparatively mild and more fragrant aroma, possibly due to lowered concentrations of FFAs and also higher level of esters, as further discussed. Although the concentrations of FFAs in Parmigiano-Reggiano are lower than in Romano, short-chain FFAs are still the most important contributors to the overall aroma of this cheese.

19.3.2 Volatile compounds in the neutral fraction

In addition to FFAs, other volatile compounds in Parmesan cheese have also been studied extensively (Barbieri *et al.*, 1994; Careri *et al.*, 1994; Meinhart and Schreier, 1986; Rafecas *et al.*, 1986; Manning and Moore, 1979; Dumont *et al.*, 1974). The numerous compounds identified in the neutral fraction of Parmesan cheeses can be categorized according their chemical classes: esters, ketones, aldehydes, alcohols, lactones, phenols, and sulfur compounds. These important classes of aroma compounds are mainly derived from three major metabolic pathways: lactose catabolism, lipid catabolism, and protein catabolism.

Esters

Esters are common components of cheese flavor where they are determined to be the main constituents of the neutral fraction for Parmesan and Grana Padano cheeses. Esters are formed from the esterification of alcohols and FFAs, through either enzymatic or chemical reactions during cheese aging. A wide variety of enzymes can be involved in the esterification processes. The esterase activities of the LAB, along with pregastric esterases, are responsible for high levels of esters in Parmigiano-Reggiano and Grana Padano cheeses (Fenster *et al.*, 2003; Hosono *et al.*, 1974). This class of volatile compounds accounts for approximately 41% of the total neutral volatile aroma compounds identified (Moio and Addeo, 1998) and 19% of the total chromatographic area (Barbieri *et al.*, 1994) for Parmesan cheese.

Methyl, ethyl, propyl, butyl, and isobutyl esters of even-numbered carbon chain acids from C2 to C16 are found in Parmesan cheese (Barbieri *et al.*, 1994; Careri *et al.*, 1994; Meinhart and Schreier, 1986). The esters with the highest concentrations in Parmesan cheese are the ethyl esters of shorter-chain even-numbered fatty acids, C4 through C10 (Meinhart and Schreier, 1986), where

Table 19.4 Aroma attributes and sensory thresholds of some volatile esters commonly found in Parmesan cheese

Compound	Aroma attributes	Threshold (ppm)	Media
Ethyl acetate	solvent, fruity	0.5–12.2	water
		22	oil
Ethyl propanoate	pineapple, fruity	0.01	water
Ethyl butanoate	pineapple, banana, melon	0.015	water
		0.6	oil
Methyl hexanoate	pineapple, fruity	0.05	water
Ethyl hexanoate	pineapple, banana, fruity	0.001–0.012	water
		0.04–0.85	oil
Ethyl octanoate	apricot, fruity, fatty, floral	0.032–0.07	water
Ethyl decanoate	fruity, grape (cognac)	0.49	water
Ethyl dodecanoate	fruity	0.33	water
3-Methylbutyl acetate	banana, apple peel	0.002	water
Isoamyl acetate	pear, banana, powerful	0.002	water
2-Phenylethyl acetate	floral, rose, honey	19	water
		18.5	oil

ethyl butanoate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate account for 95% of the total ester concentration (Moio and Addeo, 1998). These esters have strong fruity, floral notes that are commonly described as pineapple-, banana-, apricot-, pear-, and floral-like. Aromatic esters such as 2-phenylethyl acetate, 2-phenylethyl propanoate and 2-phenylethyl butanoate are found in Parmesan cheeses and are described as having floral, rose-like aromas.

Most of these esters have very low detection thresholds (Table 19.4). Both aroma extract dilution analysis (AEDA) and OAV studies have shown that ethyl butanoate, ethyl hexanoate and ethyl octanoate are the primary esters responsible for the fruity aroma in Parmigiano-Reggiano cheese, while numerous other esters provide additional contributions (Qian and Reineccius, 2003a, b, c). When the extract of volatile compounds from block Parmesan cheese is compared to that of grated Parmesan cheese, the presence of fewer ethyl esters is observed in grated cheese, which correlates to its lack of fruity flavor (Dumont *et al.*, 1974).

Ketones

Ketones are found to be among the major components in the headspace of Parmesan cheese (Barbieri *et al.*, 1994). These compounds are the products of autooxidation of unsaturated fatty acids, a process that first generates hydroperoxides, which then decompose to form carbonyl compounds (ketones and aldehydes) along with hydrocarbons and alcohols (Dumont and Adda, 1978). The β -oxidation pathway of FFAs generally produces methyl ketones with one carbon less than the parent fatty acid. By using dynamic headspace and simultaneous distillation–extraction techniques, Barbieri *et al.* (1994) and Careri *et al.* (1994) found that methyl ketones are the major constituents in Parmesan cheese. However, the extremely high concentrations of 2-heptanone and

2-nonanone in some cheeses indicate that the corresponding fatty acids may not be the sole source for these methyl ketones. An oxidation study of ^{14}C -labeled palmitic (C16) and lauric (C14) acids by *Penicillium roqueforti* spores revealed that successive β -oxidation cycles of long-chain fatty acids are involved in the generation of short-chain methyl ketones (Okumura and Kinsella, 1985; Kinsella and Hwang, 1976; Dartey and Kinsella, 1973). The β -oxidation pathway is metabolically important since it allows some microorganisms to detoxify FFAs or metabolize FFAs for energy. At low concentrations FFAs are completely oxidized to carbon dioxide where only small amounts of methyl ketones may be produced (Margalith, 1981).

Both methyl and ethyl ketones, with carbon chain lengths from C3 to C15 and from C5 to C8, respectively, are present in Parmesan cheese. Table 19.5 shows the sensory thresholds for some of these ketones. Methyl ketones, particularly 2-heptanone and 2-nonanone with fruity, blue cheese-like aromas, are the most abundant compounds in this class and contribute to the flavor of cheese (Qian and Reineccius, 2003a; Meinhart and Schreier, 1986); these carbonyl compounds are normally dominant in mold-ripened cheeses, such as Camembert and Blue cheese (Qian *et al.* 2002, Molimard and Spinnler, 1996; Behnke, 1980); however, they may also play some small role in the aroma of Parmesan, as shown by GC/O and OAV studies (Qian and Reineccius, 2003a).

1-Octen-3-one is the most important ethyl ketone identified in Parmesan, Grana Padano, and Pecorino cheeses and contributes a strong earthy, mushroom-like aroma (Frank *et al.*, 2004). Other unsaturated ketones, such as 8-nonen-2-

Table 19.5 Aroma attributes and sensory thresholds of some volatile ketones commonly found in Parmesan cheese

Compound	Aroma attributes	Threshold (ppm)	Media
Acetone	acetone-like, pungent	500	water
		125	oil
2-Butanone	acetone-like, etheric	50	water
		30	oil
2-Pentanone	floral, fruity, wine, acetone-like	2.3	water
		61	butter
2-Hexanone	floral, fruity	0.93	water
2-Heptanone	Blue cheese, fruity, sweet	0.14	water
		1.5–15	butter
2-Octanone	fruity, musty, unripe apple, green	2.5–3.4	butter
3-Octanone	fruity, penetrating, floral (lavender)	0.028–0.05	water
1-Octen-3-one	mushroom, earthy, metallic	0.01	oil
2-Nonanone	fruity, musty, rose, tea-like	0.2	water
		7.7	cheese
2-Decanone	fruity, musty	0.19	water
2-Undecanone	floral, herbaceous, fruity	0.007–5.4	water
		3.4	oil
Acetophenone	orange blossom, sweet, pungent	0.065–0.17	water

one, are also found in Parmesan cheese (Qian and Reineccius, 2003a) along with some phenyl-substituted ketones including acetophenone and 4-methylacetophenone (Barbieri *et al.*, 1994; Meinhart and Schreier, 1986), both with strong musty, floral notes, reminiscent of orange blossoms (Adda and Dumont, 1974). Other identified ketones include 3-methyl-2-butanone (camphor-like odor), 3-penten-2-one (fruity), 4-methyl-3-penten-2-one (sweet, chemical), and geranyl acetone (rose-like) (Barbieri *et al.*, 1994; Careri *et al.*, 1994; Meinhart and Schreier, 1986); however, the sensory thresholds are not well studied for most of these compounds. The dicarbonyl compound 2,3-butanedione is reported to have a moderate flavor dilution (FD) value by AEDA analysis (Qian and Reineccius, 2003b) and contributes to the buttery note observed in Parmigiano-Reggiano cheese (Virgili *et al.*, 1994).

Aldehydes

The total amount of aldehydes present in cheese is relatively low, compared to most other classes of compounds. In Grana Padano, aldehydes represent only 0.6% of the total neutral volatiles (Moio and Addeo, 1998). Along with ketones, aldehydes are produced through the autooxidation of fatty acids. Long-chain aldehydes could be formed by α -oxidation of fatty acids. Some branched-chain and aromatic aldehydes are formed from Strecker degradation of amino acids, where decarboxylation followed by oxidation leads to aldehydes. For example, phenylacetaldehyde is a Strecker degradation product of phenylalanine. The oxidation reaction can occur without enzymatic catalysis during cheese ripening.

Acetaldehyde, pentanal, hexanal, heptanal, octanal, nonanal, decanal, tetradecanal, pentadecanal and hexadecanal are the major straight-chain aldehydes found in Parmesan cheese (Barbieri *et al.*, 1994; Careri *et al.*, 1994; Meinhart and Schreier, 1986). Unsaturated straight-chain aldehydes, such as t-2-butenal, t-2-pentenal, t-2-hexenal, t-2-heptenal, t-2-nonenal, and t,t-2,4-hexadienal have also been identified in Parmesan cheese (Frank *et al.*, 2004; Qian and Reineccius, 2003a, b; Barbieri *et al.*, 1994; Careri *et al.*, 1994). Most of these aldehydes have low FD values in Parmesan cheese (Qian and Reineccius, 2003a, b). All of these aldehydes have low sensory thresholds, and are associated with green, grass-like odors (Table 19.6). The shorter-chain aldehydes are also considered to be pungent and malty, and the longer-chain aldehydes have more fatty and citrus-type notes.

Major branched-chain aldehydes identified in Parmigiano-Reggiano cheese are 2-methylpropanal, 2-methylbutanal and 3-methylbutanal. These compounds have strong malty, green, and cocoa-like aromas along with high FD and OAV values (Qian and Reineccius, 2002a, c). Other aldehydes found in Parmesan cheese are furfural, 5-methylfurfural, and benzaldehyde; all have sweet almond-like aromas, but have weak intensities by GC/O. Phenylacetaldehyde, with its rosy, green aroma, is found to have a strong aroma intensity by GC/O and high OAV in Parmigiano-Reggiano (Qian and Reineccius, 2002a, 2003a, c), meaning that this compound is likely to play a key role in providing Parmigiano-Reggiano cheese with some of its floral-type aroma characteristics.

Table 19.6 Aroma attributes and sensory thresholds of some volatile aldehydes commonly found in Parmesan cheese

Compound	Aroma attributes	Threshold (ppm)	Media
Acetaldehyde	pungent, penetrating, fruity	0.025	water
		0.0002	oil
Propanal	pungent, acrid, solvent	0.037	water
		0.009	oil
2-Methylpropanal	malty, cocoa, green, pungent	0.002	water
2-Methylbutanal	cocoa, coffee, almond, malty	0.002–0.14	oil
3-Methylbutanal	malty, cocoa	0.013	oil
Butanal	pungent, malty, green	0.018	water
Pentanal	malty, apple, green	0.012–0.07	water
Hexanal	grassy, green, tallow	0.009–0.05	water
		0.19–0.3	oil
t-2-Hexenal	green, sweet, leafy, apple	0.017–0.05	water
		0.42	oil
Heptanal	fatty, green, woody, fruity	0.031–0.25	oil
Octanal	Fatty, citrus	0.001	water
		0.056	oil
Nonanal	citrus, green, fatty, floral	0.002	water
		1	oil
Decanal	waxy, floral, citrus	0.002	water
Dodecanal	citrus, powerful	0.0005–0.002	water
Furfural	sweet, almond, penetrating	3	water
Phenyl acetaldehyde	floral, hyacinth, green	0.002	water

Alcohols

Numerous alcohols are found in Parmesan and other hard Italian-style cheese varieties. This class of compounds is comprised of primary, secondary, and tertiary straight-chain alcohols along with various branched-chain and ring-structured alcohols. Alcohols are generated through a variety of fermentation pathways. Many of the primary straight-chain alcohols are originated from the action of alcohol dehydrogenase on the aldehyde-products of fatty acid and amino acid metabolism in microbes (Behnke, 1980).

In addition to ethanol, other primary straight-chain alcohols such as 1-propanol and 1-butanol, with fruity-type and green aromas, are also abundant in cheese. In Grana Padano cheese (Moio and Addeo, 1998), primary straight-chain alcohols with lengths of C4 through C7 have been identified and are described as having medicinal, green, and woody aromas (Table 19.7). Most of these alcohols have high sensory thresholds and only contribute only slightly to cheese aroma. Longer straight-chain primary alcohols have very little aroma, if any. However, 1-octen-3-ol, with a mushroom-like aroma similar to its corresponding ketone, is an important aroma contributor to aged cheese due to its low sensory threshold (Karahadian *et al.*, 1985a, b). This compound appears in significant quantities late in the cheese ripening process; however, an excessive concentration of 1-octen-3-ol produces a defective metallic-like aroma (Moio and Addeo, 1998).

Table 19.7 Aroma attributes and sensory thresholds of some volatile alcohols commonly found in Parmesan cheese

Compound	Aroma attributes	Threshold (ppm)	Media
Ethanol	alcoholic	100–200	water
Propanol	alcoholic, fruity, sweet	9–45	water
Butanol	fruity, green, medicinal	0.5–7.5	water
3-Methylbutanol	alcoholic, green, floral, malty	0.025–4.7	water
Pentanol	green, fusel oil, woody	4.5	water
Hexanol	green, floral	0.05–2.5	water
Heptanol	fatty, sweet, green	0.52–2.4	water
2-Butanol	fruity	5.1	water
2-Pentanol	fruity, green, fusel oil	8.5	water
2-Heptanol	fruity, brassy, herbaceous	0.41	water
1-Octen-3-ol	mushroom	0.001	water
Furaneol	caramel, burnt sugar	0.031	water
2-Phenylethanol	honey, spicy, floral	0.12–1	water

While alcohols may have only limited roles in the aroma of hard Italian-style cheeses, they are important for the formation of esters, which have a much greater contribution to the aroma of Parmesan cheese.

Secondary alcohols are also prevalent in hard Italian-style cheeses, being products of the enzymatic reduction of their corresponding methyl ketones. With linear chain lengths of C4 through C9, the secondary alcohols found in Parmigiano-Reggiano and Grana Padano in the greatest quantities are 2-butanol, 2-pentanol, and 2-heptanol (Moio and Addeo, 1998; Barbieri *et al.*, 1994). The odors of these secondary alcohols have been described as fruity, green, fusel oil-like, and earthy. However, their contribution to overall aroma is likely to be low since all of these compounds have weak aromas in Parmigiano-Reggiano cheese (Qian and Reineccius, 2002a, 2003b). Additionally, since the shorter-chain secondary alcohols have high sensory thresholds, any contribution they may have toward aroma is likely to be negligible (Meinhart and Schreier, 1986).

Some branched-chain alcohols are also found in Parmesan cheese. 3-Methylbutanol, with a nail-polish, green and floral note, has a moderately high FD value by AEDA (Qian and Reineccius, 2003b); therefore, it may contribute to the overall aroma. Branched-chain alcohols can be formed through Strecker degradation of amino acids. In this process, oxidative deamination will convert amino acids, such as valine or leucine, to α -keto acids then, following decarboxylation, aldehydes will be formed and reduced to their corresponding alcohols (Fox *et al.*, 2000).

Furaneol, a ring-structured alcohol, has a strong caramel-like aroma in Parmesan cheese (Qian and Reineccius, 2002a, 2003a). Furaneol was also found in Grana Padano cheese (Moio and Addeo, 1998) and has a strong aroma in the same type of cheese (Frank *et al.*, 2004). However, the detection of this compound is highly dependent on the isolation technique employed in the analysis. Since furaneol is very polar and has a high boiling point, ineffective extraction

will underestimate the importance of this compound to Parmesan (Qian and Reineccius, 2003a).

Lactones

Lactones are cyclic esters that usually have pronounced fruity aromas associated with peaches, apricots, and coconut (Table 19.8). These compounds are formed by intramolecular esterification of hydroxyacids where the loss of water results in ring closure (Fox *et al.*, 2000). Lactones are also formed in the ruminant mammary gland from the hydrolysis of saturated fatty acids and subsequent cyclization of the free hydroxyacids (Dumont and Adda, 1978). Both γ - and δ -lactones are present in milk and therefore occur in all types of cheese, forming spontaneously when γ - and δ -hydroxyacids are released from triglycerides. In Parmesan cheese, the major lactones are δ -octalactone, γ - and δ -decalactone, and γ - and δ -dodecalactone (Barbieri *et al.*, 1994; Meinhart and Schreier, 1986), while some penta-, hexa-, hepta-, and also tetradeca-, lactones are found as well. Lactones have low volatilities, therefore studies employing headspace techniques usually do not report the presence of these compounds. However, a study using high vacuum distillation found that lactones represented about 0.1% of the total neutral chromatographic area of Grana Padano (Moio and Addeo, 1998).

Lactone concentration in cheese is directly related to their concentration in milk, which is affected by the type of feed, cow, and season (Fox *et al.*, 2000) as well as the way the cheese is manufactured. Since heating can cause hydroxy-

Table 19.8 Aroma attributes and sensory thresholds of some major lactones commonly found in Parmesan cheese

Compound	Aroma attributes	Threshold (ppm)	Media
γ -Hexalactone	coconut, fruity, sweet	1.6–13 8	water oil
γ -Heptalactone	coconut, fruity, nutty	0.52 3.4	water oil
δ -Octalactone	coconut, animal	0.4–0.57 0.1–3	water oil
γ -Octalactone	coconut, fruity	0.095 3.5	water oil
γ -Nonalactone	coconut, peach	0.065 2.4	water oil
δ -Decalactone	coconut, apricot	0.1–0.16 0.4–1.4	water oil
γ -Decalactone	coconut, apricot, fatty	0.005–0.09 1	water oil
δ -Dodecalactone	fresh fruit, peach	0.1–1 0.12–10	water oil
γ -Dodecalactone	peach, butter, sweet, floral	0.007 1	water oil

acids to lactonize (Boldingh and Taylor, 1962), cheeses made with high cooking temperature, such as Parmesan and Grana Padano, have elevated amounts of lactones. While Romano cheese curds are cooked at lower temperatures, this cheese often has lower concentrations of lactones. Lactone concentration does not increase with maturation of the cheese (Moio and Addeo, 1998). However, most lactones are present at low concentration (ppb), and because their sensory thresholds in lipid matrix are high (ppm), these compounds will have low OAVs, indicating that lactones likely have little contribution toward aroma.

Phenols

Phenolic compounds are important for some varieties of aged Italian-style cheese, especially Pecorino Romano. Aromatic amino acids are the precursors for these compounds and are converted via microbial metabolism (Gummalla and Broadbent, 2001; Ummadi and Weimer, 2001; Lopez and Lindsay, 1993; Brewington *et al.*, 1973). The odor qualities of phenolic compounds are described as smoky, unclean, medicinal, cowy, sheepy, and barnyard-like. At concentrations close to their sensory threshold levels, these compounds can provide a desirable background note; however, once the concentration becomes too high, the aroma tends to become unpleasant (Ha and Lindsay, 1991a). Substituted phenols, such as *p*-methylphenol (commonly known as *p*-cresol) or 2-isopropyl-5-methylphenol (thymol), have much stronger aromas than unsubstituted phenol. Pecorino Romano, made from sheep's milk, contains much higher levels of *p*-cresol, *m*-cresol, and 3,4-dimethylphenol than Romano made with cow's milk, where the sheep-like aromas of these compounds confirm that their presence is important to characterize cheese made with ovine milk (Ha and Lindsay, 1991b). Parmesan cheese contains fewer phenolic compounds (Barbieri *et al.*, 1994; Ha and Lindsay, 1991b; Meinhardt and Schreier, 1986), where the low concentrations of *p*-cresol, *m*-cresol, and guaiacol may provide some positive notes for the background aroma of this cheese.

Sulfur compounds

Many sulfur compounds have been identified in numerous varieties of cheeses including Parmesan cheese (Frank *et al.*, 2004; Qian and Reineccius, 2003a, b, 2002a; Barbieri *et al.*, 1994) and they play a key role in overall cheese flavor (McSweeney and Sousa, 2000; Molimard and Spinnler, 1996; Urbach, 1993). Sulfur compounds in cheese are from the degradation of sulfur-containing amino acids such as methionine and cysteine (McSweeney and Sousa, 2000; Weimer *et al.*, 1999). Both enzymatic and chemical degradation produces volatile sulfur compounds (Seefeldt and Weimer, 2000; Gao *et al.*, 1998; Shankaranarayana *et al.*, 1971; Grill *et al.*, 1967). The occurrence of some sulfur compounds is also thought to be related to the season in which the milk is procured, presumably due to differences in feed (Shooter *et al.*, 1999; Manning *et al.*, 1976). Most sulfur compounds are described as having strong alliaceous, cabbage or very ripe cheese aroma (Cuer *et al.*, 1979) and have very low odor thresholds (Table 19.9), thus are considered as important flavor contributors.

Table 19.9 Aroma attributes and sensory thresholds of some volatile sulfur compounds commonly found in Parmesan cheese.

Compound	Aroma attributes	Threshold (ppm)	Media
Methanethiol	rotten cabbage, fecal	0.002 0.00006	water oil
Dimethyl sulfide	boiled cabbage	0.0003 0.0012	water oil
Dimethyl disulfide	cabbage-like, strong onion	0.00016	water
Dimethyl trisulfide	very ripe cheese, garlic	0.00001 0.0025	water oil
Methional	boiled/baked potato	0.0002 0.0002	water oil

The most important sulfur compounds identified in cheese include hydrogen sulfide, methanethiol, dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide and methional. Manning and Moore (1979), using a headspace method, studied the volatile compounds of Parmesan cheese along with several other hard cheeses and found that hydrogen sulfide and methanethiol are important aroma indicators. Although hydrogen sulfide is found in cheese, its role is controversial with respect to flavor contribution (Urbach, 1993). The detection and measurement of hydrogen sulfide is very difficult due to its extremely low boiling point (-60°C); therefore, traditional aroma extraction and concentration techniques often miss this compound.

Through GC/O analyses, methanethiol has a 'very strong' aroma in Parmesan cheese, while 'mild' aromas are perceived in both Grana Padano and Pecorino cheeses. Dimethyl sulfide is 'just perceivable' in Parmesan. Dimethyl disulfide is 'very strong' in both Parmesan and Grana Padano, while dimethyl trisulfide is 'extremely strong' in Parmesan, Pecorino, and Grana Padano cheeses (Frank *et al.*, 2004; Qian and Reineccius, 2002a). Dimethyl trisulfide has a garlic and overripe cheese aroma. 3-Methylthiopropional, or methional, is found to be a key aroma compound of various cheeses (Qian *et al.*, 2002; Milo and Reineccius, 1997; Preininger and Grosch, 1994; Preininger *et al.*, 1994) including Parmesan cheese (Qian and Reineccius, 2003a; Qian and Reineccius, 2003b; Barbieri *et al.*, 1994). This sulfur compound has a distinctive baked potato-like aroma. Methional may be generated by Strecker degradation of methionine. Methional can be oxidized to its acid form where corresponding esters can then be formed. Ethyl 3-methylthiopropionate has been found as a trace component among the volatiles from Parmesan cheese (Meinhart and Schreier, 1986).

19.3.3 Basic compounds: pyrazines

The basic fraction of Parmesan cheese contains mostly heterocyclic nitrogen-containing compounds, such as alkylpyrazines. These compounds have characteristic nutty, roasted, and cocoa-like aromas. Pyrazines may be formed through

either Maillard reactions or microbial synthesis (Dumont and Adda, 1978; Morgan, 1976). There are many variations of pyrazines identified in hard Italian-style cheeses, including the 2,3-, 2,5-, and 2,6-dimethylpyrazine isomers, along with other pyrazines containing differing methyl and ethyl constituents. For example, in the basic fraction of Parmigiano-Reggiano cheese, 2,3-dimethylpyrazine, 2,6-dimethylpyrazine, 3-ethyl-2,5-dimethyl pyrazine, trimethylpyrazine, 5-ethyl-2-methylpyridine, 5-ethyl-2,3-dimethylpyrazine, and 6-ethyl-2,3,5-trimethylpyrazine are all identified for the baked, nutty aroma of this cheese (Qian and Reineccius, 2002a; Barbieri *et al.*, 1994). Similarly, Grana Padano cheese has been found to contain 2,3-dimethylpyrazine, 2,6-dimethylpyrazine, trimethylpyrazine, 3-ethyl-2,5-dimethylpyrazine and 2-methyl-3,5-diethylpyrazine (Moio and Addeo, 1998). Many of these nitrogen-containing compounds contribute a strong nutty aroma in hard Italian-style cheese (Frank *et al.*, 2004; Qian and Reineccius, 2002a; Moio and Addeo, 1998; Meinhart and Schreier, 1986). More studies are needed to understand the contribution of pyrazines to the nutty aroma of cheeses.

19.4 References for production methods

A comprehensive online reference for the rules and regulations that are applied to the production of Parmigiano-Reggiano, including the feeding program of the cows to the specific manufacturing procedures, and the classification of the final product, can be found at the official website of the Consorzio del Formaggio Parmigiano-Reggiano at <http://www.parmigiano-reggiano.it>. For similar information on the manufacture of Grana Padano and Pecorino Romano, visit the Consorzio per la Tutela del Formaggio Grana Padano at <http://www.granapadano.com> and the Consorzio per la Tutela del Formaggio Pecorino Romano at <http://www.pecorinoromano.net>.

19.5 References

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Low temperature hard cheeses and semi-hard washed cheeses

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20.1 Introduction

This chapter covers the production of low temperature hard cheeses, specifically Cheddar, and semi-hard washed cheeses such as Gouda and Edam. Special emphasis focuses on the flavor development of these cheeses, and future trends in methods of manufacture of full and low-fat cheeses with improvement of flavor and quality.

The classification of cheeses is extremely complicated, due to the broad range of cheese varieties and variants; however, attempts are being made to better discriminate each variety, though no scheme has been universally accepted (Fox *et al.*, 2000). Cheese has been traditionally categorized based on composition, most commonly being moisture content (McSweeney *et al.*, 2004, Scott *et al.*, 1998a). Hard cheeses can be characterized by texture as extra-hard, hard or semi-hard, with some overlap of varieties between many countries (Fox *et al.*, 2000, McSweeney *et al.*, 2004). Overall, extra-hard cheeses typically have 51% or higher water-in-fat-free substance (WFF) and greater than 60% fat-in-dry-matter (FDM), while hard cheeses have 49–55% WFF and 40–60% FDM, and semi-hard cheeses contain 53–63% WFF and 25–50% FDM (Scott *et al.*, 1998a). Table 20.1 provides more detail on the general composition of selected types of hard cheeses.

In one of the most popular books on cheese, Frank Kosikowski (Kosikowski and Mistry, 1997) writes, ‘Cheddar cheese originated many decades ago in the little village of Cheddar, England, from where it spread throughout the world. The unique step of “cheddaring” was standardized to a common commercial

Table 20.1 General composition and ripening conditions of selected hard type cheeses

Cheese type	Country	Type of milk	Starter	Curd cooking (scalding)	Curd washing and/or whey off	Ripening conditions	Maturation time	Moisture (% max.)	Fat (%)	FDM (% min.)	Salting	pH	Texture
Extra hard													
Parmesan	Italy	Semi-skilled raw milk; gravity creaming (2.0–2.8% fat)	1% mixture <i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricum</i> and <i>Streptococcus thermophilus</i>	53–55°C	Whey off when acid whey from curd reaches 0.19%	16–18°C 85% ERH	>2 years	30.8	28.4	32.0	2.6%: Brine-salted 14–15 days, dry salt rub each day	5.5–5.6	Hard, grainy
Romano (Pecorino)	Italy	Sheep's milk raw or thermized	Thermophilic starter (commercial or whey based)	45–48°C	Whey off at 0.22% TA	15–18°C 75–80% ERH	8–12 months	31.0	29.0	38.0	5.5%: dry-salting 30–60 days	5.3–5.4	Firm but harsh
Sbrinz	Switzerland	Full-fat raw cow's milk (3.75–3.9%)	0.3–0.5% Natural thermophilic sour whey starter (lactococci) to 2°C	54–56°C	Curd washing at 4% by volume cold water to whey	16–20°C	6–12 months	31.0	32.0	47.0–50.0	Dry- or brine-salted 18–22 days	5.0–5.5	Hard, grainy when broken
Hard (with eyes)													
Emmental (Swiss)	Switzerland	Raw cow's milk (3.0–3.1% fat)	Mixed thermophilic starter of <i>Sc. thermophilus</i> , <i>Lactobacillus</i> species and added <i>Propionibacterium freudenreichii</i> subsp. <i>shermanii</i>	54°C	Whey off by stirring curds and collecting in woven cloth into a hoop and running off whey	Cool room: 10–14 days 10–15°C 90% ERH Hot room: 3–6 weeks 20–22°C 80–83% ERH Matured: 7°C	4 months	41.0	30.5	43.0	1.2%: brine-salted and dry-salted	5.6	Numerous large, round holes 1–2 cm diameter, firm

Table 20.1 Continued

Cheese type	Country	Type of milk	Starter	Curd cooking (scalding)	Curd washing and/or whey off	Ripening conditions	Maturation time	Moisture (% max.)	Fat (%)	FDM (% min.)	Salting	pH	Texture
Gruyère	France	Cow's milk (3.2% fat)	0.5–0.7% <i>Streptococcus thermophilus</i> and <i>Lactobacillus helveticus</i> mixture and added <i>Propionibacterium freudenreichii</i> subsp. <i>shermanii</i>	52–54°C	Whey off by lifting curds from whey by a steel strip covered with cloth	Surface flora: 2–3 weeks 10°C, 2–3 months 15–18°C 90–95% ERH, matured at 12–15°C	8–12 months	39.0	30.0	45.0	1.1%: rubbed into cheese surface and immersed in brine 2–6 days	5.7	Large eyes, surface flora
Hard Cheddar	UK	Pasteurized whole cow's milk (factor)	1.5–3% <i>Lc. lactis</i> subsp. <i>cremoris</i> or <i>Lc. lactis</i> subsp. <i>lactis</i>	37–39°C	Whey off by removing whey when curd is dry	4–8°C	0.5–2 years	39.0	32.0	48.0	1.5%: sprinkled on curd	5.4	Uniform, closed, firm
Cantal	France	Cow's milk fresh with natural heat (3.1% fat)	1–3% mixed mesophile lactic starter	No cooking, whey drained and cheddared	Whey off by pressing curd in vats with racks or plates	8–10°C	3–6 months	33.0	26.0–30.0	45.0	1.8–2.5%: dry-salted	5.7–5.85	Firm, pliable, curd fissures
Cheshire	UK	Cow's milk	Mixed lactic organisms	32–35°C	Whey off when acidity reaches 0.21–0.23%	6–8°C	6–9 months	44.0	35.0	48.0	1.85%: dry-salted	4.9	Open short silky to granular
Idiazábal	Northern Spain (Basque)	Raw ewe milk (6% fat)	None or natural culture in milk	38°C (coagulation) cooled to 25°C	Coagulum broken and ladled into molds	10–12°C Matured in caves, smoked in beechwood kilns	2 months, further matured for <1 year	33.2	37.8	45.0	Brining or dry-salting 2 days	5.03	Firm, dry texture, few small eyes

Leicester	UK	Cow milk	Mesophilic starter	35–37°C	Curds are pitched and whey is drawn at 0.155–0.17% acidity	10–15°C	4–8 months	42.0	33.0	48.0	1.8–2.0%: dry-salted	6.5	Close, dry and buttery with some granular openings
Manchego	Spain	Ewe milk – artisanal (raw milk) or commercial (pasteurized)	Artisanal: no culture Commercial: 2% lactic starter culture and 0.5% thermophilic culture	36–40°C	Whey off before placed in molds and pressing	10–12°C 85% ERH	10–12 months	35.5	33.6	57.0	1.5%: brine-salted 2 days, dry-salting 1 day or both	5.05	Slightly pliable, firm to hard
Roncal	Northern Spain	Raw ewe milk	Indigenous flora of the milk	37–40°C	Whey off by slowly removing whey and pressing curds on side of vat	Smoked and ripened 10–12°C	6–8 months	29.4	38.8	60.0	Dry-salted or brining for 1 day	5.10	Hard, pore, without eyes
Semi-hard Caerphilly	UK	Pasteurized cow's milk (3.2–3.5% fat)	0.25% mixed cultures	32–34°C	Drawing whey at 0.2–0.212% acidity, curd scooped to conical mass at corners and sides of vat	10–13°C Rapid maturation	10–14 days	46.0	34.0	48.0	1.5%: dry salted (1%), pressed curds brine salted	5.4	Close with open spaces
Colby	USA	Cow's milk	1.5% of <i>S. lactis</i> and/or <i>S. cremoris</i>	31–39°C Stirred curd Cheddar type	Whey drained to curd level, curd washed with cold water to 26°C	3–4°C	2–3 months	42.0	29.0	50.0	Dry salted	5.2–5.4	Higher moisture, softer cheese, open texture
Edam	Netherlands	Semi-skimmed cow's milk (≈2.5% fat)	0.3–1% DL culture	50–60°C	Whey off by placing flat plates to consolidate curd, whey is taken off	12–14°C	6–30 weeks	45.0	24.0	40.0	2.0%: brine-salted 2–4 days	5.0	Small eyes, firm, elastic, pliant

Table 20.1 Continued

Cheese type	Country	Type of milk	Starter	Curd cooking (scalding)	Curd washing and/or whey off	Ripening conditions	Maturation time	Moisture (% max.)	Fat (%)	FDM (% min.)	Salting	pH	Texture
Gouda	Netherlands	Pasteurized cow's milk	0.5–1% mesophilic DL starter, nitrate added to prevent growth of <i>Clostridium</i> spp. (prevent 'late glass blowing')	36–38°C	Hot water wash to remove lactose to reduce acidity after curds molded	15°C	2–3 months to <2 years	45.0	28.5	48.0	2.0%: brine-salted 3–5 days	5.3–5.5	Small eyes, smooth and creamy, firm and flexible, round or oval gas holes in curd
Monterey Jack	USA (California)	Whole cow's milk	1% meophilic culture	39°C	Whey drained until 2.5 cm remains above curd, cold water added to reduce curds/whey to 0.030°C	8–10°C	5–7	44.0	25.0	50.0	2.5%: dry-salted	5.3	Mechanical openings, soft texture, plastic texture
Semi-hard (internally ripened with molds)													
Stilton	UK	Cow's milk (3.8% fat)	Mesophilic DL starter (<0.04%) and <i>P. roquefortii</i> spores	26–30°C (kept warm for 7 days for whey drainage)	Cured ladled into cloths placed in Stilton sink, whey allowed to escape from bags and bags tightened every hour	Cool room: 6–7 weeks 13–15°C 85–90% RH, pierced 2–3 weeks mold growth, stored 5°C	6–15 months	42.0	33.0	48.0	3.5%: dry-salted	5.2	Soft, open texture

Roquefort	France	Raw ewe's milk (8% fat)	Acidified by indigenous microflora and <i>P. roquefortii</i>	Curds not cooked, mixed with spores of <i>P. roquefortii</i>	Whey off by draining off whey to curd depth and stirred as necessary	Ripened in limestone caves in Southern France, 5–10°C 95% RH	5–10 months	45.0	31.0	50.0	3.5%: dry-salted (3 days)	6.4	Soft, fatty texture, close curd sometimes short and crumbly
Gorgonzola	Italy	Pasteurized cow's, sheep's or goat's milk	1–2% starter culture and <i>P. roqueforti</i> added separately	Curds not cooked	Stirred curd, whey removed to curd level, curd scooped into coarse cloth on draining tray	4°C	5 months	42.0	27.0	48.0	Salt rubbed into rind 2–3 days	5.8–6.0	Smooth firm surface, soft
Blue Cheese	USA	Cow's milk (1–1.5% fat)	<i>P. roqueforti</i>	Curds not cooked/ scalded (or at low temperatures below 2°C)	Stirred curd, then whey off by draining whey and allowing curd to compact in drainers or molds	10–16°C RH (that favors mold growth)	4–9 months	46.0	29.0	50.0	4.5%: dry-salted (on surface or during milling before molding)	6.5	Soft texture, no or few irregular holes

Source: adapted from Buch Kristensen (1999), Fox *et al.* (2000), Gobbetti (2004), McSweeney *et al.* (2004) and Robinson and Wilbey (1998a, b).

practice by Joseph Harding in 1857 to repress the growth of gas-forming spoilage organisms.’ Today the popularity of Cheddar cheese prevents it from having a protected designation of origin. Cheddaring, in the lingo of cheese making, means piling and re-piling blocks of warm curd in the cheese vats for about two hours or until a designated acidity is measured in the curd (Kosikowski and Mistry, 1997). Moisture control, flattening of the curd, expulsion of whey and proper texture development of this particular cheese are also attained in this step. Again, according to Kosikowski, ‘In 1867 Robert McAdam popularized this concept of cheese making in upper New York State, leading directly to the evolution of the vast American Cheddar cheese industry.’

Once arriving in America, coloring was often used for Cheddar cheese. This is often done by the use of food colorings, such as Annatto, which is extracted from the tropical achiote or annatto tree (*Bixa orellana*). The active ingredient is bixin, which becomes norbixin when extracted with alkali. The origins of this practice are clouded in the annals of history, but the three leading theories appear to be to allow the cheese to have a consistent color from batch to batch, to assist the purchaser in identifying the type of cheese when it is unlabeled, or to identify the cheese’s region of origin (European or American).

Cheddar cheese today includes a number of varieties based on slight variations in processing conditions, usually based on water addition during processing. The main varieties of Cheddar cheese include reduced or low-fat Cheddar, stirred curd or granular cheese (no cheddaring step, used for bulk pressed cheeses), and washed curd varieties (Colby and Monterey) (Lawrence *et al.*, 2004). The most common variations of Cheddar cheese are the stirred curd variants Colby and Monterey (or Monterey Jack). The manufacture of Colby and Monterey Jack originates in the United States, with similar processing as Cheddar until after cooking. These cheeses have a softer, plastic texture compared to Cheddar since the stirring of the curd prevents the development of curd structure, resulting in higher moisture content, accelerated ripening times and milder flavor (Fox *et al.*, 2000). Other variants of Cheddar include the varieties Leicester, Double Gloucester, Derby and Dunlop, which are well known only in their region of manufacture due to poor keeping quality, but differ from Cheddar only in higher moisture and lower calcium content (Lawrence *et al.*, 1993).

The fermentation of Cheddar is done by the starter culture, which is lactococci and sometimes a combination of flavor adjunct bacteria. It is a cheese that requires rennet, cooking of curd, drain of whey, and cheddaring. Once the piling and re-piling of the curd (cheddaring) reaches the required acidity, the blocks of cheese are ‘milled’ (cut to small cubes), salted and pressed. The flavor of Cheddar cheese is directed by the starter culture combination added and the ripening temperature.

A schematic of the most essential physical and biochemical changes occurring during cheese making can be found in Fig. 20.1.

A final point in this section is the selection of milk for these kinds of cheeses. Cheddar, Edam and Gouda cheeses are traditionally made from cow’s milk. It is commonly known that high solids and high fat milk is preferred for these

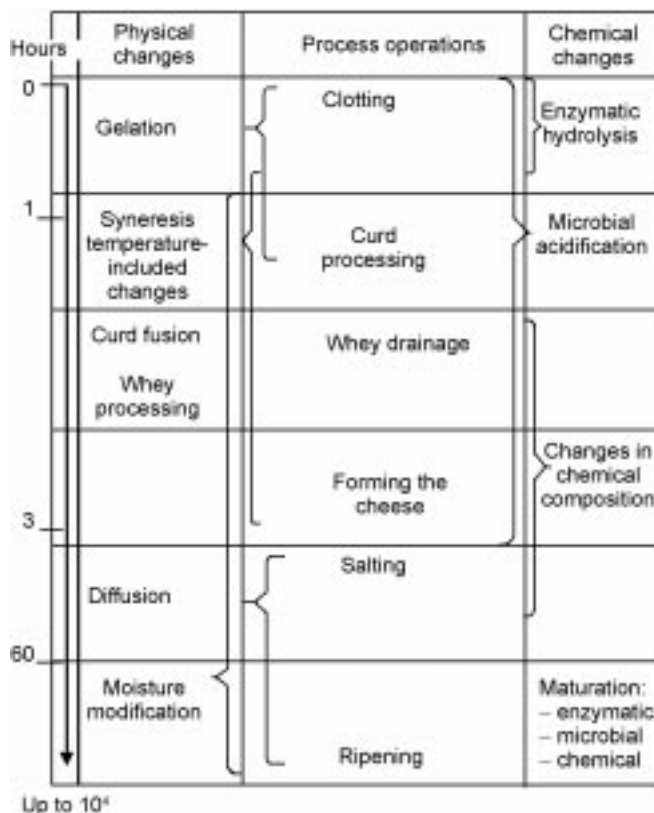


Fig. 20.1 Scheme of the most essential physical and biochemical changes occurring during the transformation of milk into cheese. Simplified example; the time scale is not linear. Adapted from Walstra *et al.* (1999c).

cheeses. Milk from the Jersey breed of cows has had some preference among cheese makers due to its higher solids and fat content. A very important trend in modern cheese manufacturing is the standardization of milk. Requirements of quality and homogeneous production throughout the year, have given place to different methods of pre-arranging the composition of the milk for cheese. The use of different commercial dairy products, such as milk powder, milk protein or even milk permeate, is valuable means of standardizing components in cheese milk (Mistry *et al.*, 1996). This cheese component standardization helps in obtaining a more homogeneous quality, especially since these kinds of cheeses are ripened, which changes the composition and structure of the cheese (Battistotti *et al.*, 1984, Walstra *et al.*, 1999a, b). When the cheese ripens, the chemical constituents that remain in the curd undergo chemical changes that determine the acidity, aroma, flavor and texture of the finished cheese (Battistotti *et al.*, 1984).

In summary, the main variations applied to manufacturing cheese include milk selection, standardization, heat treatment and pre-acidification, starter and

inoculum percentage, coagulation and type of coagulant, curd making, scalding temperature, washing of the curd, size and shape of the cheese, pressing, resting, salting, ripening temperature and time, and covering the cheese rind and packing (Walstra *et al.*, 1999b).

20.1.1 Low temperature hard cheese – Cheddar cheese

Cheddar cheese embodies a cheese made using pasteurized whole cow's milk and resulting in a cheese with firm consistency, no holes, and a flavor described as mild or pungent depending on age (Davis, 1976). The US Food and Drug Administration (FDA) standard of identity for Cheddar cheese (CFR 21.133.113) states that Cheddar cheese may be classified as a food prepared by any procedure which produces a finished cheese with a minimum milk fat of 50% by weight of the solids, and a maximum moisture content of 39% by weight (FDA, 1993). Cheddar cheese has a pH between 5.2 and 5.3 and is usually cured for 2–10 months (Walstra *et al.*, 1999b). Factors which affect the flavor of Cheddar cheese include short peptides, free amino acids, volatile compounds and fat content (Walstra *et al.*, 1999b). Fat content is an especially important contributor to consistency and flavor, acting as a solvent for hydrophobic flavor compounds, while lypolysis and formation of ketones from free fatty acids also contribute to flavor (Walstra *et al.*, 1999b).

Production of Cheddar cheese

There are many challenges in producing Cheddar cheese, mainly due to the long maturation period needed for desired flavor formation. This phase may result in the acquisition of off-flavors (Lawrence *et al.*, 1993). However, over time, changes in Cheddar cheese manufacture by incorporating computer control systems have allowed faster methods to control and change parameters during process/production cycles, thus providing better process control and consistent quality of cheese (Johnson and Lucey, 2006). In addition, the availability of reliable starter cultures has enabled cheese makers to improve the regulation of the chemical composition of these cheeses, in effect producing higher quality products (Lawrence *et al.*, 1993). There are six crucial stages in cheese making that must be followed for manufacturing of hard and semi-hard cheeses (Davis, 1976; Lawrence *et al.*, 1993). These stages comprise:

1. Acidity of renneting, involving the coagulation of milk containing a starter culture.
2. Cutting the coagulum into cubes, heating and stirring the cubes, thus producing a desired level of acid.
3. Removal and isolation of the whey curd.
4. Cheddaring the cubes of curd into large slabs.
5. Milling the cheddared curd.
6. Salting and pressing of the curd, which leads to proper matting of the curd to ultimately form a ripened cheese product.

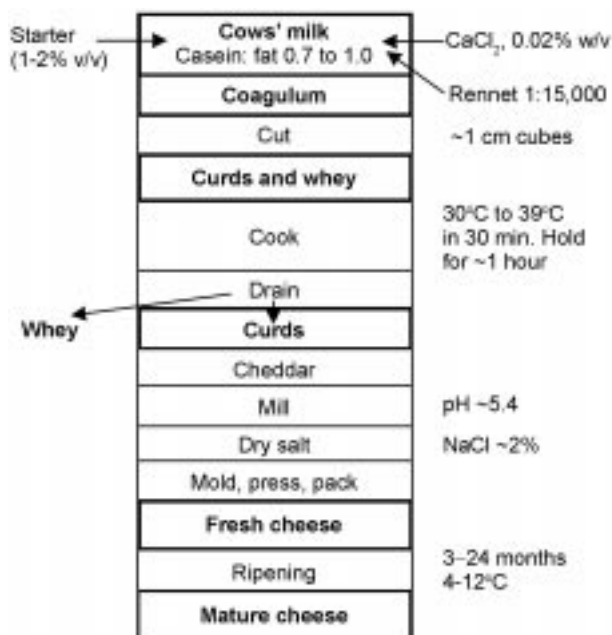


Fig. 20.2 Flow diagram for the manufacture of Cheddar cheese. Adapted from Fox and McSweeney (2004).

A flow diagram for the overall manufacturing process of Cheddar cheese is presented in Fig. 20.2.

The most characteristic feature of Cheddar cheese is ‘cheddaring’, or piling the curd, which involves cutting, turning and piling the curd in order to drain off excess whey, an extremely labor-intensive stage of processing (Davis, 1976). This process results in the formation of the fibrous texture in the curd, which occurs at $\text{pH} \leq 5.8$, developing acid in the curd and losing calcium phosphate from the protein matrix (Lawrence *et al.*, 1993). Other cheese varieties, such as Colby, also incorporate the cheddaring process into their manufacture, the only difference being in pH, moisture and calcium levels (Lawrence *et al.*, 1993).

20.1.2 Semi-hard washed cheeses – Gouda and Edam

Gouda and Edam cheeses are traditionally the two main Dutch-type cheeses produced in the Netherlands. These cheeses have a semi-hard to hard consistency, and a smooth texture with a flavor intensity that ranges greatly (Walstra *et al.*, 1993). The FDA standard of identity for Edam cheese (CFR 21.133.138) states that Edam cheese may be classified as a food prepared by any procedure which produces a finished cheese with a minimum milk fat content of 40% by weight of the solids, and a maximum moisture content of 45% by weight. The FDA standard of identity for Gouda cheese (CFR 21.133.142) must follow all the same requirements for Edam cheese, except that the minimum milk fat

content is 46% by weight, and the maximum moisture content is 45% by weight (FDA, 1993). If the dairy ingredients used for Gouda cheese are not pasteurized, the cheese must be cured at a temperature not less than 35°F for at least 60 days (FDA, 1993). The pH of these varieties can range from pH 5.0 to 5.6 (Walstra *et al.*, 1993). The variation in the character of each semi-hard cheese can depend on maturation time and environment, use of different starters, degree of acidification during curd making, whether the milk is pasteurized or not pasteurized, and contamination from microorganisms (Walstra *et al.*, 1993).

Production of Gouda and Edam cheeses

The manufacture of semi-hard cheeses has changed over time, resulting in variations of traditional varieties. In order to achieve the familiar texture and flavor characteristics of various Dutch-type cheeses, maintaining similar compositions of each cheese variety is integral. Gouda cheese was traditionally made from fresh unskimmed milk into large loaves (4–12 kg) in a cylindrical shape and matured for 6–60 weeks. Edam, on the other hand, was made from a mixture of skimmed evening milk and fresh morning milk, resulting in a cheese with 40% fat in dry matter (FDM); it was then aged for at least six months; Edam cheese tends to have a shorter texture than Gouda (Walstra *et al.*, 1993). In more recent manufacture, Gouda is typically made into 2.5–30 kg loaves and matured

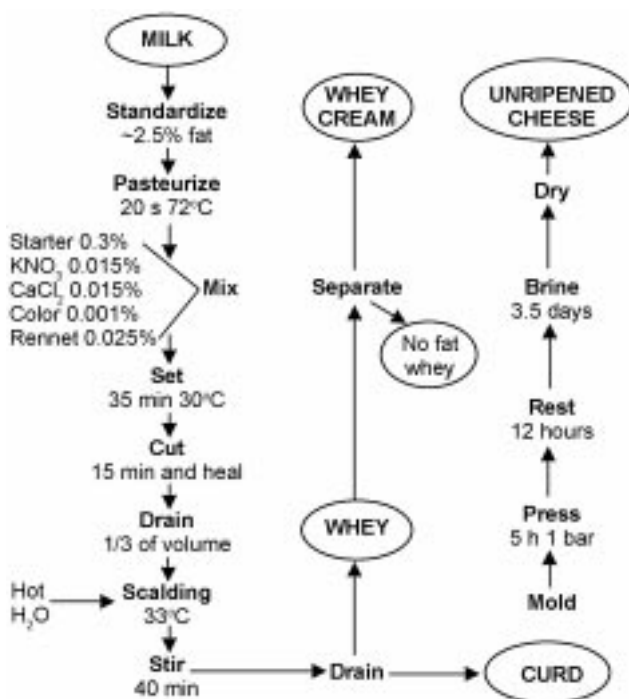


Fig. 20.3 Flow diagram for the manufacture of Edam cheese (until curing) in a fairly traditional method. Adapted from Walstra *et al.* (1993).

for 1–20 months, resulting in a cheese of 49% FDM, with 59% water content of fat-free cheese. In comparison, Edam cheese is made into 1.7–2.5 kg loaves and matured for 1–15 months, resulting in 41% FDM and 59% water content of fat-free cheese (Walstra *et al.*, 1993). Figures 20.3 and 20.4 show the flow diagrams for the main process step for the manufacture of Edam cheese and Gouda cheese, respectively.

During maturation of Dutch-type varieties, lactose, fat and protein contribute to the development of cheese properties. Unique to these varieties, the inclusion of citric acid induces eye formation; the carbon dioxide is typically produced from starters containing mesophilic lactococci and leuconostocs (Walstra *et al.*, 1993). Semi-hard cheeses are pressed to have a closed rind that is salted in brine and matured from one week to two years; this process allows for significant proteolysis, contributing to the development of cheese flavor. Renneting enzymes, milk proteinases and starter culture enzymes facilitate proteolysis

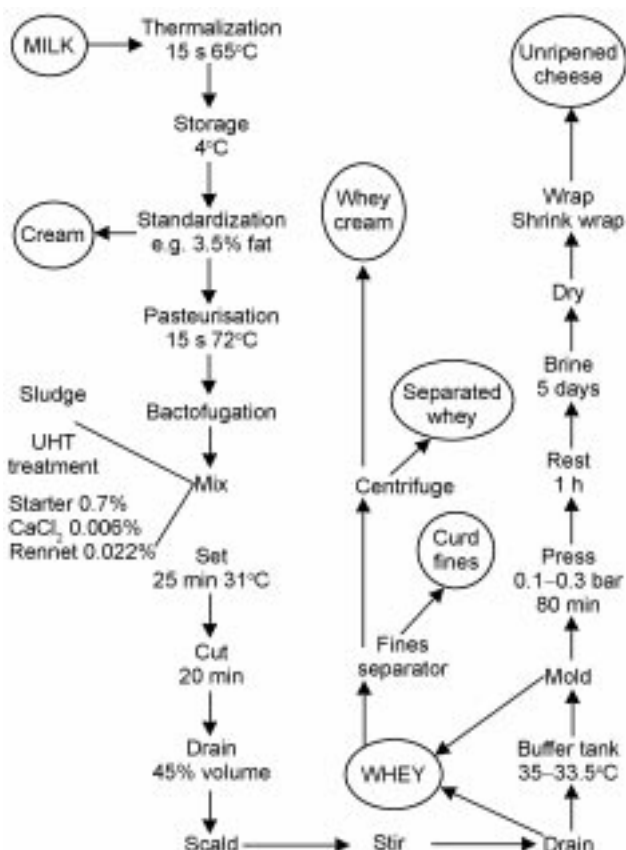


Fig. 20.4 Flow diagram for the manufacture of Gouda cheese (until curing) by a modern method. Adapted from Walstra *et al.* (1993).

during the entire manufacture process. Chymosin rapidly degrades α_{s1} -casein at the beginning of maturation, approximately 80% being hydrolyzed within one month, while β -casein is degraded much more slowly, with 50% remaining after six months; the optimum condition for hydrolysis is pH $\sim$ 5 with NaCl content about 4% in the moisture (Visser, 1977). In general, enzyme activity in these varieties is not favorable due to the pH and NaCl content; for example, plasmin activity is substantially lower in these conditions (Noomen, 1978). The overall variation in cheese composition and manufacturing techniques for hard and semi-hard cheese varieties will collectively determine the flavor development and characteristics of the final cheese product.

20.2 Flavor development of low temperature hard cheeses and semi-hard cheeses

20.2.1 Flavor development of Cheddar cheese

The exact mechanisms and specific chemical reactions involved in the formation of Cheddar cheese flavor are a complex process that is not completely understood (Lawrence *et al.*, 1993, Urbach, 1995). There are hundreds of volatile compounds that have been identified by several groups, providing various hints as to some important contributing factors that may effect flavor formation (Maarse and Vischer, 1989). This process is initiated with the hydrolysis of the casein network in the coagulum where α_{s1} -casein is cleaved by chymosin and β -casein is hydrolyzed by plasmin. This hydrolysis of the casein matrix provides the initial substrates for the formation of flavor and aroma compounds by the starter culture and non-starter bacteria that are present during ripening (Harper and Kristoffersen, 1970). Many abiotic factors, including ripening temperature, salt content, pH, redox potential, and moisture guide the development of Cheddar cheese flavor. The biotic factors of milk fat, proteolysis, starter cultures and non-starter bacteria types also add to the complexity of the flavor development process in cheese generally, and specifically with Cheddar cheese (Lawrence *et al.*, 1993). Proteolysis contributes not only to changes in texture during ripening, but to the release of flavor components bound to proteins. These components include non-volatile, water-soluble fractions such as peptides, amino acids, and salts in the cheese (Aston and Creamer, 1986).

The presence or absence of fat affects the detection of flavor. When the percentage of fat in dry matter is reduced, the characteristic Cheddar flavor is also lowered; cheeses made with FDM below 50% did not result in flavors expected from higher fat Cheddar cheeses (Ohren and Tuckey, 1969). Comparing the changes in flavor and volatiles of full-fat and reduced-fat Cheddar cheeses, after a period of six months' maturation, the full-fat cheese developed a characteristic Cheddar flavor that was consistently stronger than the reduced-fat cheese, while bitterness was more intense for the reduced-fat cheeses (Dimos *et al.*, 1996). Utilizing gas chromatography and headspace analysis for flavors and volatiles, dimethyl sulfide was the only volatile compound that showed a regular

increase over the storage time that was higher for the full-fat cheese, signifying the lower solvent power of reduced-fat cheeses but does not take part in more of an intense Cheddar flavor (Dimos *et al.*, 1996). Studies show that there is a positive correlation between the methanethiol content and the intensity of Cheddar flavor; headspace analysis indicated that the concentration of methanethiol is lower in the reduced-fat cheese and may relate to the lack of flavor in this variety (Dimos *et al.*, 1996).

The redox potential affects proteolysis and other biochemical reactions needed for the production of compounds associated with cheese flavor (Ganesan *et al.*, 2006). Sulfur-containing compounds that accept and donate hydrogen during the oxidative ripening process determine the formation of typical Cheddar cheese flavor via thiol groups. The lack of flavor in cheeses may be caused by the absence of active thiol groups (Kristoffersen, 1973). Casein has relatively few sulfur-containing amino acids that remain in the curd after pressing. Ironically, methionine increases in cheese during ripening to a concentration beyond that found in casein, indicating that the bacteria in the matrix are producing this amino acid (Fox *et al.*, 1993, Kristoffersen, 1973). The same bacteria that produce volatile sulfur compounds that are important in flavor also metabolize methionine.

Glutathione is a tripeptide that contains cysteine with a reactive sulfur group and serves multiple functions in cells, but is primarily involved in redox modulation (Urbach, 1995). Steele's group (Steele, 1995) found that lactococci accumulate glutathione but do not degrade it. When added in a cheese slurry system, reduced glutathione is oxidized immediately and leads to increased proteolysis (Harper and Kristoffersen, 1970). For example, the presence of glutathione in a cheese slurry increases β -casein hydrolysis and leads to increased flavor, which is directly proportional to the hydrolysis of β -casein, and confusingly results in more flavor products derived from α -casein (Urbach, 1995). In conjunction with the addition of glutathione, diacetyl can be added to fresh curd slurry, enhancing flavor development, but cannot solely form cheese flavor; both of these compounds are necessary for the production of Cheddar flavor and may suggest a beneficial method as an accelerated ripening technique (Barlow *et al.*, 1989; Singh and Kristoffersen, 1972; Urbach, 1995).

The addition of starter cultures is essential for the production of cheese flavor. Urbach (1995) provides an excellent review on the importance of lactic acid bacteria to flavor compound formation in dairy products, which contains a detailed list of volatiles isolated from Cheddar cheese. Lactic acid bacteria specifically contribute to the flavor of cultured milks and fresh cheese, producing acetaldehyde and diacetyl compounds during the conversion of lactose to lactic acid (Urbach, 1995). In most cases when cheeses are matured, a portion of the starter culture lyses a few weeks after manufacture. Once this occurs, enzymes are released into the cheese matrix and have access to proteins and peptides to reduce bitterness. These enzymes are responsible for the basic cheese flavor in Cheddar cheese (Chapman and Sharpe, 1990; Law and Sharpe, 1973; Urbach, 1995).

Acidic compounds in the volatile fraction of Cheddar cheese have an important role in flavor development as well (Wijesundera and Urbach, 1993). Urbach's group notes that acidic polar compounds or compounds with boiling points not lower than that of butyric acid interact with methanethiol, producing a typical Cheddar cheese odour (Wijesundera and Urbach, 1993). Sulfur compounds are considered necessary components of all cheeses; therefore, mechanisms of production of sulfur-containing compounds are important. Early studies suggested that the formation is mediated by lactic acid bacteria (Urbach, 1995). Subsequent work indicates that this biochemical process is mediated by two metabolic pathways depending on the organism (see Weimer *et al.*, 1999; Seefeldt and Weimer, 2000). Isolation of volatile sulfur compounds from Cheddar cheese resulted in the detection of H₂S, COS, CH₃SH, dimethyl sulfone and sulfur dioxide (Urbach, 1993). The presence of H₂S when found in cheese headspace functions as an indicator that native milk enzymes crucial for flavor production are still active (Urbach, 1995).

The addition of starter cultures must be carefully managed during manufacture to prevent undesirable off-flavors, such as bitterness, and to control variability of the final product (Lawrence *et al.*, 1993). When carefully selected and used, the starter culture can affect Cheddar flavor development as well as the attainment of the appropriate pH, moisture content and redox potential in the cheese (Lawrence *et al.*, 1993).

Low levels of non-starter flora, post-pasteurization, have been shown in some studies to be beneficial to flavor development when cheeses were made in open vats, accelerating flavor development (Law, 1984; Law *et al.*, 1976; Law and Sharpe, 1973). The most prevalent organisms are typically lactobacilli or pediococci or both, and seem to benefit flavor development while maintaining a low oxidation–reduction potential (Urbach, 1995). However, excessive growth may cause sour off-flavors and unacceptable quality formed from by-products such as acetic acid during metabolism (Lawrence *et al.*, 1993). Researchers have found that compounds present in Cheddar cheese such as lactic acid, carbon dioxide, sodium chloride, diacetyl, –SH compounds, low amounts of specific amino acids and peptides, and possibly non-starter flora attribute to flavor development (Lawrence and Thomas, 1979; Lindsay and Rippe, 1986; Morris, 1978).

20.2.2 Flavor development of Gouda and Edam cheeses

Many semi-hard cheeses such as Dutch-type varieties are made with either raw milk or pasteurized milk (Walstra *et al.*, 1993). When semi-hard and hard cheeses are made from raw milk, ripening time is increased and the cheese tends to have a more intense flavor, which also differs qualitatively, compared with cheese made from microfiltered or pasteurized milk (Buchin *et al.*, 1998). A study on the influence of pasteurization and fat composition of milk on flavor characteristics of semi-hard Morbier cheese found that indigenous microflora in raw milk cheeses were characterized by having higher amounts of numerous

alcohols, fatty acids and sulfur compounds, whereas pasteurized milk cheeses had higher amounts of ketones (Buchin *et al.*, 1998). Not only does processing affect flavor, but the cows' diet modifies the chemical composition of milk, which influences the sensory quality of milk products (Forss, 1993; Keen and Wilson, 1993). Pasture-fed cows typically have a different fatty acid composition and volatile compounds compared to cows fed grain or hay (Forss, 1993; Wilson and Reinbold, 1965). Buchin *et al.* (1998) found that pasture fed cows have higher concentrations of short-chain and unsaturated fatty acids, while hay-fed cows have higher concentrations of long-chain and saturated fatty acids. The hay-fed cows showed slightly more diversified characteristics in flavor than pasture-fed cows with higher milk and milk aromas, terpenes and lower amounts of hydrocarbons (Buchin *et al.*, 1998). In the flavor formation of Dutch-type cheeses, products from the breakdown of lactose and citric acid such as lactic acid and carbon dioxide and diacetyl, along with *para*- κ -casein and lipids, are integral for proper flavor development (McSweeney, 2004; Walstra *et al.*, 1993). As mentioned previously, proteolysis of the paracasein is essential in flavor formation, resulting in various taste perceptions including bitter, broth-like and sweet compounds (Mulder, 1952). The influence of microflora, milk origin, thermal processing (pasteurized or non-pasteurized milk) and biochemical reactions during ripening can contribute to the aromas and flavors in semi-hard cheeses.

20.2.3 Improving flavor of low temperature hard cheeses and semi-hard cheeses

Cheese ripening is a complicated process that involves microbiological and biochemical changes in the curd that give each variety their characteristic flavor and texture. Microbiological changes include metabolic changes in starter culture cells, growth of non-starter lactic acid bacteria (NSLAB), and in some varieties growth of a secondary microflora such as molds in mold-ripened cheeses (McSweeney, 2004). Biochemical changes during ripening involve primary actions, such as metabolism of residual lactose, lactate and citrate, lipolysis and proteolysis, followed by secondary actions of the metabolism of fatty acids and amino acids, contributing to production of volatile flavor compounds (McSweeney, 2004). Factors are manipulated to provide accelerated ripening for flavor improvement of hard and semi-hard cheeses. For example, accelerated ripening techniques have included the addition of enzymes (proteinase, lipase, β -galactocidase), elevated temperatures, adding cheese slurries and genetically modified starters (heat-shocking, $\text{lac}^- \text{prt}^-$ strains), and use of flavor adjunct cultures (Bartels *et al.*, 1987a, Grieve and Dullely, 1983; Law, 1984). This is highlighted by Weimer *et al.* (1997) who used combinations of starter cultures and flavor adjunct bacteria to produce low-fat cheese with various flavor profiles depending on the starter culture mixture.

Aroma compounds can be produced from cheese microorganisms such as lactic acid bacteria, coryneforms yeast and *Geotrichum candidum*, which all are

strain-dependent from amino acids (Yvon and Rijnen, 2001). Understanding how these cheese microorganisms catabolize amino acids offers another option in controlling aroma formation. It is important to control transamination and elimination reactions, because they initiate the two pathways that lead to formation of aroma compounds from amino acids, initiated by the action of aminotransferases to convert an amino acid to an α -keto acid; however, other catabolic pathways such as deamination or carboxylation also occur to yield other end products from amino acid metabolism (Yvon and Rijnen, 2001). Production of α -keto acids may be a rate-limiting step in the development of volatile flavor compounds (Curtin and McSweeney, 2004); accordingly, various studies have added α -ketoglutarate directly to cheese in an effort to either enhance the bacterial aminotransferase activity (Yvon *et al.*, 1998) or bypass this step completely. For example, α -ketoglutarate added to cheese curd resulted in better aroma compound development compared to untreated control cheeses (Banks *et al.*, 2001; Ur-Rehman and Fox, 2002).

Flavor adjunct cultures, often isolated from the NSLAB population and secondary microflora, are extensively reviewed as a means for accelerated ripening of cheese. Non-viable (attenuated) adjuncts and viable (non-attenuated) adjuncts are typically added to supplement the natural microflora in cheese milk, resulting in a higher quality product (El Soda *et al.*, 2000a). The predominant NSLAB species are *Lactobacillus* strains, most commonly *L. casei*, *L. paracasei*, *L. plantarum*, and *L. curvatus* (El Soda *et al.*, 2000a). Pediococci and enterococci are also NSLAB, but are found in smaller amounts, and micrococci (non-NSLAB) also contribute to flavor formation (Bhowmik and Marth, 1990, Fox *et al.*, 1998). The use of live cells during cheese ripening is extremely important, as the LAB work intracellularly through metabolic activities; they affect the changes in cheese quality during maturation (El Soda *et al.*, 2000a). Studies on incorporating Cheddar cheese with homogenates of *Lactobacillus casei* subsp. *casei* L2A and/or with live cells of the same *Lactobacillus* species at the time of renneting or salting, found that after nine months the cheeses increased by 42.8% in flavor intensity and reduced ripening time by almost 50% compared to the control cheese (Trépanier *et al.*, 1993). Gouda cheese produced with the addition of freeze-shocked adjunct cultures of *L. helveticus* showed better flavor quality than cheeses made with the untreated adjunct cultures (Bartels *et al.*, 1987b).

There is an alternative method commonly known as attenuated adjuncts addition. The attenuated adjuncts are cultures with all their microbial enzymes, and these organisms are isolated from ripened cheese, treated for inactivation of their fermenting ability, and then added to cheese milk. In this way, these adjuncts are well distributed and retained in the coagulum; the major advantage of this procedure is that the adjuncts are capable of ripening the cheese without making an overload of lactic acid (El Soda *et al.*, 2000a).

When the enzymes contained in the intracellular matrix of the inactive organisms are released through autolysis, contact is made with desired substrates and this allows improvement of flavor and texture during maturation (Chapot-

Chartier, 1996; Crow *et al.*, 1995; El Soda *et al.*, 2000b; Lortal *et al.*, 1997). El Soda *et al.* (2000a) suggest that the selection of adjunct cultures should be based on enzyme profiles (level and enzyme specificity) and autolytic properties. For example, research has shown that intracellular enzymes from non-starter adjunct cultures have strong capabilities of enhancing cheese quality and flavor formation due to specific aminopeptidolytic action on casein (El Abboudi *et al.*, 1991; Habibi-Najafi and Lee, 1996); lactobacilli have a high intracellular enzyme activity to degrade hydrophobic amino acids and reduce bitter peptides, 5–100 times higher than lactococci (El Abboudi *et al.*, 1991; El Soda *et al.*, 2000b; Habibi-Najafi and Lee, 1996). Autolytic properties of a cell for both starter and non-starter LAB are strain dependent, and environmental and physiological conditions also affect autolysis rates (Chapot-Chartier, 1996; Crow *et al.*, 1995; Kang *et al.*, 1997; Lortal *et al.*, 1997; Riepe *et al.*, 1997). For example, sodium chloride at a 0.2 M concentration has enhanced the autolysis of lactococci (Kang *et al.*, 1997).

Micrococci and pediococci have been particularly investigated because they are present naturally in milk and may survive pasteurization; they may be added to improve and enhance flavor in Cheddar cheese (Bhowmik and Marth, 1990). Micrococci are the predominant microflora of raw milk, the main source being from the udders of cows, but they are transmitted from dairy utensils, milking machines, air and dust (Abd-El-Malek and Gibson, 1948). A study suggests that micrococci are reported to comprise 16–68% of the non-starter bacterial population in Cheddar cheese (Feagan and Dawson, 1959). By supplementing a starter culture with a strain of *Micrococcus* and aging Cheddar cheese for three weeks, researchers reported an improvement in flavor score from 0.5 to 2.0 points over the control (Deane and Anderson, 1942). Micrococci have highly proteolytic, peptidolytic, and esterolytic activities, with other metabolic effects such as the production of methanethiol (Cuer *et al.*, 1979); all have a positive effect on accelerated ripening utilizing adjunct starter cultures (Bhowmik and Marth, 1990).

Investigation into the presence of pediococci in Cheddar cheese gives some indication of its prevalence and some contribution to flavor improvement (Franklin and Sharpe, 1963; Law *et al.*, 1976; Litopoulou-Tzanetaki *et al.*, 1989). Pediococci may not have an effect on flavor formation when incorporated independently (Law *et al.*, 1976), but studies have shown that combination with streptococci strains HP and K of *S. cremoris* improved Cheddar flavor during early stage ripening (Dacre, 1953). Pediococci may be used through their ability to hydrolyze milk protein and produce acetate and diacetyl metabolites (Nunez, 1976); in addition, their poor growth and limited acid production may help in improving flavor and accelerated ripening (Bhowmik and Marth, 1990). However, pediococci production of D(–)-lactate with formation of calcium lactate forms white crystals on the cheese which may be undesirable to consumers (Bhowmik and Marth, 1990; Thomas, 1986). Therefore more research is needed to improve the uses of pediococci in cheese. Deliberately adding specific adjunct or non-starter bacteria to cheese can help to increase the rate and

intensity of the development of cheese flavor, specifically that of Cheddar cheeses (Thomas, 1985; Urbach, 1995).

The action of lipolysis in internally bacteria-ripened cheeses such as Gouda, Cheddar, Edam and Swiss is considered low compared to mold-ripened and Italian varieties (Tungjaroenchai *et al.*, 2004). Varieties made from pasteurized milk do not contain strong lipolytic agents, therefore it is the action of enzymes from starter and non-starter microflora that contribute to lipolysis (Collins *et al.*, 2003; McSweeney, 2004; McSweeney and Sousa, 2000). In Cheddar cheese, lipolysis occurs by lipase enzymes from the starter culture, secondary flora and lipases naturally present in milk (Bhowmik and Marth, 1990). Short-chain fatty acids produced by lipolysis and bacterial metabolism play a major role in cheese flavor (Jeon, 1994). Common short-chain fatty acids found in Cheddar cheese that constitute Cheddar flavor include butyric, caproic and capric acid formed from milk fat degradation (Urbach, 1997).

The use of adjunct cultures in Cheddar cheese slurries has displayed an increase in the rate of lipolysis, with variation in lipolytic activity among different strains (El-Soda *et al.*, 1992; Madkor *et al.*, 1999, 2000). Tungjaroenchai *et al.* (2004) examined the influence of adjunct cultures (*Lactococcus lactis* ssp. *diacetylactis*, *Brevibacterium linens* BL2, *Lactobacillus helveticus* LH212, *Lactobacillus reuteri* ATCC 23272) on volatile free fatty acid production in reduced-fat Edam cheese, and found that *L. lactis* ssp. *diacetylactis* showed the highest activity, with acetic acid being the most prevalent acid detected throughout six months of ripening. Lipolysis plays an integral role in ripening, though excessive levels of lipolysis can result in rancid cheeses (Collins *et al.*, 2003).

Proteolysis may well be the most important initial biochemical event in cheese ripening as a mechanism to provide substrates for further catabolic processes by the starter culture, flavor adjunct, and NSLAB present in the matrix. It is a slow process and may be the rate limiting factor in ripening, therefore methods to increase proteolysis have been researched (Ardö and Pettersson, 1988; Birkeland and Abrahamsen, 1987; El Soda and Pandian, 1991; Fox *et al.*, 1996; Wallace and Fox, 1997; Wilkinson, 1993). This process softens cheese texture due to the hydrolysis of the casein matrix of the curd, as well as lowering the water activity from changes in water binding by newly formed carboxylic acid and amino groups by hydrolysis (McSweeney, 2004). The release of certain amino acids during proteolysis such as glutamic acid, methionine and leucine relates to the development of flavor in Cheddar cheese (Puchades *et al.*, 1989), leucine and methionine being the main contributors to cheesy flavor found in the water-soluble fraction of Cheddar cheese (Aston and Creamer, 1986; Kowalewska *et al.*, 1985). The importance of methanethiol as a precursor to other sulphur compounds and contribution to flavor intensity in Cheddar cheeses (Adda *et al.*, 1982; Hemme *et al.*, 1982) has led to the discovery of an enzyme that is capable of producing methanethiol from methionine in *L. cremoris* B78, which gives an indication that this enzymatic production could be achievable in Cheddar and Gouda cheeses (Alting *et al.*, 1995). Aroma development can also be controlled by regulating amino acid degradation during cheese ripening; this is important

because the concentration of various key aromas will determine the final flavor of the cheese (Yvon and Rijnen, 2001). The major precursors of aroma compounds from amino acid degradation include aromatic amino acids (phenylalanine, tryptophan, tyrosine), methionine and branched-chain amino acids (valine, leucine, isoleucine), contributing to aroma formation (Yvon and Rijnen, 2001). To further investigate how proteolysis is affected by adding free amino acids, Wallace and Fox (1997) added cas-amino acids to Cheddar cheese and observed that over six months of ripening, cheeses with intermediate levels of added amino acids ($21\text{--}42\text{ mmol kg}^{-1}$) developed a flavor and texture superior to either the controls or the cheeses with the highest level of cas-amino acids (63 mmol kg^{-1}) and had a higher level of all peptides (Wallace and Fox, 1997). If successful methods for accelerated ripening of cheeses can be determined, there will be economic advantages for cheese manufacturers with decrease of maturation time and manufacturing costs (Yvon and Rijnen, 2001).

20.3 Membrane filtration processes in cheese manufacture

Cheeses have been made using retentate from ultrafiltered milk, which gives economic advantages compared to current manufacturing processes (Spangler *et al.*, 1990). This has been used as a tool to standardize milks to precise casein, fat, serum protein and lactose contents for cheeses (Johnson and Lucey, 2006). When using highly concentrated retentate, there is less syneresis, which is important in texture development and more whey proteins retained in the cheese, adding nutritional value and increased yield (Kosikowski, 1974; Wingerd, 1971). Spangler *et al.* (1990) studied the manufacture of Gouda cheese from ultrafiltered milk and found that at a $5\times$ retentate concentration, 0.007% rennet extract (based on unconcentrated milk volume) and coagulation temperature of 38.9°C , bitterness was minimized in UF Gouda and gave a cheese more similar in moisture, Instron hardness and proteolysis to conventional Gouda cheese.

Accelerated ripening techniques have been used in the manufacture of reduced-fat Cheddar cheese from condensed milk. Brandsma *et al.* (1994) found that the utilization of condensed milk at 1.8- to 2.0-fold concentration provided advantages in producing good flavor with little bitterness and increased cheese yield. The use of condensed milk combined with elevated temperature (11°C) and enzymes added from proteases and lipases resulted in a reduced fat Cheddar cheese with firm, crumbly body but with higher Cheddar flavor score than the control cheeses (Brandsma *et al.*, 1994). However, researchers observed the development of surface crystals and off-flavor after 12 weeks of ripening; therefore, careful selection of enzymes by balancing the proteases and lipases may help to obtain satisfactory cheese flavor and texture.

Microfiltration is a means to separate milk fat globules according to size, obtaining a milk with a larger amount of small globules similar to homogenization, using ceramic microfiltration membranes with pore sizes of $2\text{--}5\text{ }\mu\text{m}$ (Goudedranche *et al.*, 2000). Homogenization of the milk or cream is used to

increase the surface area of fat globules, possibly improving body and texture by creating a fat extending effect, which gives better texture in reduced-fat Cheddar cheese (Drake and Swanson, 1995; Emmons *et al.*, 1980; Metzger and Mistry, 1994, 1995). Commercial application of ultrafiltered milk or microfiltered milk can be used by cheeses that do not have standard identity, but cheeses such as Cheddar, Mozzarella and Swiss must have special permission to use milk concentrated by membrane filtration processes, and its use must be indicated on the ingredient label (Johnson and Lucey, 2006).

20.4 Microencapsulation technology in accelerated ripening of cheeses

Microencapsulation technology has been a method used for accelerated ripening of cheeses. Benech *et al.* (2002) has incorporated the use of liposome-encapsulated nisin Z combined with *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis* UL719 and *Lactobacillus casei* ssp. *casei* L2A adjunct cultures in Cheddar cheese ripening. Nisin is a preservative that is a bacteriocin used in foods that inhibits pathogens and spoilage microorganisms in cheese without disruption of the cheese-making process (Benech *et al.*, 2002). In addition to being a preservative, nisin also induces lysis of some starter cultures, which releases intracellular enzymes, therefore accelerating casein hydrolysis which is associated with flavor development (Morgan *et al.*, 1997). The group found that incorporating *Lb. casei* and the nisinogenic culture produced a debittering effect and improved cheese flavor and quality in Cheddar cheese (Morgan *et al.*, 1997). Microencapsulation is currently being applied in the research of biological systems, pharmaceutical applications in drug delivery and vaccines, and is especially used in the cosmetic industry (Thompson, 2003). This type of technology offers many opportunities in the food industry, which may provide improvements in cheese ripening and many other food applications.

20.5 New technological innovations for reduced-fat cheeses

Accelerated ripening is especially important for low-fat Cheddar cheese manufacturers, since they have been challenged in finding ways to develop a low-fat cheese which embodies the characteristics of flavor and texture similar to full-fat cheese (Lawrence *et al.*, 1993). Dietary guidelines recommend that consumers control caloric intake, and the portion of fat contributing to calories. In the United States, guidelines state that no more than 30% of caloric intake should come from fat. Demand for more low-fat product choices in the marketplace has caused accelerated manufacturing of low-fat cheeses (Mistry, 2001). Reduced and low-fat cheeses have achieved only very limited success commercially. This can be partially explained by the fact that when fat is removed during cheese making, problems occur with lack of texture, aroma and

flavor (Mistry, 2001; Rodriguez, 1998). Alternatives employed in low-fat cheese making have included changing traditional processing techniques, selection of starter cultures, using additives consisting of stabilizers and fat replacers, and using technological innovations (Mistry, 2001; Rodriguez, 1998).

These new technological innovations include removal of fat by a novel processes using physical separation and supercritical fluid extraction (Rodriguez, 1998). A recent study conducted by Nelson and Barbano (2004) involved a physical fat removal process after the aging of Cheddar cheese to produce a reduced-fat Cheddar cheese by centrifugation. By investigating the efficiency of fat removal at various temperatures, gravitational forces and durations of applied force, a process was developed that decreased grams of saturated fat per serving of cheese from 6.3 to 3.1 grams, with flavor intensity at least as intense as full-fat cheeses (Nelson and Barbano, 2004). Researchers found that there was a positive linear relationship between temperature and fat removal from 20 to 33°C, while the most efficient parameters of 30°C and 23,500g for 5 minutes removed 50% of the fat (Nelson and Barbano, 2004). When comparing the flavor intensities of three different aged Cheddar cheeses, than applying the fat removal process to each cheese, the flavor intensity of the reduced-fat cheeses increased as well as the rate of flavor release when made from higher intensity full-fat cheeses, therefore the reduced-fat cheese had at least as intense a flavor as the full-fat untreated cheese (Nelson and Barbano, 2004). In a follow-up study utilizing the novel process to remove fats, sensory evaluation of cheeses and cheese fat made from the novel fat removal process and aged for 9 or 39 months found that compared to full-fat cheese, the reduced-fat reformed cheese had a very similar flavor profile to the full-fat cheese, but had lower intensities of milk fat/lactone and sulfur flavors; this confirms that researchers were able to successfully remove 50% of the fat from the cheese and retain the characteristic Cheddar cheese flavor (Carunchia-Whetstine *et al.*, 2006).

Supercritical fluid extraction (SFE) has been used for various applications in the food industry, with limited studies on use on cheese. The principle of supercritical fluid extraction consists of utilizing a fluid such as carbon dioxide that is brought to a specific temperature-pressure combination, allowing it to attain supercritical solvent properties for the selective extraction of fat from the sample matrix (Nielsen, 2003). Carbon dioxide is chosen as the most ideal solvent used in extraction, because its low critical temperature allows lipids to be removed from the food component without being thermally degraded during processing (Rizvi *et al.*, 1994). The critical point for carbon dioxide to become a supercritical fluid is 31.06°C and 7.386 MPa or 73.8 bar (Rizvi *et al.*, 1994). A recent study on the use of SFE in determining total fat and fat-soluble vitamins in Parmigiano cheese and salami, found that the quantity extracted and collected by supercritical carbon dioxide was statistically equivalent to Soxhlet extraction (Perretti *et al.*, 2004). The determination of fat-soluble vitamins by HPLC suggests that the extractability of vitamins using SFE was comparable to official methods, especially for α -tocopherol due to the absence of light and oxygen and the use of a lower temperature than in typical methods (Perretti *et al.*, 2004).

There are official methods using supercritical fluid extraction, such as the determination of total fat in oily seeds (AOCS, 1996), with possibilities for expanding the use of SFE technology for different analytical purposes or for food processing. Supercritical fluid extraction has been used as a new alternative to extract aromas from cheeses. Larráyo *et al.* (1999) have used SFE to extract volatile fractions of unsmoked Idiazábal ewe's milk cheese, for the identification and quantification of the volatile fractions of cheese. Roncal cheese aromatic extracts were also obtained by SFE in different solvent conditions, while using sensory methods (ranking, similarity and matching tests) to assess the representativeness of aromatic extracts (Larráyo *et al.*, 2000). Researchers used pure liquid carbon dioxide at extraction conditions of 50°C and 109 bar and 20 minutes for the static and dynamic phase. They found that ewe's milk whey cheese was an adequate support for assessing sensory qualities of aromatic extracts of a ewe's cheese, and a 2:1 mixture of *n*-hexane:acetone yielded the most representative aromatic extracts of Roncal cheese. Ultimately this technique is suitable for use in producing specific and representative aromatic extracts from different Protected Designation of Origin (PDO) cheeses (Larráyo *et al.*, 2000).

Yee (2006) utilized SFE technology to develop reduced-fat grated cheese products, while retaining similar characteristic flavor profiles of full-fat versions. Mild Cheddar cheese aged over 60 days and Parmesan cheese aged over 10 months were used as samples in the study. The most efficient process parameters for fat removal from Cheddar cheese were 40°C, 200 bar and 1000 grams CO₂, while for Parmesan cheese the values were 35°C, 350 bar and 1000 grams CO₂ (Yee, 2006). After SFE treatment at optimized parameters, at least 45.48% of the fat was removed from the Cheddar samples and at least 39.74% from the Parmesan cheese samples. Gas chromatography-mass spectroscopy was utilized to characterize the flavor compounds of each cheese sample. The Cheddar samples analyzed by GC-MS showed higher concentrations of methanethiol, dimethyl disulfide and dimethyl trisulfide in SFE Cheddar cheese, compared to full-fat Cheddar cheese. However, hydrogen sulfide was higher in full-fat Cheddar than in SFE-Cheddar. SFE-treated Parmesan cheese had higher concentrations of dimethyltrisulfide and methional than full-fat Parmesan cheese, but was lower in methanethiol and had similar amounts of dimethyl disulfide, key contributors to Parmesan cheese aroma. According to a triangle difference test, panelists were able to significantly discriminate between the SFE Cheddar cheese and the full-fat version, but could not discriminate between the SFE Parmesan cheese and the full-fat version. Acceptability tests indicated that SFE Cheddar cheese had a lower acceptability compared to full-fat and low-fat commercial products, while SFE Parmesan cheese had higher acceptability compared to all other versions. This study suggests that SFE technology can be used in the dairy industry to develop low-fat cheese products, which retain flavor compounds that are not typically fully developed with alternative methods of low-fat cheese processing. Research in supercritical fluid extraction has been conducted for potential uses in food, agriculture, analytical supercritical fluid

chromatography and the petrochemical and chemical industries (Rozzi and Singh, 2002), and with its potential application to cheese manufacture this could be an interesting sector for research and advancement.

20.6 Future trends

The new emerging trends used in the development of hard and semi-hard cheeses may provide alternatives for flavor and texture improvement of these popular cheeses, creating products that will satisfy consumer taste buds and allow cheese makers to develop high quality cheeses.

New technological advances on improving flavor and quality of hard and semi-hard cheeses have focused on methods for making cost savings during cheese manufacture utilizing filtration systems, incorporating microencapsulation technology into the cheese matrix for accelerated ripening, and various methods for improving flavor and texture of reduced-fat cheeses to accommodate consumer demand for healthier and better tasting food choices.

20.7 References

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21

Soft-ripened and fresh cheeses: Feta, Quark, Halloumi and related varieties

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21.1 Introduction

Some 8000 years ago cheese is thought to have originated in what is now known as the Middle East (Kosikowski, 1982). Homer (ninth to eighth century BC) wrote about a cheese from sheep's or goat's milk manufactured by cyclops Polyphemus, which is probably the ancestor of Feta cheese, and Pliny (first century AD) referred to 'sour milk' cheeses, likely the ancestors of Domiati cheese. These cheese types may be considered as the forerunners of various cheese types that evolved through the centuries. The types of soft-ripened cheeses preserved or stored in brine (pickled cheeses) manufactured today either by small-scale, but in the last decade mainly by large-scale, mechanized and standardized methods in Eastern Europe, the Balkans and the Middle East, can be classified as follows (Abd El-Salam and Alichanidis, 2004):

- Acid-coagulated (Mish cheese)
- Rennet-coagulated, salting of curd (Feta, Telemes, Beyaz peynir, Brinza, Bli-sir-U-kriskama, Bjalo, Chanakh, Akawi, Baida, Iranian white cheese)
- Rennet-coagulated, salting of cheese milk (Domiati, Dani, Gibna bayda).

21.2 Feta and related cheeses

21.2.1 Manufacture

Raw sheep milk or a mixture of sheep and goat milk is still used for cheese production at home. However, pasteurized standardized (ratio casein/fat = 0.7–0.8/1) sheep or a mixture of sheep and goat (up to 30%) milk and starter cultures were adopted in commercial Greek dairies. The milk is cooled to 32°C, rennet is

added and inoculated with starter culture. The curd is cut (2–3 cm cubes) and transferred into perforated molds for draining (14–16°C) without pressing. The curd is subsequently cut into blocks and salted by coarse salt until it contains about 3–3.5% salt. The cheese remains in the same room for about 15 days. During this period, a slime of surface microflora develops, which contributes flavor to the cheese during ripening. The cheese blocks are transferred to barrels or tins with brine (6–8%) for continued ripening during cold storage (2–5°C) (Anonymous, 1998; Abd El-Salam *et al.*, 1993; Abd El-Salam and Alichanidis, 2004; Anifantakis, 1991; Zygouris, 1952).

21.2.2 The surface microflora and its possible effect on cheese ripening and flavor

The predominant surface microflora of fresh cheese (~4 days, when the microbial slime starts appearing) is composed of lactic acid bacteria (LAB), yeast and salt-tolerant microbes (Fig. 21.1). *Leuconostoc*, *Lactococcus lactis* and *Lactobacillus plantarum* constitute the predominant LAB microflora developed at day 4, while at the end of ripening in the warm room the LAB microflora is composed of lactobacilli with *Lb. plantarum* being the predominant species. Aminopeptidase activities are largely due to lactobacilli, while lipolytic activities are either not detected or weak in these organisms (Tzanetakis and Hatzikamari, 1994). The predominant yeast species at 4 days are *Saccharomyces cerevisiae* and *Debaryomyces hansenii*, while at the end of ripening in the warm room *S. cerevisiae* is the predominant yeast. Yeast also contains Leu- and Val-aminopeptidase that is higher than the Cys- aminopeptidase activity. In addition, their esterase/lipase activity is higher than the esterase activity, and for some strains only lipase activity is found (Tzanetakis *et al.*, 1996). The halotolerant

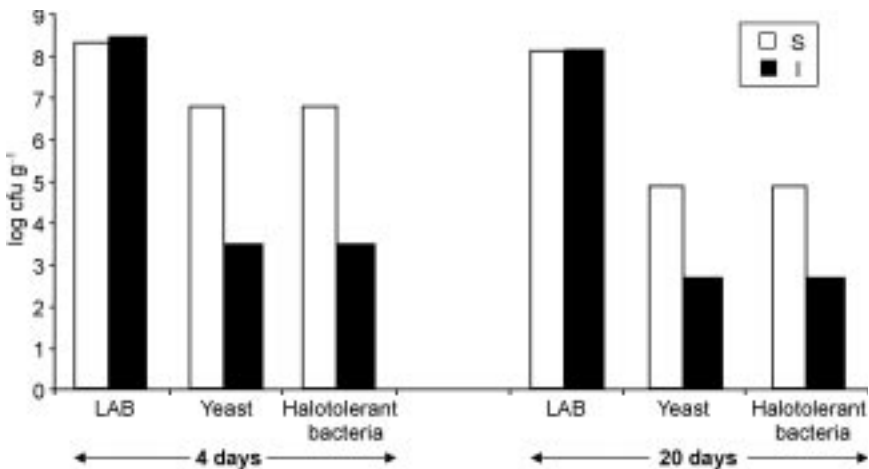


Fig. 21.1 Evolution of the microflora in Feta cheese surface (S) and interior (I) during ripening in the warm room.

surface microflora is composed of staphylococci, micrococci, enterococci and coryneform bacteria. *Staphylococcus saprophyticus* predominates the halo-tolerant bacteria content on the surface of cheese at day 4, while coryneforms are commonly found in 20-day-old cheese. Staphylococci isolates from the Feta cheese surface do not exhibit any aminopeptidase activity; however, staphylococci, in general, show quite strong esterase and esterase/lipase enzyme activity (Tzanetakis *et al.*, 1995a). Thus, lactobacilli of the cheese surface may affect proteolysis, yeast participates in both proteolysis and lipolysis, and staphylococci contribute to cheese lipolysis.

21.2.3 The predominant microflora of the cheese interior and its possible effect on cheese ripening and flavor

An interesting characteristic of traditional cheeses is the predominance of their natural lactic microflora over the other microbial groups with the progress of ripening. Non-starter lactic acid bacteria (NSLAB) proliferate during Feta cheese aging and may reach numbers as high as 10^7 – 10^8 cfu g⁻¹ at the end of ripening in the warm room (Table 21.1). Facultatively heterofermentative lactobacilli is an important group throughout cheese ripening. *Lb. plantarum* is the dominant species, constituting 50% of the NSLAB population, and *Lactobacillus paracasei* subsp *paracasei* and *Lactobacillus brevis* are also often found. In addition, enterococci and pediococci are among the predominant NSLAB of Feta cheese (Tzanetakis and Litopoulou-Tzanetaki, 1992).

Lb. plantarum isolates from Feta cheese during ripening exhibit lipolytic and peptidolytic activities (Xanthopoulos *et al.*, 2000a). The amounts of amino acids accumulated in the milk are low and strain-dependent (Fig. 21.2). Strains of slow and medium acidification rates are also found. An array of peptidolytic enzymes can also be detected in *Lb. paracasei* subsp *paracasei* isolates from Feta such as endocellular dipeptidase, aminopeptidase, endopeptidase, and

Table 21.1 Log counts and distribution of lactic acid bacteria of Feta cheese throughout ripening

Counts on	After manufacture			
	Curd	15 days	30 days	90 days
MRS agar	4.59	7.88	7.95	7.65
Rogosa's acetate agar	2.41	8.08	7.77	8.03
Enterococci (%)	17.14	1.43	6.12	5.41
Pediococci (%)	7.14	17.14	4.08	–
Facultatively heterofermentative (%)	47.14	51.43	69.39	81.08
Obligately heterofermentative (%)	28.58	30.00	20.41	13.51
pH	6.45	5.19	4.59	4.56
NaCl-in-moisture (%)	nd	5.92	6.55	7.28

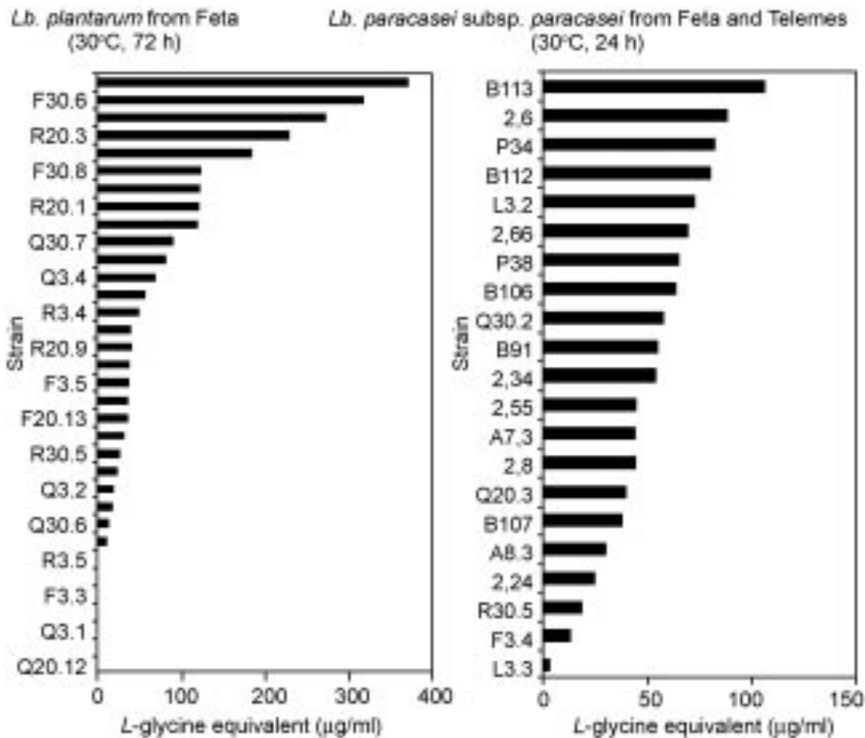


Fig. 21.2 Proteolytic activity of NSLAB isolated from Feta and Telemeas cheeses after growth in milk.

carboxypeptidase enzyme activity (Bintsis *et al.*, 2003). Their dipeptidyl aminopeptidase activity is low, except for the diagnostic substrate Gly-Pro-pNA. This is an important feature for cheese ripening since caseins have a high proline content. In addition, *Lb. paracasei* subsp *paracasei* degrade caseins (Bintsis *et al.*, 2003). The amount of amino acids accumulated in the milk after 24 hours is low and strain dependent (Mama *et al.*, 2002) (Fig 21.2). Moreover, the esterase activity increases with increasing carbon chain length (Bintsis *et al.*, 2003). Less frequently isolated during ripening of Feta are heterofermentative lactobacilli and leuconostocs that produce esterase activity that preferentially degrades short-chain fatty acids (Vafopoulou-Mastrojiannaki *et al.*, 1996). *Leuconostoc* exhibits peptidase activities and hydrolyzes the caseins (Vafopoulou-Mastrojiannaki and Litopoulou-Tzanetaki, 1996).

Pediococcus pentosaceus isolates from Feta produce acid slowly relative to other isolates and often form diacetyl and acetaldehyde when grown in milk (Litopoulou-Tzanetaki *et al.*, 1989) via aminopeptidase activity and amino acid catabolism. These organisms also have dipeptidyl aminopeptidase and dipeptidase activity for peptides containing proline (Vafopoulou-Mastrojiannaki *et al.*, 1994). *P. pentosaceus* strains also exhibit carboxypeptidase activities with significant inter-strain differences. Their overall proteolytic activity on β -casein

is considerably higher than on α_{s1} -casein. Quite low esterase activities are found in *P. pentosaceus* (Vafopoulou-Mastrojiannaki *et al.*, 1994) compared to other organisms.

Enterococci isolates from Feta are also poor acidifiers and some of them exhibit low proteolytic activity (Sarantinopoulos *et al.*, 2001; Ambadoyiannis *et al.*, 2004). Low peptidase activities are also exhibited by enterococci, and their esterolytic system is rather complex; enterococci seem to preferentially degrade substrates with low molecular weight fatty acids (Sarantinopoulos *et al.*, 2001). The main volatile compounds produced by enterococci are acetaldehyde, ethanol and acetoin (Sarantinopoulos *et al.*, 2001).

Therefore, the predominant LAB microflora throughout ripening of Feta cheese may contribute to proteolysis and flavor development of the cheese by virtue of an active proteolytic system, the ability to form carbonyl compounds, their lipolytic activity and their acidifying abilities. It is interesting to note that marked variability within predominant LAB isolates from Feta concerning their activities of biotechnological importance may serve as a basis for the selection of strains with physiological properties of biotechnological interest appropriate to be used as adjunct starters.

21.2.4 Improving the flavor of Feta cheese by the use of starters

The influence of the various starter combinations on the microbiological, physicochemical and sensory characteristics of Feta cheese has been studied in several investigations (Litopoulou-Tzanetaki *et al.*, 1993; Sarantinopoulos *et al.*, 2002; Vafopoulou-Mastrojiannaki *et al.*, 1990).

A high acidification rate of the starter culture in order to lower the pH of the cheese to pH ~4.8 within 18–20 hours, is necessary for successful manufacture of Feta cheese (Abd El-Salam and Alichanidis, 2004). When thermophilic starter cultures, combinations of mesophilic LAB (*Lactococcus lactis* subsp *lactis*, *Lactobacillus casei*) and adjunct cultures (*Enterococcus durans*, *Leuconostoc cremoris*) are used to make Feta cheese, significantly lower pH values are attained in the cheese (Litopoulou-Tzanetaki *et al.*, 1993). However, during the later stages of ripening the adjunct *E. durans* population accumulates higher concentrations of amino acids and small peptides, producing cheese with significantly better flavor (Litopoulou-Tzanetaki *et al.*, 1993). This was also observed with addition of *Enterococcus faecium* as the adjunct culture to Feta cheese. The cheese flavor was also improved via enhanced degradation of α_s - and β -casein and increasing concentrations of free amino acids (Sarantinopoulos *et al.*, 2002). The main volatile compounds formed were ethanol, acetate, acetaldehyde, acetoin and diacetyl, with highest amounts determined for ethanol, followed by acetate.

When *P. pentosaceus* was included in the starter culture for Feta cheese production, the cheese pH reduced to lower levels and the amount of soluble nitrogen, even in the fresh cheese, was significantly higher than the control (Vafopoulou-Mastrojiannaki *et al.*, 1990). The levels of acetaldehyde in cheese

made with *P. pentosaceus* were also significantly higher and the flavor improved. *P. pentosaceus* accelerated cheese ripening and the maturation time of Feta cheese by one month.

21.2.5 Formation of flavor compounds during ripening of Feta cheese

The flavor of typical Feta cheese is mildly rancid, slightly acid and salty and it is frequently described as flavorful and appetizing (Abd El-Salam *et al.*, 1993). Significant changes in the constituents of cheese take place during its maturation that contribute to the development of organoleptic properties. These changes are affected greatly by factors such as milk quality, pasteurization, the starter culture type and the ripening temperature (Vastardis, 1989). During ripening the lactose in Feta is rapidly fermented, thereby decreasing the pH to ~5.0 during coagulation and draining (6–8 hours), and to ~4.8 after 18–20 hours (Abd El-Salam and Alichanidis, 2004).

The low pH reached in ripening Feta cheese (~4.5) is favorable for the higher retention and proteolytic activity of chymosin (Abd El-Salam and Alichanidis, 2004), but may decrease the activity of proteolytic enzymes produced by lactococci (Law and Kolstad, 1983). In addition, salt-in-moisture content of Feta cheese rises to over 5% with the progress of ripening (Litopoulou-Tzanetaki *et al.*, 1993) and this may affect the degradation of caseins (Trujillo *et al.*, 1997). Thus, only ~18% of the total nitrogen of the cheese is soluble in water (WSN) at the end of ripening in the warm room (Katsiari *et al.*, 2000) and this percentage increases slightly during storage at 4°C for a total of two months. However, the amount of WSN is probably underestimated due to the fact that solubilized casein diffuses into the brine. The total free amino acid (FAA) concentration increases throughout aging, especially during ripening in the warm room (Katsiari *et al.*, 2000). The concentrations of the individual FAAs in the mature cheese (Table 21.2) indicate that Val, Leu, Phe and Lys are the four major FAAs in mature Feta cheese. Another abundant FAA is γ -aminobutyric acid, which is derived from glutamic acid and ornithine that has arginine as precursor. Ornithine, citrulline, α - and γ -aminobutyric acids of Feta cheese accumulate during ripening as metabolic products of microorganisms (Bütikofer and Fuchs, 1997).

Acetic acid is the dominant (40–75%) free volatile organic acid in the mature Feta cheese that impacts the cheese flavor (Abd El-Salam *et al.*, 1993; Abd El-Salam and Alichanidis, 2004; Katsiari *et al.*, 2000; Kandarakis *et al.*, 2001; Kondyli *et al.*, 2002) (Table 21.3). However, acetic acid is not a product of lipolysis and is derived early in ripening from lactose and citrate catabolism (Sarantinopoulos *et al.*, 2002). Butyric acid is also present at high levels and together with other free fatty acids (FFA) accumulates in Feta during ripening to impact the cheese flavor. The most abundant volatile compounds that also contribute to the flavor of the mature cheese include alcohols (mainly short chain), aldehydes and ketones (Kondyli *et al.*, 2002; Sarantinopoulos *et al.*, 2002). Ethanol and butan-2-ol are the main volatile compounds of mature Feta cheese.

Table 21.2 Free amino acids ($\mu\text{mol/g}^{-1}$) of Feta cheese

Amino acid	Age of cheese	
	60 days	120 days
Asp	0.44	0.64
Thr	0.37	0.55
Ser + Glu + Asn	2.47	3.24
Glu	1.03	1.40
Cit	0.00	0.01
Gly	0.49	0.72
Ala	1.10	1.41
α -Aba + Cys	0.02	0.03
Val	1.55	2.06
Met	0.35	0.52
Ile	0.68	0.91
Leu	2.55	2.94
Tyr + β -Ala	0.37	0.22
Phe	1.37	1.72
γ -Aba	1.46	1.97
Orn	0.44	0.93
Lys	1.15	1.76
His	0.37	0.40
Trp	0.03	0
Arg	1.03	0.89
Total	17.27	22.30

Source: based on Katsiari *et al.* (2000).

21.2.6 Teleme cheese

The technology of manufacture of Teleme cheese differs from that used for Feta in the procedure of draining and salting. The molded curd of Teleme is subjected to pressure to expel the whey. After draining the blocks of Teleme they are immediately immersed into brine (usually 18% NaCl for 20 hours) to allow penetration of salt. However, the manufacturing procedure may differ in the various countries of production and among manufacturers (Mallatou *et al.*, 2003). Teleme cheese was traditionally made from sheep milk, but cow, buffalo, and goat milk or mixtures are used commonly.

LAB growth and evolution in Teleme cheese from ewe milk is similar to Feta, with *Lb. plantarum* becoming the predominant species with the progress of ripening. Lactococci are present in curd and cheese until the age of up to 75 days, while leuconostocs constitute an important microbial group until 30 days of ripening (Litopoulou-Tzanetaki and Tzanetakis, 1992; Tzanetakis and Litopoulou-Tzanetaki, 1992). Isolates of *P. pentosaceus*, lactobacilli and leuconostocs from Teleme are also similar to isolates from Feta in respect of their proteolytic and lipolytic activities (Vafopoulou-Mastrogiannaki *et al.*, 1994, 1996; Mama *et al.*, 2002; Vafopoulou-Mastrogiannaki and Litopoulou-

Table 21.3 Free fatty acids (mg/100 g) and volatile compounds of Feta cheese

Free fatty acids	Age of cheese		Volatile compounds ¹	Age of cheese 180 days
	120 days	180 days		
C2:0	81.9	82.84	Diethlether	175
C4:0	1.48	1.7	Acetaldehyde	336
C6:0	6.50	7.01	Propanal	675
C8:0	2.57	3.27	Acetone	649
C10:0	3.86	5.41	Butanal	61
C12:0	12.3	12.9	Butan-2-one	159
C14:0	11.2	12.8	Ethyl acetate	507
C16:0	34.17	43.80	3-methyl butanal	160
C18:0	10.20	11.70	Ethanol	26686
C18:1	7.72	8.36	Pentanal	157
C18:2	1.44	1.47	Pentan-2-one	630
Total	173	191.08	Butan-2-ol	6727
C4–C18:2	91.40	108.2	Pentan-1-ol	312
			3-hydro butanal	52
			Hexanal	357
			Heptanal	289
			3-methyl butan-1-ol	963
			3-hydroxy butan-2-one	1477

¹ Total ion chromatographic peak areas in arbitrary units.

Source: based on Kondyli *et al.* (2002).

Tzanetaki, 1996; Tzanetakis and Litopoulou-Tzanetaki, 1989) (Fig. 21.2). When mesophilic starter cultures (*Lc. lactis* subsp *lactis* and *Lb. casei*) are used concurrently with *Ln. cremoris* and *E. durans* the pH is lower than in cheese made with thermophilic starter cultures (Tzanetakis *et al.*, 1995b). In addition, the amino acid levels in these cheeses are higher and have improved flavor. *P. pentosaceus* is also beneficial as an adjunct starter culture (Tzanetakis *et al.*, 1991). In its presence, the growth of LAB is enhanced, the pH of the cheese is significantly lower than the control and the cheese proteolysis is accelerating, resulting in a concomitant acceleration of ripening by one month.

The rate of proteolysis in Teleme cheese is much slower than in Feta cheese initially (Abd El-Salam and Alichanidis, 2004) and even though the FAA content of cheese at less than 90 days is lower than in Feta, the levels become similar in the later stages of ripening (Zerfiridis *et al.*, 1989). Milk lipase, lipases found in rennets and lipases released by the secondary flora are some of the main factors that contribute to the FFA accumulation (Abd El-Salam *et al.*, 1993). Total FFA of cheeses made from ewe, goat or a mixture of the two milks is significantly lower than the amount in cow milk cheese (Mallatou *et al.*, 2003) (Table 21.4). Acetic acid, produced mainly through the fermentation of lactate (Abd El-Salam *et al.*, 1993), is the major volatile extracted within the FFAs. Acetic acid is characteristic of pickled cheeses with a harsh but not rancid flavor and a related typical aroma (Mallatou *et al.*, 2003). In addition to acetic (C2)

Table 21.4 Acetic acid (C2) and individual free fatty acids (mg kg^{-1}) of Telemes cheeses at 60 days made from different types of milk

Free fatty acid	Ewe's milk	Goat's milk	Ewe/goat mixture	Cow's milk
C2	308 $\pm$ 47	276 $\pm$ 41	353 $\pm$ 6	371 $\pm$ 27
C4	35 $\pm$ 0	35 $\pm$ 0	36 $\pm$ 2	38 $\pm$ 2
C6	35 $\pm$ 0	35 $\pm$ 0	35 $\pm$ 0	35 $\pm$ 0
C8	55 $\pm$ 0	55 $\pm$ 0	55 $\pm$ 0	55 $\pm$ 0
C10	36 $\pm$ 2	50 $\pm$ 3	46 $\pm$ 3	43 $\pm$ 2
C12	56 $\pm$ 2	55 $\pm$ 3	55 $\pm$ 3	56 $\pm$ 3
C14	58 $\pm$ 4	45 $\pm$ 5	58 $\pm$ 7	101 $\pm$ 6
C16	183 $\pm$ 14	175 $\pm$ 18	196 $\pm$ 19	375 $\pm$ 25
C18	93 $\pm$ 10	93 $\pm$ 12	96 $\pm$ 8	120 $\pm$ 3
C18:1	140 $\pm$ 18	100 $\pm$ 15	121 $\pm$ 9	198 $\pm$ 16
C18:2	73 $\pm$ 3	72 $\pm$ 2	72 $\pm$ 2	Trace

Source: based on Mallatou *et al.* (2003).

other FFA present in Telemes cheese are palmitic (C16) and oleic (C18:1) (Mallatou *et al.*, 2003; Vafopoulou-Mastrogiannaki *et al.*, 1989).

21.2.7 Beyaz peynir cheese

Beyaz peynir is a Turkish white-brined cheese made from sheep, goat or cow milk. The cheese has a salty and acid flavor. Substantial amounts of cheese are still made from raw milk (Erkmen, 2000). The LAB microflora of mature cheese is composed of *Lc. lactis* subsp *lactis*, *Lb. plantarum* and *Lb. paracasei* subsp *paracasei*, while enterococci constitute a significant portion as well (Durlu-Ozkaya *et al.*, 2001).

The LAB isolated from Beyaz peynir have limited capabilities to produce acid, produce FFA, and degrade proteins. Aminopeptidase activity of the isolates is also weak, resulting in relatively low amino acid accumulated in the milk (Durlu-Ozkaya *et al.*, 2001). The mature cheese (120 days) contains 853 $\text{mg } 100 \text{ g}^{-1}$ and 698 $\text{mg } 100 \text{ g}^{-1}$ FAA for sheep and cow milk cheese, respectively (Ücuncü, 1981). The total FFA content increases with ripening and the amount in 30-day cheese is 63.8 $\text{mg } 100 \text{ g}^{-1}$ with acetic acid being found at the highest concentration (Akin *et al.*, 2003).

21.2.8 Domiati cheese

Domiati cheese is the most popular variety made in Egypt. The best quality Domiati cheese is made from buffalo milk. In the usual method of cheese making, salt is added to milk prior to renneting. The salted (8–15%) milk is renneted at 38°C and after coagulation (at 2–3 hours) it is ladled into molds to drain (at 12–24 hours). The cheese is either consumed fresh or pickled in salted whey from the same cheese (Abou-Donia, 1991; Abd El-Salam *et al.*, 1993). To

avoid the use of an extensive quantity of salt and to retain the typical flavor of Domiati, pasteurization and addition of starter cultures is now used (Abou-Donia, 1991; Abd El-Salam and Alichanidis, 2004). Single or a mixture of starter cultures containing lactococci, lactobacilli and enterococci have been tried by several investigators (Abou-Donia, 1991).

Fresh Domiati is characterized by high pH (6.0–6.5), while the pH of the pickled cheese declines to as low as 3.3 (Abd El-Salam *et al.*, 1993). The LAB microflora isolated from the surface is composed of lactococci and lactobacilli, with *Lc. lactis* subsp *lactis*, *Lb. casei* and *Lactobacillus delbrueckii* subsp *bulgaricus* being the dominant species (Abou-Donia, 1991). Helmy (1960) reports that Domiati with a low salt content (7.5% in the milk) has lactococci as the dominant LAB, which are later replaced by lactobacilli. In the higher salt cheese (15% in milk), micrococci shared the dominance with the lactobacilli. Enterococci are also found in Domiati cheese (Hemati *et al.*, 1997). The enterococci isolates from Domiati exhibit high esterolytic activities.

Abou-Donia (1991) pointed out that the flavor of Domiati is affected by milk acidity, starter culture type, homogenization, pasteurization, additives, ripening temperature and storage temperature. During ripening soluble proteolysis products diffuse into the brine to attain equilibrium with their concentration in the cheese (Abd El-Salam *et al.*, 1993; Abd El-Salam and Alichanidis, 2004). Glutamic acid is also converted to γ -aminobutyric acid, while arginine is converted to ornithine (El-Erian *et al.*, 1974). High levels of ammonia in the ripened cheese also contribute to the cheese flavor (Abd El-Salam and Alichanidis, 2004). The changes in the volatile fatty acids (VFA) occur mainly during the first month of ripening when maximum bacterial growth occurs (Shehata *et al.*, 1984), with acetic acid being the principal volatile acid that contributes to the flavor (El-Shibiny *et al.*, 1974).

21.3 Acid and acid/rennet-curd fresh cheeses: introduction

Fresh cheeses are manufactured by the coagulation of milk, cream or whey by acidification or a combination of acid and heat and eventually addition of rennet. Fresh cheeses are ready for consumption immediately after production. For their production, pre-treated milk undergoes slow acidification and gelation at pH 4.6–4.8. The curd is stirred and the whey is removed. The structure of the coagulum is influenced by many processing factors. The end product is either cooled and packed directly as cold-packed products (e.g. Quark and Fromage Frais) or further treated as hot-pack products (e.g. cream cheese) (Guinee *et al.*, 1993; Schulz-Collins and Senge, 2004; Farkye, 2004; Kosikowski and Mistry, 1997). Fresh cheeses manufactured by a combination of acid and heat are Queso Blanco (and Paneer), Ricotta (and Ricottone), Myzithra, Manouri and Anthotyros (Greek whey cheeses). They are made from whey or whey added by milk or in the case of Queso Blanco whole milk (Farkye, 2004; Anifantakis, 1991; Kosikowski and Mistry, 1997).

21.3.1 Flavor of fresh cheeses

The development of flavor compounds in fresh cheeses is related to mesophilic starter cultures (Spinnler *et al.*, 1997) added for acidification. The first factor involved in the flavor of fresh cheeses is the acidity. Lactic acid contributes to the formation of the 'fresh' character of the cheese along with acetaldehyde usually present at concentrations of 0.1–1.0 ppm. Other aldehydes, such as hexanal, nonanal and decanal, found at even higher levels, may also contribute to cheese freshness. Diacetyl, at usual concentrations of 1–6 ppm, contributes greatly to the aroma and the 'creamy' flavor of these cheeses. Other end products of citrate metabolism, such as acetate and 2,3-butanediol, and the short chain fatty acids butyric, octanoic and decanoic, may also participate in the formation of their organoleptic characteristics. Ethyl butyrate, as well as γ - and δ -lactones, form in the cheeses to contribute a 'fruity' or 'milky' flavor to the cheese. The 'flat' flavor is a result of the inability of the starter to form aroma compounds from citrate. 'Yogurt' or 'green flavor' is due to the formation of acetaldehyde rather than diacetyl, mainly from the metabolism of citrate-positive lactococci (Lindsay *et al.*, 1965).

21.4 Quark and other fresh cheeses

Quark is a soft white cheese with a clean, refreshing mildly acidic flavor, made from pasteurized skim milk with a LAB starter culture and a small amount of rennet. It can be fat free or contain up to 12% fat, which contributes to the overall flavor. A 12% fat Quark resembles Neufchâtel cheese. Varieties directly related to Quark are Tvorok, Fromage Frais and some other fermented milk products from the Middle East and Balkan regions (Labneh, Tan, Tulum), India (Chakka and Shirkhand) and Europe (Skyr, Ymer, Lactofil) (Schulz-Collins and Senge, 2004; Kosikowski and Mistry, 1997; Guinee *et al.*, 1993).

The flavor of cheese is the result of the action of the starter and is largely due to diacetyl and possibly acetaldehyde. These products may be flavored by the addition of sugars, fruit purées and other condiments. They are also often blended with yogurts and other fresh cheeses for the production of new fresh cheese products with different flavor attributes (Guinee *et al.*, 1993; Kosikowski and Mistry, 1997; Schulz-Collins and Senge, 2004). Filtration technology used at different stages of the manufacture of Quark-type products results in softer, smoother and creamier structures (Schkoda and Kessler, 1996; Mucchetti *et al.*, 2000). The pH-sensitive solubility of calcium is the main cause of bitterness in Quark made from UF sweet milk (Jelen and Renz-Schauen, 1989).

21.4.1 Cream cheese and related varieties

Cream cheese is a slightly acid-tasting product with a mild diacetyl flavor made from standardized, homogenized, pasteurized milk and a starter culture, containing citrate fermenting *Lc. lactis* subsp. *lactis*. After acidification to pH

4.5–4.8, the coagulum is gently agitated, heated and concentrated (Guinee *et al.*, 1993; Kosikowski and Mistry, 1997; Schulz-Collins and Senge, 2004). Neufchâtel, Petit Suisse, Gervais or Fromage Frais à la crème are also comparable to cream cheese. By adding various flavors, spices, herbs and fish, cream cheese types of diverse flavor are produced.

21.4.2 Cottage cheese

Cottage cheese is a mildly acid cheese, to which cream and salt are added at a later stage of production. It may also contain vegetables, chives, pineapple, olives, spices, and condiments. Cottage cheese varies widely in flavor, which may be bland to relatively high in acid, taste and aroma. This is achieved using several methods for controlling the amount of flavor. Strains of lactococci, which are least susceptible to agglutination, are used for acid production, or direct acidification by food-grade acids is commonly applied. In addition, low CO₂-producing citrate-fermenting lactococci or leuconostoc strains are added for the production of diacetyl (Kosikowski and Mistry, 1997; Farkye, 2004; Guinee *et al.*, 1993).

Diacetyl at ~2 ppm (Hempenius *et al.*, 1965), and especially a diacetyl: acetaldehyde ratio of 3–5 (Lindsay *et al.*, 1965), are desirable for a good cottage cheese flavor. Ratios of <3 or >5 result in flavor defects of green or harsh flavors, respectively. Lactic acid (124–452 mg kg⁻¹) contributes to the acidic taste, while formic, acetic, propionic and butyric acid contribute to the aroma of Cottage cheese (Brocklehurst and Lund, 1985). Bitter, fruity or fermented, malty, musty, sour, stale and unclean flavor defects result from the activity of microbial contaminants. Acid flavor, and sometimes bitterness, results from the action of starter culture activity (Stone and Naff, 1967).

21.4.3 Anevato cheese

Anevato is a traditional spreadable Greek cheese made from raw goat or ewe milk or mixtures of the two. Traditionally, shepherds with large flocks of goats and sheep produced Anevato cheese. They renneted milk obtained in the morning just before taking the cattle out for feeding and the curd was floating on the surface and it was ready to be drained on their return late in the afternoon.

LAB dominates the microbial population in the traditional cheese manufactured throughout the whole lactation season. Lactococci dominate in the cheese until 15 days of storage and *Lc. lactis* subsp. *lactis* (mostly citrate-positive strains) is the most abundant species (Hatzikamari *et al.*, 1999). The isolates of *Lc. lactis* subsp. *lactis* may differ considerably in respect of their acidifying, caseinolytic, aminopeptidase and esterase/lipase activities. Using selected 'wild' isolates as the starter culture to make cheese from raw milk improved the microbiological quality of the cheese. However, cheeses from heat-treated milk lacked the typical flavor of raw milk cheese, despite the use of starter cultures isolated from traditional cheese (Xanthopoulos *et al.*, 2000b).

21.4.4 Queso Blanco

Queso Blanco-type cheeses are creamy, highly salted and acid in flavor, semi-soft fresh cheeses, produced in Central and South America. In the traditional method, milk is heated to boiling and acid whey is added under continuous stirring, until coagulation is completed. Heating at 85°C for 5 minutes results in a very desirable quality cheese (Parnell-Clunies *et al.*, 1985). The hot milk is acidified with HCl, H₃PO₄, lactic, tartaric, citric or glacial acetic acid, or fruit juices or acid whey concentrate (Kosikowski, 1982; Farkye, 2004; Farkye *et al.*, 1995), under gentle agitation. The curd is left to settle and after whey removal, salt (2–2.5%) is added to the curd, which is then pressed. The pH of Queso Blanco decreases from 5.2 to 4.9 during storage (Farkye, 2004). Major volatile compounds contributing to the flavor and aroma of Queso Blanco cheese are acetaldehyde, acetone, ethyl, isopropyl and butyl alcohols, as well as acetic, propionic and butyric acids (Siapantas, 1967; Guinee *et al.*, 1993).

21.4.5 Ricotta

Ricotta is a soft and creamy cheese with a delicate texture and pleasant slight caramel flavor. It is made either by heating acidified (pH 5.9–6.0) whey with some milk, or from whole or standardized (1–2% fat) milk (Kosikowski and Mistry, 1997). A temperature of 80°C and 0.30% titratable acidity is the usual combination for whole milk. While being held cold for 24–48 hours acidification is achieved by addition of a starter culture, acetic acid, citric acid, phosphoric acid or acid whey powder. Ricotta cheese made with acetic acid has a flavor differing from that made by starter cultures, with a rich nutty flavor attributed to the starter culture. The best precipitant in terms of flavor is lactic acid starter; however, good quality acid whey powders, which are natural lactic acid starter fermented products, give excellent flavor to the cheese as well (Guinee *et al.*, 1993; Farkye, 2004; Kosikowski and Mistry, 1997). Ricotta cheese usually has a short shelf-life (3 weeks at 4°C) and is susceptible to microbial spoilage by mold, yeast and coliform bacteria that results in a sour fermented flavor.

21.4.6 Myzithra, Anthotyros and Manouri

Myzithra, Anthotyros and Manouri are Greek whey cheeses made from many types of whey, but mainly from whey obtained during manufacture of cheeses made from sheep milk or a mixture of sheep and goat milk. The Greek whey cheeses are made without acidification of the whey, by heating at 88–92°C (Zygouris, 1952; Anifantakis, 1991). Better quality Myzithra is made by adding whole milk (3–5%) to the whey during heating. Anthotyros is a specific type of Myzithra initially manufactured only in Crete with whey derived from Kefalotyri cheese whereby a small amount of sheep or goat milk is added (Anifantakis, 1991). Manouri is a high-fat version of Myzithra, traditionally originating from Western Macedonia, Northern Greece. Historically it is

obtained during the production of Batzos, a semi-hard cheese from goat milk. However, Manouri is the main product of this cheese-making process (Lioliou *et al.*, 2001). The high pH of the cheese seems to favor microbial growth and the numbers increase significantly to high levels during storage for 20 days. *Enterobacteriaceae*, yeast and staphylococci constitute a significant portion of the surface microflora, but only limited degradation of the cheese constituents occurs. However, high levels of microorganisms on the surface and visible growth deteriorate the quality of the cheese.

21.5 Rennet coagulated semi-hard fresh cheeses

21.5.1 Halloumi cheese

Halloumi is a semi-hard cheese made principally in Cyprus from sheep or goat milk or mixture of the two. The raw milk is coagulated as for Feta cheese. The blocks of the cheese (10 × 10 × 3 cm) are heated at 92–95°C in the whey extracted during draining. Subsequently, they are folded in half and sprinkled with a mixture of coarse salt and finely chopped mint (Papademas and Robinson, 2000, 2001, 2002; Robinson, 1991; Abd El-Salam and Alichanidis, 2004). The fresh product has a characteristic aroma that is unique to this cheese. If not sold immediately, the cheese is stored in salted whey (10–12% NaCl). Due to increasing demands for exports, pasteurized cow's milk is now used for its production and the flavor of the various cheese types vary greatly (Papademas and Robinson, 2001).

E. faecium dominates the microflora in fresh cheese from sheep milk, but it is replaced by lactobacilli. In mature cow cheese the LAB microflora is composed of lactobacilli (Papademas and Robinson, 2000). Yeast is also found in mature Halloumi and exhibits activities of esterase and lipase enzymes. Both lactobacilli and yeast have Leu-aminopeptidase activity, and thus have positive impact on the flavor of the cheeses (Papademas and Robinson, 2000). Proteolysis is more active in the ovine cheese, which also has higher overall concentrations of VFA in both the fresh and the mature cheese (Table 21.5). Acetic acid is the dominant VFA in fresh and especially in mature cheese (Papademas and Robinson, 2000). The concentrations of forage-based terpenes and related compounds that could be associated with flavor are different in cow milk than in a mixture of sheep and goat milk cheeses. Terpenes, such as α -pinene, β -pinene, copaene, thymol, α -caryophyllene, β -caryophyllene and δ -cadinene are found in the sheep and goat cheeses above their reported flavor threshold values, thereby contributing to the overall flavor of these cheeses (Papademas and Robinson, 2002).

21.5.2 Batzos cheese

Batzos is a semi-hard, low-fat traditional Greek cheese made from either raw goat milk or raw sheep milk as a by-product during production of Manouri. The goal is to obtain fat-rich, high quality Manouri or a large quantity of butter from

Table 21.5 Mean free volatile fatty acid (FVFA) (mg kg^{-1}) contents of fresh and mature commercial Halloumi cheeses $\pm$ standard deviations

FVFA	Fresh		Mature	
	Ovine	Bovine	Ovine	Bovine
Ethanoic	189.19 $\pm$ 77.69	162.50 $\pm$ 33.95	1093.50 $\pm$ 55.35	1079.75 $\pm$ 77.53
Propanoic	107.81 $\pm$ 16.3	129.76 $\pm$ 15.68	60.94 $\pm$ 7.86	72.50 $\pm$ 7.14
2-Methylpropanoic	42.45 $\pm$ 13.60	12.5 $\pm$ 0	39.58 $\pm$ 9.55	16.67 $\pm$ 7.22
Butanoic	28.13 $\pm$ 13.26	21.88 $\pm$ 4.42	36.84 $\pm$ 18.50	22.92 $\pm$ 3.61
3-Methylbutanoic	68.81 $\pm$ 16.8	52.95 $\pm$ 17.92	31.25 $\pm$ 8.84	26.53 $\pm$ 15.58
Pentanoic	46.88 $\pm$ 13.26	0	88.98 $\pm$ 19.73	25.00 $\pm$ 0.00
Total	483.27	379.59	1356.25	1,248.03
Lactic acid	516.000 $\pm$ 393.16	2050.21 $\pm$ 89.65	13,925.00 $\pm$ 331.38	11,137.19 $\pm$ 140.73

Source: based on Papademas and Robinson (2000).

the whey for use in production of this cheese. For this purpose, the milk is vigorously 'hit' during coagulation and thus a large proportion of fat is transferred to the whey (Zygouris, 1952). Some cheese makers apply mild heat before placing the curd in cheesecloth and hanging it to drain and ripen. The next morning, the ripened curd is cut into slices and salted with coarse salt. The cheese is either consumed fresh (usually fried, plain or with eggs) or placed in tins with brine and stored in cool rooms with commercial cheeses using additional advances today (Anifantakis, 1991; Anonymous, 1998).

LAB and *Enterobacteriaceae* are the dominant groups of microorganisms in cheese from either raw sheep or raw goat milk (Nikolaou *et al.*, 2002; Psoni *et al.*, 2003). In cheese from sheep milk enterococci dominate in the spring, whereas lactobacilli are the most abundant LAB in the summer production. *E. faecium* is the dominant species in the spring, and *Lb. paracasei* subsp *paracasei* dominates in the cheese made during the summer (Nikolaou *et al.*, 2002). In this cheese α_s -casein is degraded at a faster rate than β -casein and both caseins are hydrolyzed more rapidly in the spring than in the summer. Moreover, the FAA content is higher in summer cheese than in spring cheese and the milk fat is degraded more in the summer than spring. In cheese from goat milk, enterococci were abundant in winter, while in spring and summer lactobacilli isolates were found more frequently. *Lc. lactis* subsp *lactis* was the most frequently isolated species in the cheese (Psoni *et al.*, 2003). The *Lc. lactis* subsp *lactis* and *E. durans* isolates from goat milk cheese display acidifying and proteolytic activities when grown in milk, and exhibit peptidase activities on synthetic substrates. Their caseinolytic activity is significant with preference towards α_s -casein. When isolates of *Lc. lactis* subsp *lactis* from the traditional cheese were used as starter to make cheese from goat milk, due to high salt-in-moisture content ($>6.5\%$) in the cheese, both α_s -casein and β -casein undergo slight degradation during storage. In general, the ripening changes were not affected by the presence of starter culture and its contribution to flavor seems to be limited (Psoni *et al.*, 2006).

21.6 Sources of further information and advice

21.6.1 Key books

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- KOSIKOWSKI F V and MISTRY V V (1997), *Cheese and Fermented Milk Foods. Volume I: Origins and Principles*, F V Kosikowski, L.L.C., Westport, CT.
- ROBINSON R K and TAMIME A Y (1991), *Feta and Related Cheeses*, New York, Ellis Horwood.

21.6.2 Major trade/professional bodies

Feta cheese

- <http://greekproducts.com/greekproducts/fetacheese>
- <http://fiascofarm.com/dairy/feta.html>

Teleme cheese

- <http://www.arvanitis.gr>

Beyaz Peynir cheese

- <http://www.tahsildaroglou.com>

Domati cheese

- <http://www.luresext.edu/goats/library>

Quark

- <http://www.mothernature.com/library/ency>
- http://www.carbery.com/food_ingredients/application/dairy/quarg

Cream cheese

- http://www.kraft.com/archives/brands/brands_cream.html

Cottage cheese

- <http://www.explorewinsconsin.com/westbycooperativecreamery>
- www.graftonvillagecheese.com

Anevato, Batzos, Manouri

- www.kourellas.gr

Queso Blanco

- <http://www.wisdairy.com/cheeseinfo/mastercheesemaker/mc>

Ricotta

- <http://babcock.cals.wisc.com/artisan/crave-report.en.html>
- <http://www.realontario.ca>

Halloumi

- <http://myweb.cytanet.com.cy/cmio/hallumi.html>

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Cheeses with secondary cultures: mould-ripened, smear-ripened and farmhouse cheeses

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22.1 Introduction

In a small proportion (~10%) of the cheese varieties sold, the surface is covered by a layer of mould, yeast and bacteria (Fig. 22.1) (Bockelmann, 1999; Cantor *et al.*, 2004; Chamba and Irlinger, 2004; Spinnler and Gripon, 2004). These aerobic microorganisms impact on the appearance, flavour and texture development of cheeses substantially, which usually leads to shorter ripening periods of several weeks rather than months. Apart from the influence of the physical and chemical parameters of the cheese milk, the starter and non-starter lactic acid bacteria, these secondary cheese cultures contribute significantly to the complexity of cheese manufacture. Maintaining a high level of hygiene as well as a profound knowledge of the needs of a typical surface flora are essential, because the cheese surfaces are exposed to an unsterile environment during ripening. Undesirable contaminants will grow immediately if the balance of the cheese surface microflora is disturbed. Surface ripened cheeses are not mass-produced like Gouda or Cheddar cheese. They are traditionally produced on small scale in a farmhouse environment in many European countries. Production is more industrialised in countries like Denmark, France, Germany and the Netherlands; however, most cheese companies are small- to medium-sized enterprises.

A number of primary groups of surface ripened cheeses exist that are characterised by a surface ripening microflora consisting of mould, yeast and red smear bacteria. These include the following:

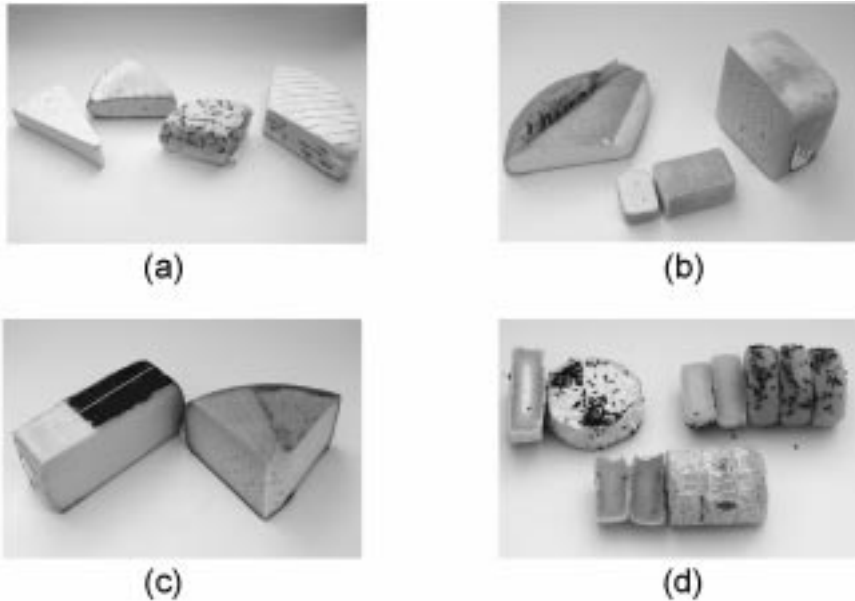


Fig. 22.1 Appearance of several surface ripened cheese varieties. (a) Mould cheeses (from left): typical Camembert cheese with a uniform white *P. camemberti* surface, a white mould cheese with visible growth of smear bacteria, Gorgonzola cheese with the typical wet-sticky surface, and a blue-veined Cambozola-type cheese showing growth of both *P. camemberti* and *P. roqueforti*. (b) Smear cheeses (from left): Chaumes-type and Limburg-type soft-cheese, semi-soft Tilsit-type cheese. The surface is covered only by paper or foil and is usually sticky. In case of strong *G. candidum* growth (Limburg cheese) the surface can appear quite dry. (c) Smear semi-hard cheeses produced with mesophilic lactic starters (left) and thermophilic lactic starters (right). The surface is quite dry because of the cover of wax and foil and the long ripening time (>10 weeks). The brand names were removed from the waxed areas. (d) Acid curd cheeses, traditional 'light' products with less than 1% fat (from left): surfaces covered by *P. camemberti*, *G. candidum* and smear bacteria. Caraway spices are usually added to all varieties (surface and core).

1. Mould-ripened cheeses, such as Camembert and Brie
2. Bacterial smear ripened cheeses (i.e., red smear cheeses) such as Tilsit and Romadour
3. Intermediate products like Pont l'Évêque.

Blue-veined cheeses, such as Roquefort and Gorgonzola, are special surface-ripened varieties, where the surface is extended into the core by piercing the green cheeses with needles, allowing the growth of *Penicillium roqueforti* on these inner surfaces. Understanding the microbial ecology of the cheese surface is essential for starter culture development, which makes it possible to control surface ripening and to improve flavour. In case of mould-ripened cheeses the microbiological situation is rather simple. A variety of well-characterised fungal cultures is sold by all of the starter culture companies. The microflora of smear

cheeses is much more diverse, but only a few species are commercially available, which does not reflect the complexity of their surface flora.

Can the flavour of surface ripened cheeses be improved? Many cheese experts will probably say no, because the flavour of these varieties is already much more intense and manifold compared to 'conventional' cheeses. It seems rather unlikely to improve the flavour of a Camembert, Tilsit or Gruyère cheese, especially when produced from raw milk. However, improvement could also mean a better control of the still highly undefined surface microflora, especially of red smear cheeses. Here, further improvement of flavour by traditional means, such as the recycling and use of the natural, undefined house microflora taken from mature cheeses (called 'old-young smearing'), is problematic because of the apparent hygienic risk of spreading contaminations not only with *Listeria monocytogenes*, but with increasingly antibiotic-resistant enterobacteria and enterococci as well (Terplan *et al.*, 1986; Rudolf and Scherer, 2001; Teuber, 1999; Teuber and Perreten, 2000). For 'conventional' cheeses, accelerated cheese ripening is a primary issue to improve the flavour. Probably because of the low price, many mass-produced cheeses have a rather flat taste due to the short ripening periods applied. Ripening intervals of surface ripened cheeses are rather short, too, but the flavour is still rather intense due to the aromatic properties of the aerobic surface flora, especially of smear cheeses. Thus, one improvement for consideration is further development of milder varieties, which would make them acceptable for more consumers and will likely increase sales of surface ripened cheese varieties as a group. A large number of 'modern' (milder) products have already been developed in the last few decades (blue-veined cheeses with 'milder taste' and smear cheeses with 'less smell but the same great taste'). In the current chapter emphasis is placed on improved control of the surface flora: the typical compositions for surface ripened cheese varieties are described and strategies to use tailor-made surface starter cultures are presented.

22.2 Mould ripened cheeses

Apart from the obvious need to work under very hygienic conditions, when surface ripened cheeses are produced, the surface colonisation of mould cheese does not seem to be so problematic as for smear cheeses. The uniform white appearance of mould cheeses indicates that the surface flora is quite homogeneous, containing mostly the microorganisms added as the starter culture (Lund *et al.*, 1995). After either adding *Penicillium* spores to the cheese milk or spraying them onto the cheese surface, a dense layer of mycelium, which develops within days, protects the surface of Camembert-like cheeses from contamination with pathogenic bacteria or undesirable mould. However, at the same time, the adventitious yeast and smear flora (if present) can develop and can contribute to the organoleptic properties of mould cheeses (Spinnler and Gripon, 2004). *Penicillium camemberti* is responsible for development of the typical aroma and texture of most varieties. In the nineteenth century, blue-grey coloured moulds (*P.*

album and *P. glaucum*) were predominant on mould-ripened cheeses. White variants of *P. camemberti* have been used since 1910, because they are more acceptable to consumers. For blue-veined cheeses, piercing with needles creates aerobic areas inside the cheeses, which allows growth of *P. roqueforti*. Strains from both species are selected according to morphological and physiological properties. For *P. roqueforti*, pigmentation is another selection criterion.

The composition of the surface microflora is complex when raw milk is used. Apart from the starter culture and non-starter lactic acid bacteria and the secondary surface starter cultures (*P. camemberti*, *P. roqueforti*), the variable endogenous raw milk flora is very important for the intense taste and full flavour of these cheese varieties. The presence of the yeasts *Debaryomyces hansenii*, *Kluyveromyces lactis*, *Saccharomyces cerevisiae*, *Geotrichum candidum* and other species is typical of raw milk cheeses, but they are present in variable numbers on cheeses made from pasteurised milk (Roostita and Fleet, 1996). Bacteria known from the surface of smear cheeses are also found in raw milk cheeses (e.g. staphylococci, coryneform bacteria, enterococci and enterobacteria). A recent analysis of German Camembert cheeses produced from pasteurised milk found that the bacterial groups and yeasts mentioned above are found on mould cheeses (Table 22.1) as

Table 22.1 Surface microflora of three German Camembert cheeses produced from pasteurised milk

Three batches of cheese (July, August, September 2005) from three producers (A, B and C) were analysed. All producers used *P. camemberti* as only secondary cheese culture. For sampling, thin slices of the surface were cut. Moulds and yeasts were analysed on YGC agar, coryneforms on modified milk agar, staphylococci on SK agar, enterococci on kanamycin esculin agar, and enterobacteria on VRBD agar (Hoppe-Seyler *et al.*, 2000). Single coryneform isolates and staphylococci were classified by ARDRA (Hoppe-Seyler *et al.*, 2003, 2004) and were shown to be *B. linens* (orange) and *M. gubbeenense* (yellow). Beige coryneforms (e.g. *Corynebacterium casei*) were not detected. Staphylococci were identified as *S. equorum*, few isolated enterococci as *E. faecium* and *E. durans*, and enterobacteria as *Serratia* spp.

	Cell counts (cfu cm ⁻²)		
	(A) 60% FID	(B) 30% FID	(C) 12% FID
<i>P. camemberti</i>	10 ⁴ , 10 ⁵ , 10 ⁵	10 ⁴ , 10 ⁵ , 10 ⁶	10 ⁴ , 10 ⁵ , 10 ⁵
<i>D. hansenii</i>	nd, 10 ⁶ , 10 ⁵	nd, nd, 10 ⁵	nd, nd, nd
<i>G. candidum</i>	10 ⁴ , 10 ⁵ , 10 ⁴	nd, nd, nd	nd, nd, nd
Other yeasts	10 ⁴ , 10 ⁵ , nd	nd, nd, nd	nd, nd, nd
Orange coryneforms	10 ⁵ , 10 ⁶ , 10 ⁶	nd, nd, 10 ⁶	nd, 10 ⁴ , nd
Yellow coryneforms	10 ⁵ , 10 ⁵ , 10 ⁶	nd, nd, nd	nd, nd, 10 ³
Staphylococci	10 ⁴ , 10 ⁴ , 10 ²	10 ² , <10 ² , 10 ²	10 ³ , 10 ³ , 10 ³
Non-coryneform cocci or rods	10 ⁵ , 10 ⁵ , 10 ⁶	10 ³ , 10 ⁵ , 10 ⁵	10 ³ , 10 ⁴ , 10 ⁴
Enterococci	na, 10 ³ , <10 ²	na, 10 ³ , <10 ²	na, <10 ² , <10 ²
Enterobacteria	na, 10 ⁴ , 10 ²	na, <10 ² , <10 ²	na, <10 ² , <10 ²

na = no analysis, nd = not detected among colonies counted, detection limit = 10² cfu cm⁻², FID = fat in dry matter.

adventitious microorganisms from the house microflora as well as starter cultures.

In some countries, such as France, raw milk cheeses have a long tradition and consumers appreciate the strong flavour of these cheese varieties. However, food safety can be a problem, since the microbial quality of the raw milk and the necessary high hygienic standards of production and ripening conditions are not easily maintained. Studies on the microbiological composition of raw milk from selected farms in the Camembert region of Normandy found considerable numbers of samples to be contaminated with pathogens, apart from the usual non-starter bacterial flora (Desmaures *et al.*, 1997). Pointing out the need for high raw milk quality is of critical importance for the manufacture of safe products. Mould-ripened cheeses in Europe are usually produced from pasteurised milk, which helps to ensure a high level of food safety but leads to less aromatic flavours. Perhaps this is not a disadvantage, since the majority of consumers seem to prefer mild-aromatic products.

22.3 Smear ripened cheeses

Bacterial smear-ripened cheeses have a long tradition (Bockelmann, 2003). Without any knowledge of the bacterial nature of the surface flora, a large variety of smear cheeses was produced long before 1900 (Fox, 1993). Among many other varieties, Gruyère, Tilsit, Limburg, Romadour, Chaumes, and the acid-curd varieties 'Harzer Roller', or 'Handkaese' are well known in European markets. When green cheeses, produced from raw milk – an important source for surface microorganisms – are exposed to air with a high relative humidity (>95%) they naturally develop a smear layer on the surface. Probably the first scientific papers on smear cheese ripening were published by Weigmann (1898) and Laxa (1899), who stated that lactic acid degradation by 'Saccharomycetes' and 'Oidium' yeast (now known as *Debaryomyces hansenii* and *Geotrichum candidum*, respectively) was necessary for smear development. For some soft cheese varieties they already described a bacterial smear consortium consisting of yellow cocci (*Micrococcus* spp. or *Staphylococcus* spp.), proteolytic orange *Bacterium linens* (*Brevibacterium linens*) and lemon rods (likely *Microbacterium gubbeenense* or *Arthrobacter nicotianae*). No beige-pigmented *Corynebacterium* species were mentioned, which is not unusual for the soft cheese varieties studied (Bockelmann *et al.*, 2003). These aerobic microorganisms have a strong impact on the appearance, flavour and texture development of the cheeses, which usually leads to short ripening periods of several weeks rather than months. Smear cheeses are generally known for their intense sulphurous and ammoniacal aroma.

Smear ripened cheeses can be produced from any kind of rennet curd or acid curd (e.g. quarg). They can be divided into (semi-)soft (moisture 45–55%), semi-hard (moisture 45–50%), and hard cheeses (moisture 35–45%). In general, cheeses are salted by brining in $\geq 18\%$ sodium chloride. Small soft cheeses like Limburg and Romadour are brined for 1.5–4 hours, the larger semi-soft and hard

cheeses for 24–72 hours or longer. Alternative traditional manual methods include wiping small cheese blocks or wheels with cloths soaked in brine or rubbing salt onto the surface. A much more recent dry salting method is automated spraying of cheeses with dry salt vapours, which was established for small soft smeared cheeses.

After salting, the cheeses are smeared with salt water containing yeast and bacteria. Traditionally, the smear is applied to the cheese using a rotating brush, which is wetted when moving through the smear liquid placed at the bottom of the machine. For soft cheeses spraying machines are used instead. The smear liquid is made in water containing 3% salt or more (sometimes by adding part brine solution) to mirror the salinity of the cheeses. Often it is inoculated by commercially available surface starter cultures, such as *D. hansenii* and *B. linens* (Bockelmann, 1999). Recommended inoculation concentrations are $\sim 10^4$ cfu ml⁻¹ in the cheese milk, or 10^6 cfu ml⁻¹ in the smear, or 10^{11} cfu 100 kg⁻¹ of cheese. Cheese producers do not rely on these cultures alone. The potential sources of typical smear microorganisms are cheese milk, cheese brines, the air of ripening rooms, ripening shelves and the human skin. Since the introduction of pasteurisation, which has considerably improved food safety, the cheese milk flora have less influence on the surface microflora of cheeses (Holsinger *et al.*, 1997).

To solve this problem mature cheeses are treated in the same machine before green cheeses are smeared (sprayed), which is called ‘old–young’ smearing because part of the ‘old’ flora from mature cheeses is retained in the smear liquid and is subsequently brushed (sprayed) onto the surface of green cheeses. The associated hygienic problems are obvious: saprophytic or pathogenic bacteria as well as moulds can become part of the house microflora and can persist over long periods of time by this in-house contamination cycle (Terplan *et al.*, 1986).

A certain low level of contamination with enterobacteria, pathogens like *Listeria monocytogenes* and other contaminants can be assumed for most old–young smeared cheeses. For reasons largely unknown, pathogens sometimes grow to high cell numbers, only then posing a risk for consumers. Not the pathogenicity, but the (transferable) multiple antibiotic resistances are matters of concern, when enterobacteria and enterococci are contaminating the cheese surface (Teuber, 1999; Teuber and Perreten, 2000). Therefore old–young smearing is being more and more criticised and efforts have been initiated to establish alternative methods, i.e. functional defined surface starter cultures to meet the continuously increasing hygiene demands of European guidelines and regulations.

An alternative strategy used by some cheese producers is to use cheese brines to adjust the salinity of the smear liquid and to avoid old–young smearing. Since the cheese brines contain all the microorganisms necessary for cheese ripening, this is a possible approach (see Section 22.4). Since the concentrations of the microorganisms are quite low, however, an increased level of care is necessary, such as more frequent and shorter brushing intervals.

Correct handling and storage of smear cheeses during ripening is essential. Ripening temperatures range from 12°C to 18°C, and the humidity should be at least 95% for fast smear development. Excessive ventilation should be avoided. In addition, repeated turning of cheeses and surface treatment by repeated smearing (brushing) are important for successful ripening. Typical ripening times are 2 weeks for the soft Limburg/Romadour varieties (200–500 g), 1–6 months for semi-soft Tilsit-type cheeses (2.5–3.5 kg), and 6–12 months for hard cheeses, like Gruyère (>2.5 kg).

Apart from the influence of starter culture and non-starter lactic acid bacteria, smear cheese ripening is influenced by metabolic activities of the surface microflora. Surface ripening begins with the growth of yeast (e.g. *D. hansenii*, *G. candidum*, *Kluyveromyces marxianus*, *Candida krusei*), which utilise residual lactose, galactose and lactate. Acid- and salt-tolerant staphylococci (i.e. *Staphylococcus equorum*) may grow in the first few days of ripening. *Corynebacterium casei* and *Corynebacterium mooreparkense* are also acid- and salt-tolerant, which explains the high populations observed in all stages of ripening (Bockelmann *et al.*, 1997c; Brennan *et al.*, 2002). With lactate degradation, the surface pH of the cheese is elevated to above pH 6, and *B. linens* and other coryneform bacteria (e.g. *Microbacterium gubbeenense*, *Arthrobacter nicotianae*) also grow (Eliskases-Lechner and Ginzing, 1995b). Eventually the whole surface of the cheese is covered by coryneform bacteria, staphylococci and yeast (Table 22.2) (Bockelmann, 2002; Eliskases-Lechner and Ginzing, 1995a).

22.3.1 Surface colours

Smear cheeses possess a dry or sticky surface that is beige or light brown (semi-soft, hard cheeses) or more orange (soft cheeses), the latter sometimes having streaks of white from *G. candidum* growth. For soft cheeses, the use of orange carotenoid food colorants is common to enhance the orange appearance (Fig. 22.1(b), left). Orange colours may be caused by *B. linens* when present in high populations. For some cheese varieties, orange patches are not caused by *B. linens* but by high concentrations of orange-pigmented staphylococci *S. saprophyticus* and *S. equorum* (Bockelmann *et al.*, 2002). Red pigments can be liberated by yellow-pigmented *M. gubbeenense* or *A. nicotianae*. Their yellow pigments turn into a reddish colour under alkaline and proteolytic conditions, both being common conditions during smear cheese ripening (Bockelmann *et al.* 1997a). The mechanism of how a more orange (soft cheeses) or more red surface colour (semi-soft cheeses) is developed is not yet understood. The authors speculate that it may be due to the percentage of the yellow coryneforms in the surface flora and the level of proteolysis on the surface.

22.3.2 Microflora of semi-soft cheeses

Due to their natural presence in cheese brines, *D. hansenii* and cream- or orange-pigmented *S. equorum* are always found on semi-soft cheeses, with highest cell

Table 22.2 Surface cell counts of several types of smear cheeses

Red smear cheeses were analysed routinely over several years with more than 20 samples for each cheese variety and four samples for Gruyère-type raw milk cheeses. Plating of coryneforms on modified milk agar (Jäger *et al.*, 2002) allowed a quite reliable pre-classification according to different cell morphology and colony colour, which was confirmed for single isolates by ARDRA classification. The restriction patterns were compared to type strains of the species (Hoppe-Seyler *et al.*, 2003, 2004). Most beige-coloured colonies belonged to the species *Corynebacterium casei*, yellow colonies to *Microbacterium gubbeenense* (some to *Arthrobacter nicotianae*), and orange colonies of irregular rods to *Brevibacterium linens*. Staphylococci (beige and orange colonies) were generally classified as *Staphylococcus equorum*. Sometimes *S. xylosus* and *S. saprophyticus* were identified. The latter species was typical for the surface microflora of acid curd cheeses. Bacterial groups, which were not found (–), might have been below 1% of the surface cell counts on modified milk agar and thus could not be analysed by plating methods.

Surface microflora	Gruyère-type	Tilsit-type	Chaumes	Limburg/Romadour	Acid curd cheese
Yeasts (cfu/cm ⁻²)	<10 ⁴	10 ³ –10 ⁶	10 ⁴ –10 ⁷	10 ⁴ –10 ⁷	10 ⁷ –10 ⁸
Smear bacteria (cfu/cm ⁻²)	10 ⁸ –10 ⁹	10 ⁸ –>10 ⁹	10 ⁸ –>10 ⁹	10 ⁸ –>10 ⁹	10 ⁸ –>10 ⁹
Beige, beige-reddish <i>Corynebacterium</i> spp.	50–>90%	50–>90%	<50–70%	10–>60%	20–>90%
Yellow <i>Arthrobacter</i> , <i>Microbacterium</i>	<1–2%	<1–5%	1–30%	2–>50%	–
Orange <i>B. linens</i>	2–10%	0.1–15%	<1%	<1–15%	0.1–90%
Orange, motile <i>Halomonas</i> sp.	–	–	–	30–40%	–
<i>Staphylococcus</i> spp.	<1–1%	0.1–5%	0.1–5%	<1%	1–90%
<i>Micrococcus</i> spp.	–	–	0.1–20%	–	–
Non-coryneform rods	0–70%	–	–	–	–
Enterococci	<10 ⁶	<10 ³	<10 ⁵	<10 ⁴	<10 ⁶
Enterobacteria	<10 ⁶	<10 ⁵	<10 ⁶	<10 ⁴	<10 ⁶

densities in the first week of ripening (Table 22.3) (Jaeger *et al.*, 2002). If commercial staphylococci (trade name *Micrococci*) are used for smearing, *Staphylococcus xylosus* is often detected on the cheese surface (Bockelmann and Hoppe-Seyler, 2001). A certain percentage of yellow-pigmented *M. gubbeenense* and *A. nicotianae* is detected. Beige or beige-reddish pigmented corynebacteria are the most abundant in the surface flora. The dominant species of all coryneforms, practically always present in the surface microflora, is *Corynebacterium casei*, a species that was classified as *Corynebacterium ammoniagenes* in older reports (Bockelmann *et al.*, 2005). Lower numbers of *C. mooreparkense* (*C. variabile*) and *C. flavescentis* are also frequently isolated from semi-soft cheeses. In older reports *B. linens* is generally called the ‘typical red smear bacterium’ before all other species because of the bright orange pigments. It is one of the best studied cheese bacteria, the strong sulphur metabolism and the bacteriocins specific for *L. monocytogenes* have been studied in detail (Eppert *et*

Table 22.3 Development of the surface flora of experimental semi-soft smeared cheeses

The surface starters consisted of *D. hansenii*, *S. equorum*, *C. casei* (beige), *M. gubbeenense* (yellow) and *B. linens* (orange). After four weeks of ripening (15C, >96% relative humidity) the cheeses were waxed and foiled and were further ripened at the same temperature at a lower humidity. The bacterial colonies retrieved from modified milk agar (mMA) matched the colony and cell morphology of the smear bacteria used. Several batches of cheese, which were ripened, showed a variable flora composition at all sampling points. The high *Staphylococcus* counts at the beginning and the end of ripening and the high counts of *C. casei* seemed to be a general phenomenon. In some batches the cell counts of yellow and orange coryneforms were reversed. The variability of the surface flora composition between batches was as high as observed for commercial smear cheeses, too (Table 22.2). The detection limit of smear species was around 1% of the cell counts on mMA (– = not detected, <1% = single colonies on overgrown plates detected).

	Ripening time					
	1 week	2 weeks	4 weeks	6 weeks	9 weeks	15 weeks
Yeast (cfu cm ⁻²)	7×10^7	3×10^7	4×10^6	1×10^6	1×10^4	1×10^8
Bacteria (cfu/cm ⁻²)	1×10^9	5×10^9	3×10^9	7×10^9	4×10^8	1×10^9
Staphylococci	92%	25%	4%	1%	<1%	97%
Beige(-reddish) coryneforms	8%	71%	62%	71%	51%	3%
Yellow coryneforms	–	2%	33%	26%	43%	–
Orange coryneforms	–	2%	<1%	–	<1%	–
Other bacteria	–	–	–	1%	5%	–

al., 1997; Valdes-Stauber and Scherer, 1996; Weimer *et al.*, 1999). However, the cell densities of *B. linens* on the semi-soft cheese surface are rather limited. Even with no *B. linens* detected on selective agars (<0.1% of the microflora), cheeses can be of normal appearance and flavour (Bockelmann *et al.*, 1997c). A low level of contaminating bacteria (enterococci, enterobacteria) is always expected on the surface of smear cheeses (Gianotti, 1999) (Table 22.2).

22.3.3 Microflora of soft cheeses

The surface microflora of soft cheeses shows distinct differences (Table 22.2). The yeast flora consists of two dominant yeasts, *D. hansenii* and *G. candidum*. Apart from cream-coloured coryneform bacteria (*Corynebacterium* spp.), a high percentage of yellow coryneform *M. gubbeenense* or *A. nicotianae* is observed (Table 22.2). Usually at 20–50% of the flora the percentage can be near 100% of total cell counts for some factories (Bockelmann *et al.*, 2003). *B. linens* and *Staphylococcus* spp. show normally low cell densities, as observed for semi-soft cheeses. Often, staphylococci are not detected (Bockelmann *et al.* 2003). Typical of French Chaumes cheese are orange *Micrococcus* spp., microscopically being large diplococci (Bockelmann and Hoppe-Seyler, 2001). The presence of a high percentage of salt-tolerant, motile rods (*Halomonas*

spp.) is typical for a single German producer of Limburg and Romadour cheeses, these bacteria having always been detected on cheeses over several years (Table 22.2) (Bockelmann *et al.*, 2005). For soft cheeses, the degree of contamination with enterococci and enterobacteria is usually higher than for semi-soft cheeses.

22.3.4 Microflora of farmhouse, raw milk and acid curd cheeses

In the farmhouse environment any smear or mould cheese can be produced. In general, the hygienic standards are lower compared to commercially produced cheese, leading to a stronger influence of the farm-specific or 'house microflora' on cheese ripening. This approach often results in a rather intense aroma in the final cheese when compared to commercially produced cheeses of the same style. The most intense and full flavour is probably achieved when raw milk is used for the production of surface ripened cheeses. The level of contamination of farmhouse cheeses or raw milk cheeses (e.g. with enterococci and enterobacteria) is quite variable, often even lower than for 'industrial' cheeses produced from pasteurised milk (Burri, 1999; Gianotti, 1999).

The variability of the surface flora of a typical smear-ripened Irish farmhouse cheese was described by Brennan *et al.* (2002). Interestingly, the surface microflora were quite similar to the species found on 'industrial' soft and semi-soft cheeses. Of 400 isolates, 10 were coagulase-negative staphylococci. They were not further classified, but they most likely belonged to *S. equorum*, though the non-food grade *S. saprophyticus* may also have been present (Bockelmann *et al.*, 1997c, 2002, 2005; Hoppe-Seyler *et al.*, 2004). Of 390 coryneform isolates 50.2% were *C. casei*, 26% were *C. mooreparkense*, 12% were *M. gubbeenense* and 9.3% were unidentified. These new species were recently described by Brennan *et al.* (2001a,b). *C. mooreparkense* is quite similar to *C. variabile* and *M. gubbeenense* to *A. nicotianae*, thus these new species names may perhaps apply to species classified differently in older papers (see Hoppe-Seyler *et al.*, 2003). Five isolates were classified as *Corynebacterium flavescentis*. Interestingly, *B. linens* was added as a starter culture but was not recovered from the cheese using normal plating procedures. The authors speculate that early growing staphylococci may have inhibited the starter strain. However, it may well be that the proportion of the *B. linens* strain was less than 0.1–1% of total cell counts and could not be detected by these plating techniques. Such low amounts of *B. linens* were reported to be quite common for various cheeses (Bockelmann *et al.*, 1997c; Bockelmann and Hoppe-Seyler, 2001). Brennan *et al.* (2002) indicate that the high percentage of *C. casei* and *C. mooreparkense* in the microflora could be due to their lactate degrading ability and because they are acid and halotolerant (e.g. growth at pH 4.9 with 8% salt added). Using pulsed field gel electrophoresis (PFGE) the authors determined that the bacterial flora was dominated by single clones of the three adventitious species of coryneforms – *C. casei*, *C. mooreparkense* and *M. gubbeenense*. The PFGE patterns of the 37 unidentified isolates were also similar, indicating that they

were a single strain. A recent study with four Irish smeared farmhouse cheese varieties show the presence of 14 different bacterial species on the cheese surface; for each cheese between four and nine species were found in substantial numbers (Mounier *et al.*, 2005). Apart from the known smear species, *A. arilaitensis*, as first described by Irlinger *et al.* (2005), was dominant on three cheese varieties, together with *C. casei*.

Quite different from all rennet-type cheeses are the traditional German 'sour milk' (i.e. acid curd) cheeses that originated as a farmhouse cheese product. They are produced from low-fat quarg (>30% dry mass) that uses *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* as the starter culture. Originally, the initial acidification was performed spontaneously or by mesophilic lactic acid starter cultures. Salting of acid curd cheeses is performed by mixing quarg with ripening salts (NaCl, NaHCO₃, CaCO₃) to obtain the appropriate salinity and pH. Ripening of acid curd cheese is usually restricted to 2–3 days in the factory. Ripening progresses during transport and cold storage in food markets over the shelf life of 35–45 days after packaging. For acid-curd cheeses, a different 'old–young' inoculation step is included with the same effect as old–young smearing. A special batch of cheeses (called 'culture cheese') is ripened for two weeks before adding it to fresh acid curd to initiate ripening (Bockelmann *et al.*, 2002).

The different technology results in significant differences in the appearance, flavour and surface microflora (Fig. 22.1(d), Table 22.2). The white opaque colour of the quarg turns into a translucent light yellow-brown proceeding from the surface to the core. The colour is similar to milk, when caseins are solubilised with citrate under alkaline conditions. A chemical nature of this colour change, not proteolysis, is the likely cause of this colour (Bockelmann *et al.*, 2002). As the colour changes to yellow-brown the crumbly quarg texture changes to smooth and elastic. For acid-curd cheeses the smear flora is visible near the end of the shelf life (35–45 days). Often orange patches are not caused by *B. linens*, but by pigmented staphylococci (*S. saprophyticus*). Different yeast species (*Kluyveromyces marxianus*, *Candida krusei*, *C. utilis*, *C. lipolytica* and *Trichosporon asahii*) are frequently identified. Most common in analyses are *K. marxianus* and *C. krusei*, which were shown to be essential for a typical ripening process (Engel and Roesch, 1995; Bockelmann *et al.*, 2002). *K. marxianus* prefers to utilise galactose and lactose, while *C. krusei* prefers lactate as the primary carbon source. The deacidification properties of both yeasts are important for smear development.

On typical cheeses the major part of the microflora consists of beige-pigmented coryneform bacteria (Table 22.2). When used as surface starter, sometimes *B. linens* may form nearly 100% of the total surface cell density. Also important are the naturally growing staphylococci, usually *S. saprophyticus*. This is an important contaminant of acid-curd cheeses and is often the dominant species of the surface flora (Bockelmann *et al.*, 2002).

22.4 Microflora of cheese brines

Cheese brines, which are not pasteurised regularly (e.g. in Tilsit factories), develop a typical salt-tolerant microflora with a composition depending on the house microflora (Bockelmann, 2002). Highest concentrations are observed for yeast (*D. hansenii*) and staphylococci (*S. equorum*), which can be detected at 10^4 – 10^6 cfu ml⁻¹ in aged brines (Jaeger *et al.*, 2002; Wyder and Puhon, 1999). Smear bacteria (*B. linens*, *Corynebacterium* spp.) and other bacteria and yeast (*Candida versatilis*) are usually found at lower cell counts (10–100 cfu ml⁻¹; Bockelmann *et al.*, 2005). Sometimes cheese brines can be contaminated with *Penicillium*, *Mucor* and other moulds, especially when herbs are added to the cheese milk (e.g. caraway or wild garlic *Allium ursinum*; Bockelmann, unpublished results). Since the surface of smear cheeses is not covered by any artificial means (wax, foil) after brining, it can be imagined that the brine microflora have a significant impact on the development of the surface microflora and product quality of smear cheeses. According to results of Jaeger *et al.* (2002) high concentrations of *D. hansenii* and *S. equorum* ($>10^6$ cfu ml⁻¹) in cheese brines show a beneficial effect on deacidification in the first two days of ripening and a mould-inhibiting effect on the cheese surface. Obviously, no effect is present when dry salting is used, or for acid curd cheese, when quarg is mixed with solid ripening salts. The control of the brine microflora is widely neglected. This is surprising since it is quite common to use volumes of cheese brines to prepare smear liquids of cheeses.

22.5 Sensory description

Muir *et al.* (1995) devised a protocol for characterising the aroma of hard and semi-hard cheeses. The samples were adequately described in terms of nine aroma attributes: overall intensity, creamy/milky, sulphurous/eggy, fruity/sweet, rancid, cowy/unclean, acidic, musty and pungent. With statistical analysis, descriptors such as *aroma intensity*, *musty*, *pungent* and *fruity* were most meaningful and clearly separated blue-veined cheeses from the rest of the cheeses. Unfortunately, white mould-ripened cheeses were not included in the studies.

22.5.1 Proteolytic and lipolytic enzymes

Penicillium camemberti and *P. roqueforti* possess several extracellular proteolytic enzymes, a metalloproteinase and an aspartic proteinase with a preference for hydrophobic and aromatic amino acid residues (Chrzanowska *et al.*, 1993, 1995; Gripon *et al.*, 1980), including extracellular carboxypeptidase activity (Auberger *et al.*, 1995). Due to the extracellular location, these enzymes contribute to cheese-ripening by liberation of amino acids and have a debittering effect in cheeses. An intracellular prol-aminopeptidase from *P. camemberti* and an aminopeptidase from *P. camemberti* cleave a wide range of substrates

and debitter the peptide fraction from a peptic digest of casein (Fuke and Matsuoka, 1993; Matsuoka *et al.*, 1991). An extracellular carboxypeptidase and aminopeptidases with similar specificity have been purified from *Geotrichum candidum* (Auberger *et al.*, 1997).

The effect of rennet, the starter culture and the mould flora was studied by comparing the ripening of Camembert cheese with that of rennet-free and starter culture-free cheeses. The effect of mould proteinases was marked by degradation of β -casein when levels of mould proteinases increased after more than two weeks of ripening. The further breakdown of large peptides to small peptides and amino acids was attributed to the proteolytic system of lactic acid bacteria (Takafuji and Charalambous, 1993). Other studies have confirmed these results in various cheeses. In Camembert, α_{s1} -casein was degraded first by chymosin, as the content of the extracellular fungal proteinases increased within the first 10 days of ripening; degradation products of α - and β -caseins were detected. The changes proceeded from the surface to the centre of the cheeses. The most abundant amino acids were glutamic acid, serine and proline. It is clear that ripening proceeds from the surface of the cheese to the centre (Iwasawa *et al.*, 1996). The proteolytic role of yeast in the surface flora is thought to be minimal compared to other organisms present.

Amino acid conversion was reviewed by Hemme *et al.* (1982) and Curtin and McSweeney (2004). In blue-veined cheese and Camembert, citrulline and ornithine are formed by conversion of arginine. Glutamic acid is decarboxylated to γ -aminobutyric acid. Tyramine, histamine and tryptamine are formed by decarboxylation of amino acids. The presence of *B. linens* on cheese surfaces should be beneficial, since these bacteria possess deaminases, which metabolise biogenic amines (Leuschner and Hammes, 1998). The importance of *B. linens* for cheese ripening is stressed by the fact that the bacteria can liberate large quantities of volatile sulphur compounds from cysteine and methionine (Weimer *et al.*, 1999).

Lipids are hydrolysed extensively in mould-ripened cheeses; 5–20% of triglycerides are degraded, depending on the type and age of cheese (Gripon, 1993). The strong and intense lipolytic activity during ripening of Gorgonzola cheese was studied by Contarini and Toppino (1995). All triglycerides with various chain lengths were degraded at similar rates. Higher concentrations of diglycerides than monoglycerides were observed, with neither component being accumulated during ripening. The concentrations of free fatty acids were closely correlated to cheese age, with high variations of concentrations between individual samples. These compounds are the substrates necessary for the production of carbonyl compounds characteristic for the aroma of blue cheese.

Penicillium camemberti possesses an extracellular lipase, with an alkaline pH optimum (Alhir *et al.*, 1990; Lamberet and Lenoir, 1976), which is produced together with mycelial growth after several days of ripening. *Penicillium roqueforti* possesses two lipases, one with an acidic and one with an alkaline pH optimum (for a detailed review see Gripon, 1993). The specificities of the two enzymes were found to be different *in vitro*, the alkaline lipase being more active on milk fat. The

presence of two lipases in *P. roqueforti* may account for the differences in the aroma of *P. camemberti*-ripened cheeses and blue-veined cheeses.

22.5.2 Aroma of mould ripened cheeses

Strains can be grouped into aromatic groups, useful in their selection for cheese making. Traditional raw milk Camembert is much more aromatic due to the additional raw milk microflora. Yeasts contribute to a rose-like odour (2-phenyl-ethanol) at the beginning of ripening. The volatile flavour compounds liberated by *P. camemberti* are mainly methyl ketones and the corresponding secondary alcohols, fatty acids, and the alcohols (3-methylbutanol, 2-methylbutanol, 3-octanol and 1-octen-3-ol), which contribute to the basic flavour of Camembert-like cheeses (Jollivet *et al.*, 1993). Primary alcohols are important aroma compounds. 3-methylbutan-1-ol has an alcoholic-floral note and is present in significant quantities in Camembert cheese. The alcohol 1-octen-3-ol is important for the mushroom note in the typical Camembert flavour (Jollivet *et al.*, 1993).

Ammonia is another important element of Camembert aroma produced by *P. camemberti*, *G. candidum* and *B. linens* liberated by deamination of amino acids. Decarboxylation of amino acids liberates amines that have fruity, alcoholic or varnish-like aroma notes, and numerous amines were identified in Camembert cheese (Spinnler and Gripon, 2004). Amines are not final products but are subjected to oxidative deamination to form aldehydes. Aldehydes in cheese are transitory compounds since they are transformed rapidly to alcohols or corresponding acids.

In a study examining French blue cheeses, the volatile flavour fraction methyl ketones represented 50–75% of the total flavour profile; significant numbers of secondary alcohols and especially esters were found in Roquefort cheeses (Gallois and Langlois, 1990). It is possible to accelerate the ripening of blue cheese by 15 days with addition of extracellular enzymes from *Penicillium roqueforti*, which stimulates the formation of soluble nitrogenous compounds, free amino acids, volatile fatty acids and total carbonyl compounds (Rabie, 1989).

Sulphur compounds (methanethiol, sulphides, thioesters) are liberated mainly by coryneform bacteria including *B. linens*, which are present at least on some mould cheese varieties (Table 22.1). Yeast and mould also produce sulphur compounds, especially *G. candidum* which is known for the liberation of sulphury aromas. Sulphur compounds originate mainly from methionine metabolism, are quite volatile and have very strong aroma notes (garlic, cowshed, fecal, cabbage) that are not appreciated by many consumers. These notes are more typical for smear cheeses. In late ripening stages of mould cheeses sulphur compounds are reduced and can disappear completely (Spinnler and Gripon, 2004). Many different esters are present in mould cheeses. They are produced by yeasts early in the ripening process and they have diverse fruity and floral notes. A detailed description of flavour compounds and pathways was given by Spinnler and Gripon (2004).

22.5.3 Aroma of smear ripened cheeses

Deacidification of smear cheeses by lactate degradation and alkaline metabolites, like ammonia, cause softening of the cheese matrix. The effect of the surface flora on texture is more visible near the surface (0–5 mm depth) with a more intense and diverse protein degradation.

Volatile aromatic sulphur compounds originating from methionine and cysteine are likely to be key components of smear cheese flavour and contribute to the garlic, cowshed and fecal notes (Brennan *et al.*, 2004). The thioesters (S-methylthioacetate, thiopropanoate, thiobutyrate, etc.) are also important for the overall aroma. *B. linens* is responsible for the conversion of methionine to methanethiol, α -ketobutyrate, and ammonia. These bacteria are actively producing the very aromatic volatile H₂S, methanethiol, dimethyldisulphide, S-methylthioacetate, 4-trithiapentane and ethional. It may be beneficial for consumer acceptance of smear cheeses that *B. linens* occurs at low numbers in the surface microflora of most smear cheeses. *B. linens* is probably the most well-known producer of aroma compounds, though other smear bacteria such as *Corynebacterium glutamicum*, *Arthrobacter nicotianae*, *Staphylococcus equorum* and *Micrococcus luteus* also liberate aromatic sulphur compounds to a lower but still significant degree (Brennan *et al.*, 2004). This indicates that a balanced aroma is only possible with mixed cultures of smear bacteria growing on cheese, which was also determined in cheese model systems by Bockelmann *et al.* (1997b).

Acid-curd cheese, a typical example of which is ‘Harzer Roller’ (Fig. 22.1(d), top right), has a very strong flavour and odour and is usually bought on impulse, for immediate consumption. Shops tend to carry only a very limited range of this type of cheese, which is not regarded as having wide appeal to consumers. Low prices and frequent special offers reinforce the ‘cheap’ image of the cheese. Of all cheeses the ripening of acid-curd cheese is probably least understood, because no studies were performed on these varieties until 2001. Because of the high percentage of yeast in the core and surface of ripened acid-curd cheeses, yeast-specific aroma compounds such as alcohols and esters are liberated. The yeast (i.e. *C. krusei*) also liberates sulphur compounds, with the resulting aroma notes being further enhanced by the coryneform flora, including *B. linens* (Bockelmann *et al.*, 2002).

22.6 New developments in starter technology

The development of defined smear starters containing all relevant species is not only important for smear cheeses but contributes to an improved ripening of mould cheeses too (Table 22.1). The impact of yeasts and smear bacteria on mould cheese ripening was reviewed by Spinnler and Gripon (2004). For smear cheeses the extension of commercial bacterial cultures seems to be essential, since the old–young smearing is still common practice because of the lack of suitable cultures. Contamination of smear cheeses with *Listeria monocytogenes* periodically causes food-borne illness, and consequently economic losses for

cheese manufacturers (Rudolf and Scherer, 2001). Thus, in the long run it is essential to employ alternative ripening strategies using more defined surface cultures without contamination cycles.

22.6.1 Defined surface starters for smear cheeses

To define the requirements for a functional surface starter, essential components of the surface microflora have to be identified and the role of the species detected has to be understood. In a study of Brennan *et al.* (2002) a commercial *B. linens* strain was used for smearing but could not be detected on mature cheeses; *Corynebacterium*, *Arthrobacter* and *Microbacterium* species were predominant instead. This confirmed that a defined surface culture should consist of more than *B. linens* and that some commercial *B. linens* strains might not be suitable for smear cheese ripening, since they could not be detected on mature cheeses.

Today, reliable identification of smear species and strains is possible using a combination of biochemical identification (e.g. the API system), partial sequencing of 16S rDNA, amplified ribosomal DNA restrictions analysis (ARDRA) and FTIR spectroscopy for species, and using pulsed field gel electrophoresis (PFGE) and other methods for strain identification. However, such classification is time consuming and can be used for only a small number of isolates. A fast, rough picture of a smear microflora can be obtained by using the very rich 'modified milk agar' for plating (Hoppe-Seyler *et al.*, 2000). By colony morphology (pigmentation) and microscopy (irregular rods, clumping cocci), beige-coloured colonies indicate the presence of *Corynebacterium* spp.; orange colonies are most likely caused by *B. linens* and *Staphylococcus* spp. (e.g. *S. equorum*). Staphylococcal colonies are quite typical (larger diameter) and easy to distinguish from *brevibacteria* by microscopy. Most likely, yellow colonies can be classified as *Arthrobacter nicotianae* or *Microbacterium gubbeenense*.

Defined surface starters can be tested in a real cheese environment on a very small scale (500 g to 5 kg) (Bockelmann *et al.*, 2000). Sufficient humidity (>95%) can be provided either by a water reservoir at the bottom of the ripening cell or by placing the chambers in climatic rooms with appropriate humidity, which is the better solution. By measuring the surface pH and determination of surface cell counts, cultures can be screened for the following essential parameters: (1) fast deacidification and growth on the cheese surface, (2) development of typical smell and taste, and (3) prevention of bacterial and fungal contamination.

22.6.2 Cultures for semi-soft cheeses

On the basis of the composition of the surface of commercial Tilsit cheese, a defined surface starter consisting of five species was proposed for surface ripening (Bockelmann *et al.*, 1997b,c). Laboratory- and pilot-scale cheese trials confirmed that a smear consisting of *D. hansenii*, *S. equorum*, *B. linens*, *M. gubbeenense*, and/or *Arthrobacter nicotianae* and *Corynebacterium casei* was a

functional surface starter (Bockelmann *et al.*, 2000). The minimum concentration of bacteria in the smear solution was 10^7 cfu ml⁻¹. On a laboratory scale, cheeses were smeared once or twice in the first week of ripening, which allowed deacidification almost as fast as in the old-young smeared control cheeses (Fig. 22.2). The surface pH of 7.0 was achieved after one week with total bacterial cell counts of about 10^9 cfu cm⁻², which is sufficient to protect the surface from mould contamination. Semi-soft green cheeses (Tilsit-type) incubated in sterile brines were more sensitive to mould contamination than cheese brined in the presence of yeasts and staphylococci (Jaeger *et al.*, 2002). In these studies, coryneform bacteria were not used as brine adjuncts. Looking at recent results (Bockelmann *et al.*, 2005) an additional positive effect could be obtained if smear bacteria are inoculated into cheese brines as well (10 – 100 cfu ml⁻¹). The starter culture mentioned was tested on a pilot scale in cooperation with Arla Food, Denmark. The presence of all starter strains after eight weeks of ripening was detected by PFGE. After numerous cheese trials it can be concluded that semi-soft cheese ripening proceeds appropriately if the pH exceeds 7 and the total bacterial counts are above 10^9 cfu cm⁻² after seven days of ripening.

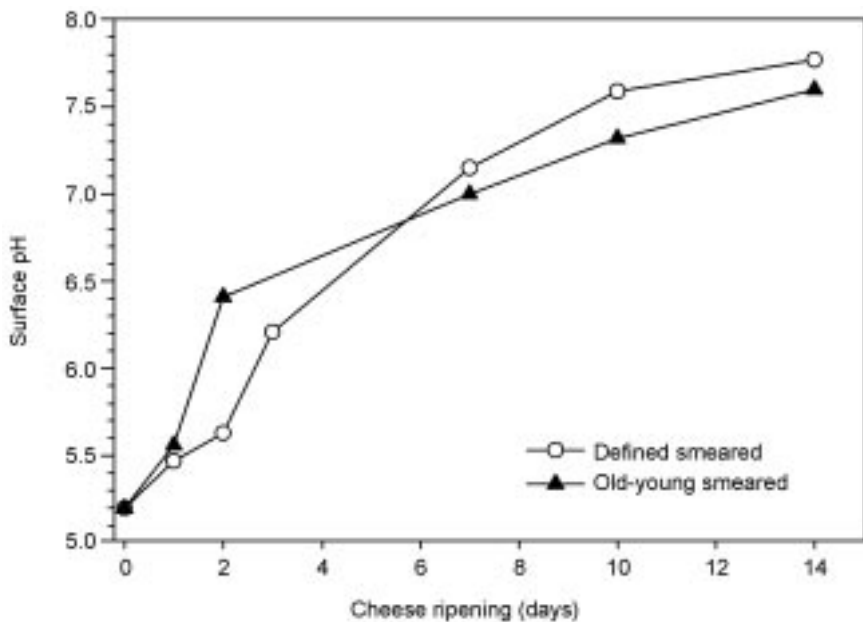


Fig. 22.2 Deacidification of the surface of Tilsit-type experimental cheeses. Both batches (four cheeses each) were ripened on a 10 kg scale. One batch was smeared with yeasts and smear bacteria of cheese origin (*Debaryomyces hansenii*, *Brevibacterium linens*, *Microbacterium gubbeenense*, *Corynebacterium casei* and *Staphylococcus equorum*). The concentration in the smear liquid was around 10^8 cfu ml⁻¹ for bacteria and 10^7 cfu ml⁻¹ for yeasts. The other batch was smeared with an old-young smear obtained from a local Tilsit cheese producer (total bacterial cell counts around 10^{10} cfu ml⁻¹. Cheese brines used for salting were pasteurised.

22.6.3 Cultures for soft cheeses

In contrast to Tilsit-type cheeses, the deacidification (and smear development) of the surface of commercial smeared soft cheeses (Limburg, Romadour-type) shows a lag phase of several days (Fig. 22.3). The typical Tilsit surface starter described above proved to be not suitable for soft cheese ripening; the deacidification was slow, and the aroma and the appearance were untypical (Bockelmann *et al.*, 2003). The presence of *Geotrichum candidum* in the cheese milk ($\sim 10^2$ cfu ml⁻¹) was found to be essential for appearance and aroma development of Limburg cheeses. Since no commercial *G. candidum* cultures are used by German cheese companies producing Limburg and Romadour cheeses, *G. candidum* obviously belongs to the typical house microflora of soft cheese companies (Bockelmann *et al.*, 2003). Different *G. candidum* strains show large differences regarding visual properties of cheeses and also development of volatile flavour, which essentially resemble that of *B. linens*.

Compared to Tilsit cheese brines, the brines of soft cheese plants have lower concentrations of yeasts, staphylococci and other smear bacteria (Bockelmann *et al.*, 2005). According to some German soft cheese producers, the cheese brines have to be sterilised quite often to avoid excessive growth of *D. hansenii* on the

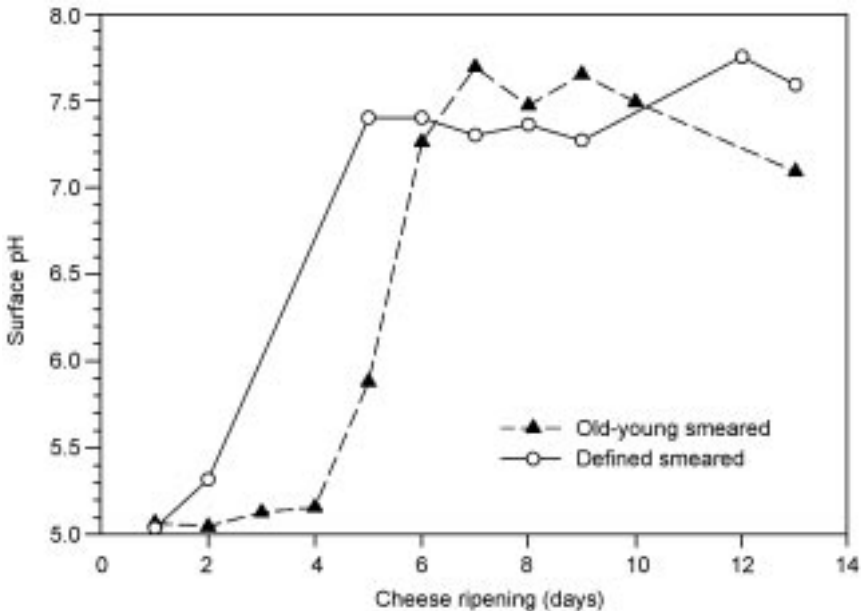


Fig. 22.3 Ripening of soft Limburg cheese. For the experimental, defined smeared cheese batch the cheese milk was inoculated with *Geotrichum candidum* (10^2 cfu ml⁻¹). The cheese brines contained *Debaryomyces hansenii* and *Staphylococcus equorum* (10^6 cfu ml⁻¹). Cheeses were smeared with *Microbacterium gubbeenense*, *Brevibacterium linens* and *Corynebacterium casei* (10^7 cfu ml⁻¹ in smear). The deacidification of commercial (old-young sprayed) cheeses was measured in a German Limburg cheese plant.

cheese surface. Two cheese brine samples obtained from Limburg cheese producers on the day before disinfection showed *D. hansenii* yeast cell counts of $>10^4$ and $>10^5$ cfu ml⁻¹, which seem to be the critical values for disinfection. Because of the beneficial effect of high concentrations of yeasts and staphylococci on Tilsit cheese ripening, the cheese brines for experimental soft cheese trials were inoculated with *D. hansenii* and *S. equorum* to a level of about 10^4 cfu ml⁻¹. As shown in Fig. 22.3, hardly any delay in deacidification was observed using the defined smear bacterial culture.

Culture development for soft cheeses concentrated on yellow-pigmented bacteria (*M. gubbeenense*, *A. nicotianae*), which were shown to produce a typical smear cheese flavour and colour in combination with *B. linens* and were predominant on the surface of commercial smeared soft cheeses (Table 22.2). The bacterial surface cell counts after one week of ripening were lower compared to experimental semi-soft Tilsit cheeses where 10^9 cfu ml⁻¹ can be expected. This may be due to fast growth of the two yeasts *G. candidum* and *D. hansenii* on Limburg cheeses in the first week of ripening, which may have retarded bacterial growth. After two weeks, the fully developed smear microflora was 10^9 cfu ml⁻¹, populations comparable to commercially produced cheeses (Bockelmann *et al.*, 2003).

In contrast to experimental Tilsit-type cheeses, unsmeared Limburg control cheeses, inoculated only with *G. candidum*, *D. hansenii* and *S. equorum* via cheese milk and cheese brines, have normal deacidification but not the typical colour and aroma development and have lower bacterial cell counts by two weeks of ripening. The bacterial flora of these control cheeses essentially consisted of the *S. equorum* used in the cheese brines. In spite of the orange pigmentation of the *Staphylococcus* strain used, the appearance of cheeses was beige rather than orange due to the low surface populations of $\sim 10^6$ – 10^7 cfu cm⁻², indicating the absence of yellow-pigmented *M. gubbeenense* or *A. nicotianae* that are important for typical colour development (Bockelmann *et al.*, 2003). To mimic the natural composition of the smeared soft cheese surface microflora, *C. casei* was added in the smear starter. The corynebacteria detected on ripened cheeses had no clear visual or aromatic effect on cheese ripening. After numerous laboratory and pilot-scale cheese trials, Bockelmann *et al.* (2003) concluded that aroma and appearance were mainly influenced by *G. candidum*, *B. linens* and *M. gubbeenense*, species that are consistently important for typical soft cheese ripening.

22.6.4 Cultures for acid curd cheese

The production of acid curd cheeses, traditionally performed by different farmhouse-type facilities, leads to the development of a typical but random yeast flora during shipment and storage, with the dominating species usually being *K. marxianus* and *C. krusei* (Table 22.2) (Bockelmann *et al.*, 2002). The use of these two species in the cheese milk (10^2 cfu ml⁻¹) and storage of the curd over 5–7 days at 15°C led to final yeast populations of about $\sim 10^7$ cfu g⁻¹ with

excellent cheese making properties from a technological and hygienic point of view (Bockelmann *et al.*, 2002). The time necessary for this 'quarg ripening' can be monitored by measuring the quarg core temperature, which is usually 1–2°C above the room temperature of about 15°C during anaerobic growth.

As for other smear cheeses, deacidification of the cheese surface is essential for ripening of the acid-curd cheeses, allowing development of the smear flora. The surface pH of acid-curd cheese reaches pH 7 after approximately one week, which is a function of *K. marxianus* and *C. krusei* (Fig. 22.4) (Bockelmann *et al.*, 2002). The addition of the food-grade *S. equorum* in the smear is done in order to simulate the presence of the naturally occurring *S. saprophyticus* contaminants, which results in further acceleration of the smear development. In contrast to other smear cheeses made with *S. equorum* or *B. linens*, if a single smear adjunct is used a homogeneous population builds during ripening, with a surface population of $\sim 10^9$ cfu cm⁻² (Bockelmann *et al.*, 2002).

The smell and taste of experimental cheeses ripened with just *K. marxianus*, *C. krusei* and *S. equorum* were quite typical with yeasty, alcoholic, ester and fruity notes with a sulphury (smear) flavour. Studies in model quarg systems

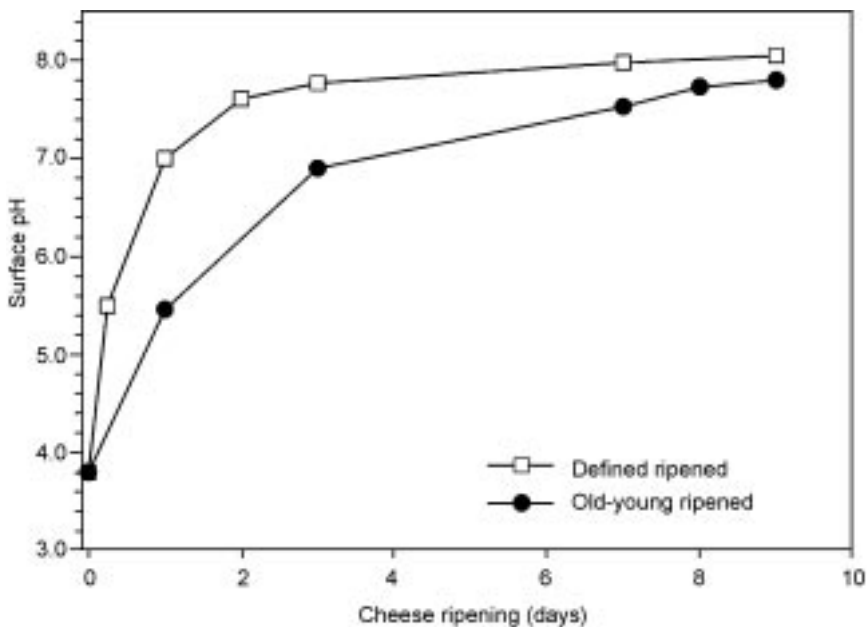


Fig. 22.4 Ripening of acid curd cheese. For the experimental cheese batches the milk was inoculated with *Kluyveromyces marxianus* and *Candida krusei* (10^2 cfu ml⁻¹). The fresh quarg (dry mass 32%) was incubated in closed plastic bags at 16°C for 7 days before the quarg was mixed with salts and the cheeses were formed. At that time the yeast counts had increased to 10^7 cfu g⁻¹ for both species. The first data point of the curves resembles the quarg pH before the addition of ripening salts, the second the surface pH of the cheeses 12 hours after the addition of salts (24 hours for the commercial cheese). *Brevibacterium linens* was sprayed on to the surface after moulding (10^6 cfu ml⁻¹, day 2).

found *C. krusei* produced smear cheese-like (i.e. *B. linens*-like) flavour compounds. With addition of *K. marxianus* alcohol- and strong ester notes (glue-like) developed. In co-culture of both yeasts a typical, mild acid-curd cheese aroma was produced (Bockelmann *et al.*, 2002). However, the sulphury flavour was more pronounced when the surface of the cheeses was sprayed with *B. linens*. The role of *Corynebacterium* species for smear and aroma development is clearly important, but the exact mechanisms of flavour development remain unclear. *Corynebacterium casei* and *C. variabile* were identified on commercial cheeses. It is possible that growth of these beige-pigmented bacteria can reduce the untypical orange surface colour of cheeses, which often is caused by high concentrations of *B. linens* and orange staphylococci.

22.7 Conclusions and future trends

A large number of mould and yeast cultures are commercially available. Strains are well characterised for many biochemical activities and visual properties. An immediate extension of the collections does not seem necessary. However, for acid-curd cheeses no adapted yeast cultures are available. The potential of smear bacteria including staphylococci is not fully used today, only a few species and strains being commercially available. There is a need to extend culture collections to include species for surface ripened cheeses. It should be noted that application of smear cultures need not be confined just to smear cheeses. The controlled use of smear bacteria for mould cheese ripening could enhance flavour development of mould cheese without changing its visual properties, especially of cheeses produced from pasteurised milk (Table 22.1) (Fig. 22.1a).

There is no doubt that the use of defined smear cultures will improve food safety. With no 'old-young' contamination cycles present in factories, which stabilise any occurring contamination, it should be possible to reduce common microbial contaminants significantly. The question remains whether the aroma and appearance of these cheeses can be kept unchanged. Initial results indicate that enhanced control of aroma development is possible. Williams *et al.* (2004) described the influence of proteolytic and amino acid converting enzymes of smear culture strains on semi-soft cheese ripening. It was shown that the enzymic activities of the cheese smear could be manipulated by surface starter composition, which influenced flavour compounds. Recent results obtained in an EU demonstration project showed that even with the use of defined surface starters the typical aromatic properties of experimental cheese matched the commercial varieties of the industrial project partners (Hannon *et al.*, 2004). One reason may be that the house microflora was detectable on these cheeses and consequently had an influence on cheese ripening.

Controlling the house microflora is one logical approach, but has proven difficult in other cheese types. Brines used for salting are one point to begin these measures in surface ripened cheeses. The isolation and identification of brine yeast and bacteria on species- and strain-level is a routine task. Currently,

the complete house microflora of cheese plants is analysed to this level. On the surface of four farmhouse cheeses, between seven and 18 strains, including non-food-grade species like *Vibrio* spp. and *Staphylococcus saprophyticus*, were found in one study (Mounier *et al.*, 2005). The highest strain variation was found for *C. casei*. These results match an analysis of the house microflora of a German Tilsit cheese factory. It is likely that 10–30 strains can simulate an almost complete house microflora on the cheese surface. Such a strain collection would be a valuable tool in case the brine or house microflora of a factory are severely disturbed. The task to isolate, identify, store and use these strains in case of need is possible today and should be practical, at least for larger cheese factories. The envisaged concentrations of about 100 cfu ml^{-1} for each strain, representing the natural levels, are quite easy to produce even for very large cheese brines, since cell counts of $>10^9 \text{ cfu ml}^{-1}$ in Erlenmeyer flasks are common growth yields for smear bacteria.

Even though of minor importance for the European cheese market, acid-curd cheeses can be very interesting foods for consumers who prefer calorie-reduced milk products. Acid-curd cheeses are traditionally produced from skim milk and still have flavourful sensory properties. Wider distribution of such cheeses is likely prevented by traditional production techniques leading to an almost random surface microflora with unavoidable levels of fungal or bacterial contamination. Bockelmann *et al.* (2002) found that new cultures (yeast and bacteria) can be used to develop products that are microbiologically stable over the shelf life of 45 days.

22.8 Sources of further information and advice

- Federal Research Centre for Nutrition and Food (Institute of Microbiology, Kiel, Germany, www.bfel.de)
- The Netherlands Dairy Research Institute (www.nizo.nl)

The starter culture companies have been subject to concentration and restructuring over recent decades. Some well-known suppliers offering yeasts, moulds and bacteria for surface ripened cheeses are (in alphabetical order): Chr. Hansen (www.chr-hansen.com), Danisco (www.danisco.com; formerly Wisby and Rhodia-Food), Degussa-Bioactives (formerly Sanofi or SKW, www.degussa.com), and Sacco srl (www.saccosrl.it).

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Producing low fat cheese

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23.1 Introduction

Prevalence in the Western World of diseases of affluence (obesity, heart disease and certain cancers) associated with imbalances in food intake and sedentary lifestyles has prompted governments of many industrialised nations to produce guidelines containing nutrition and dietary information for the general public. Dietary guidelines in the United States and most of the industrialised world recommend a reduction in total dietary fat to 30% of total energy (McDonald, 2000). As such, consumer awareness of dietary fat has increased and consequently the demand for low fat foods, including cheese, has grown substantially. The largest market for low and reduced fat foods is the United States (Hilliam, 1996) where in 1998 sales of low and reduced fat cheese accounted for approximately 20% of supermarket sales of cheese (Mistry, 2001). In Europe the market for low fat foods is relatively small and underdeveloped by comparison. The largest European market for low fat foods is the UK (Hilliam, 1996) where sales of low and reduced fat cheeses are growing at a faster rate than the mainstream full fat cheese market (Guinee *et al.*, 1998) although overall consumption of these products remains low at only 8% of total cheese consumption. In Mediterranean countries such as France, Italy, and Spain, traditional eating patterns continue to have a strong influence on the market, resulting in demand for taste and authenticity rather than calorie reduction by consumption of reduced fat foods (Hilliam, 1996). While lower fat cheese has gained some popularity, the consumer is demanding better-flavoured products, which will be needed if this market is to expand.

Although there is enormous potential for growth in the low fat cheese market, the development of this market over the past 20 years has been slow and this has

been attributed to poor consumer perception of the first generation of low fat products that were considered inadequate in terms of both taste and texture (Guinee *et al.*, 1998). Significant advances in understanding the biochemical and physicochemical characteristics of low fat variants in the past decade has led to improvements in quality of flavour, texture and functionality.

The term 'low fat cheese' encompasses a wide range of cheeses including soft, semi-hard and hard cheeses. Soft cheeses such as cottage cheese and quarg, prepared by acid coagulation using skim milk, are low fat products that have been manufactured for decades and are accepted by consumers as desirable products. Difficulties arise when attempts are made to produce low fat variants of cheeses that are popular and established as full fat varieties.

In producing low fat variants of standard fat cheeses such as Cheddar, processing parameters must be altered substantially to produce a cheese with acceptable texture. The resulting low fat variant differs markedly from its full fat counterpart compositionally. The ratio of moisture to casein, the pH and salt-in-moisture, all factors that influence the biochemical and microbiological changes, which control flavour and texture development (Fox and Wallace, 1997), differ substantially from full fat cheeses. The ripening characteristics differ between full, reduced and low fat variants, leading to substantial differences in flavour and texture, which in turn impacts consumer acceptance. Optimising the textural character of low fat Cheddar while maintaining good flavour has proved technologically challenging, but considerable advances were made in the last 10 years. Production of low fat Mozzarella with functional properties similar to those of the traditional product is the subject of much research activity, as are the flavour properties of reduced and low fat Cheddar cheese.

Low fat variants of many different cheese varieties are now available in the marketplace. Additionally, new concept cheeses that do not attempt to mimic an existing full fat variety are appearing. A ripened low fat cheese with bioactive properties was developed by a group in Finland and is in commercial production (Ryhanen *et al.*, 2001). Further advances in this area might be expected over the coming years.

23.2 Technology of manufacture

First generation low fat cheeses, especially low moisture ripened varieties, were generally characterised as having texture and flavour notes that are atypical of the equivalent full fat cheese. Low fat Cheddar cheese was frequently perceived as being dry, excessively firm and difficult to masticate. The cheeses had low flavour intensity and off-flavours such as bitter and unclean/barnyard flavours dominated the flavour profile. Use of stabilisers and fat mimetics to improve texture was noted to contribute to off-flavour development many cheese varieties. Elimination of the defects in these cheeses has proved technologically challenging. Approaches include the manipulation of processing parameters to enhance moisture levels, control of lactose levels, increasing the surface area of

the fat globules by homogenisation, selection of starter cultures, use of flavour adjunct cultures or enzymes for flavour and texture manipulation.

23.2.1 Legal standards for low and reduced fat cheese

There is currently no internationally recognised standard description for low fat cheese but there is expectation that the Codex Commission on international trade will set a maximum limit of 50% reduction of fat (on a dry-matter basis) from a referenced variety (Johnson, 2003). Legal standards for composition of low and reduced fat Cheddars are based on the legal minimum fat content of the full fat Cheddar (Johnson and Chen, 1991; Kosikowski and Mistry, 1997). Unlike full fat Cheddar there is no upper limit for moisture content of half and reduced fat Cheddar.

23.2.2 Processing parameters

Milk preparation

Milk for manufacture of low and reduced fat cheese is standardised to an appropriate fat level or casein to fat ratio depending on the fat content required in the final cheese. Casein-to-fat ratios of 2.05 and 1.28 are recommended for half fat (15.1%) and reduced fat (20.2%) Cheddar cheese, respectively (Johnson and Chen, 1991). Absolute values depend on the efficiency of recovery of fat in cheese and will be plant specific.

Standardisation of milk to appropriate casein-to-fat ratios for production of low fat cheese is generally achieved by removing fat from the milk or by adding reconstituted non-fat dry milk. However, fortification with condensed milk (Anderson *et al.*, 1993), direct ultrafiltration (McGregor and White, 1990) or fortification with dried ultrafiltrated or microfiltered retentate has been used as well (Rodriguez *et al.*, 1999; St-Gelais *et al.*, 1998; Ur-Rehman *et al.*, 2003).

Control of moisture

The processing of skim milk to Cheddar cheese using a full fat manufacturing protocol results in the production of a cheese that is too hard. The structure of the casein gel network that forms on renneting, together with moisture removal on syneresis, determine the physical properties of cheese. In a full fat Cheddar, the casein gel network entraps fat and moisture. Removal of fat from the casein network in low fat cheese results in the formation of a much tighter paracasein network, which on syneresis becomes excessively firm. To improve the textural character of low fat cheese, moisture levels in the curd must increase. Methods of enhancing moisture content include the manipulation of scald temperatures, stir times and temperatures (Banks *et al.*, 1989), the use of curd washing, or dry stirring as opposed to curd milling (Johnson and Chen, 1995) or milling curd at a higher pH (Guinee *et al.*, 1998). Reduction of fat in milk for Cheddar cheese manufacture is associated with increasing moisture and protein content on a

percentage basis, but a decrease in the concentration of moisture in the non-fat substance (Guinee *et al.*, 2000). These manipulations result in a different balance of nutrients and substrates for the starter culture during ripening that lead to changes in the flavour.

Washing or rinsing of curd with cold water is commonly practised in the USA to enhance moisture retention in curd (Johnson *et al.*, 1998). Cold washing reduces the rate of syneresis and removes residual lactose from the curd. The cold curd absorbs moisture and a higher moisture cheese with less acid is obtained. Washing also removes soluble calcium that leads to further solubilisation of calcium phosphate and an increase in casein hydration. These effects result in the development of a softer bodied cheese at a faster rate than that of a cheese of similar composition in which the curd is been washed during manufacture (Johnson *et al.*, 1998). Curd washing, however, can be detrimental to flavour development. Cheeses manufactured with a wash treatment are bland or mild in flavour and do not develop mature Cheddar cheese flavour (Johnson *et al.*, 1998). These cheeses tend to have a short shelf life of 2–4 months after which the body becomes pasty (Johnson *et al.*, 1998) and off flavours such as meaty and brothy develop (Johnson and Chen, 1995). Lowering the cooking or scald temperatures and reducing the scald and stir times (Banks *et al.*, 1989; Johnson *et al.*, 1998) can be used to obtain the desired moisture level in the final cheese. Employing a high pH at mill (5.6–5.9 for low fat Cheddar) is recommended to enhance moisture retention (Kosikowski and Mistry, 1997; Guinee *et al.*, 1998; Johnson *et al.*, 1998).

Johnson and co workers (Johnson and Chen, 1995; Johnson *et al.*, 1998) developed a process to produce quality low fat Cheddar that involves manipulation of several of the processes outlined above to optimise moisture retention. Curd formation is allowed to proceed for 50 minutes until a firm curd is obtained for cutting. The scald temperature is reduced to 37°C and the curd/whey mixture is stirred at this temperature for only 5 minutes. The curd is drained at a high pH (6.45) and is salted at a high pH (5.9). This procedure produces a low fat cheese that has a fat content of 14.5–15% (50% reduced fat cheese, 48–49% moisture, 1.9–2.1% lactic acid and a pH of 5.0–5.1 at 10 days). The texture develops within two months at 7–8°C and the cheese develops a typical mature Cheddar flavour.

Blending of skim milk curd and full fat curd following whey drainage also produces an acceptable low fat Cheddar cheese (Fenelon *et al.*, 1999).

Selection of starter and adjunct cultures

Starter cultures play an important role in cheese manufacture because of their metabolism and hence texture and flavour development. Selection of the correct starter culture is important for flavour and texture profile in full fat cheeses, but is even more important in production of low fat cheeses that are susceptible to development of off-flavours. A starter culture that produces high quality standard fat Cheddar may not be suitable for producing a quality low fat variant (Johnson and Chen, 1991).

Slower acid-producing starters are more desirable in the production of low fat cheeses, since the development of acid is a critical parameter to minimise off-flavours. As reduced and low fat cheeses contain more moisture, a slow rate of acid development during the cheese making process is essential to maintain a strong buffering capacity in the cheese and prevent development of intense acid flavours (Drake and Swanson, 1995). Ardö (1997) recommends that starter cultures used for low fat cheese manufacture, where temperature profiles differ from those in a normal fat content cheese, should be selected on the basis of temperature sensitivity, autolytic properties and proteolytic/peptidolytic activities. Autolysis should be induced at a lower temperature for a reduced fat cheese starter. Recent studies indicate that a balance between autolysis and active cultures during ripening provides compounds not found in milk or milk fat (Ganesan and Weimer, 2004). Considering the combination of the starter culture with the flavour adjunct culture, it is suggested that mixtures of culture be determined to provide the best flavour profile for reduced and low fat cheeses (Weimer *et al.*, 1997). Culture suppliers now provide starter cultures that have been specially designed for the manufacture of reduced and low fat cheeses. These cultures are characterised by slow growth, slow acid development and low proteolytic activity (Drake and Swanson, 1995; Katsiari *et al.*, 2002).

Non-starter lactic acid bacteria and adjunct cultures

During commercial cheese making a secondary non-starter bacterial population develops in the curd from adventitious lactic acid bacteria contaminants present in the milk, dairy plant and surrounding environment. These non-starter lactic acid bacteria (NSLAB) are initially present at low levels in factory-made Cheddar cheese and begin to grow after removal from the press. The adventitious NSLAB population increases to 10^6 – 10^8 cells g^{-1} after ~3 months of maturation and they maintain this level throughout the remainder of the ripening period. NSLAB counts in low fat cheese are generally considered to be lower than those of full fat cheese (Haque *et al.*, 1997; Fenelon *et al.*, 2000). Although the overall size of the NSLAB population remains relatively stable, pronounced shifts in the species complement and strain profile during ripening occur in full fat Cheddar and a similar effect would be expected in low fat Cheddar. The dominant NSLAB in Cheddar cheese are mesophilic lactobacilli, although *Pediococcus* and *Leuconostoc* spp. are also found (Peterson and Marshall, 1990). The NSLAB population is heterogeneous. Cheddar produced in several different countries around the world consistently reports the dominance of the facultative heterofermentative lactobacilli such as *Lactobacillus paracasei*, *Lb. plantarum*, *Lb. curvatus* and *Lb. casei* (Williams and Banks, 1997). Williams *et al.* (2002) reports differences in the NSLAB populations of full fat Cheddar cheeses produced in different production runs in the same factory and this variation may contribute to between-batch variations in the quality of the cheese produced in a creamery. Although Cheddar cheese can develop full mature flavour in the absence of NSLAB, as demonstrated by cheese making in aseptic vats, the NSLAB are considered to add desirable flavour notes and reduce the

harshness and bitterness associated with some starter cultures. As a consequence within the last decade there has been growing interest in the use of adjunct strains of selected NSLAB to suppress the growth of adventitious lactobacilli in full fat and low fat Cheddars. However, careful selection of adjunct cultures of lactobacilli and addition to curd at suitable levels have been successfully employed to improve flavour of low fat Cheddar. Addition of pediococci to low fat Cheddar results in a sharp aged Cheddar flavour at 6 months, whereas addition of micrococci results in intense brothiness and unclean off-flavours (Bhowmik *et al.*, 1990).

Fenelon *et al.* (2002) compared the use of six different types of starter/adjunct mixes for the production of low fat Cheddar. Each starter system contained a base starter of *Lactococcus lactis* ssp. *cremoris*. Three of the trials also included a second acidifying culture of *Lactococcus lactis* ssp. *lactis*, together with a selection of adjunct culture mixes comprising *Lb. helveticus*, *Lb. helveticus/casei*, *Leuconostoc cremoris/Lc. lactis* var *diacetylactis*/*St. thermophilus/Lb. helveticus*. The type of starter and/or the bacterial adjunct used did not influence primary proteolysis. However, variability in free amino acid levels was related to starter/adjunct systems. The principal amino acids in order of decreasing concentration were glutamic acid, leucine, phenylalanine, valine, lysine, serine and proline. Free amino acids were highest throughout ripening in cheeses made with the combined acid-producing starters (*Lc. lactis* ssp. *lactis/Lc. lactis* ssp. *cremoris*) in conjunction with the *Lb. helveticus* adjunct culture. Concentrations of low molecular weight peptides were highest in these cheeses, as were flavour-grading scores at 90 and 180 days of ripening. *Lb. casei* 7a in conjunction with selected starters produced a clean acid, non-bitter, milky, buttery low fat Cheddar (Midje *et al.*, 2000). The resultant cheese was mild in flavour, rather than having a flavour comparable to full fat mature Cheddar, but was acceptable to consumers. The starter culture mix used in manufacture comprised *Lactococcus lactis* ssp. *cremoris* SK11, *L. lactis* ssp. *lactis* biovar *diacetylactis* JV1 and *Lactobacillus casei* 7A. *Lactococcus lactis* ssp. *cremoris* SK11 was selected for its ability to produce a clean acid non-bitter Cheddar cheese, while *L. lactis* ssp. *lactis* biovar *diacetylactis* JV1 was included to produce buttery notes and acetate. To further enhance flavour and reduce bitterness, *Lb. casei* 7A was added.

Weimer *et al.* (1997) successfully used *Brevibacterium linens* as an adjunct to improve the flavour of low fat Cheddar cheese. Brevibacteria are normally found on the surface of Trappist-type cheeses and are not traditionally used as flavour adjuncts in Cheddar cheese. Beneficial effects on flavour in Cheddar are derived from the high level of methanethiol produced by these cultures (Ferchichi *et al.*, 1985; Dias and Weimer, 1998a,b). Weimer *et al.* (1977) also found that the success of the flavour adjunct culture depended on the combination with the starter culture. The Cheddar cheese best liked for flavour toggled between *B. linens* BL2 and lactobacilli depending on the starter culture used.

Tungjaroenchai *et al.* (2001) evaluated the effect of four adjunct cultures with differing levels of aminopeptidase activity on the flavour and texture of a

reduced fat Edam cheese (20% fat). Aminopeptidase activity of *Lc. lactis* ssp. *diacetylactis* was higher than that of *Lb. helveticus* LH 212, *Lb. reuteri* and *B. linens* BL2, respectively, but cheeses containing *Lb. helveticus* developed the highest levels of free amino acids. Beneficial texture effects were obtained using *Lb. helveticus* LH 212 and *Lb. reuteri*.

The flavour and texture of a low fat (9%) ewes' milk Kefalograviera-type cheese was improved significantly by selection of a commercial culture that produced acetate, diacetyl and acetoin from citrate fermentation (Katsiari *et al.*, 2002). Acetate is the dominant free fatty acid in full-fat Kefalograviera cheese and comprises 34% of all free fatty acids in the mature cheese. The selected commercial cultures improved the body and texture and greatly enhanced the flavour intensity of low fat high-moisture Kefalograviera-type cheese compared with the commercial regular starter used in full fat cheese production.

Ryhanen *et al.* (2001) developed a low fat cheese (20% fat) with bioactive properties. The probiotic cheese was developed as a new concept cheese that did not attempt to mimic an existing full fat variety. The starter culture used in manufacture comprised a 12-strain mixture that included lactococci, *Leuconostoc* sp., *Propionibacterium* and lactobacilli, in addition to *Lactobacillus acidophilus* and *Bifidobacterium* spp. The probiotic *Lb. acidophilus* and *Bifidobacterium* spp. remained viable for up to 7 months at 10^6 cfu g⁻¹, and thereby satisfied the minimum target level required for a probiotic effect.

Attenuated lactobacilli

The use of attenuated cultures increases the bacterial enzyme pool in the cheese curd without interfering with the acidification profile of the starter during cheese manufacture. Attenuated cultures are unable to grow and produce lactic acid but deliver active enzymes during cheese maturation. Attenuation of cultures for cheese making can be achieved by heat treatment, freezing, spray drying, freeze drying, lysozyme or solvent treatment (Klein and Lortal, 1999; Johnson *et al.*, 1995).

Attenuated cultures of *Lb. helveticus* were used to improve flavour development and reduce bitterness in a rindless low fat cheese (Ardö *et al.*, 1989). Enhanced flavour was obtained in low fat Gouda by the addition of lactobacilli attenuated by heat (Skeie *et al.*, 1995) and a beneficial salt-dependent debittering effect was also observed using this approach (Skeie *et al.*, 1997). Johnson *et al.* (1995) compared the effects on flavour development in reduced fat Cheddar cheese using a strain of *Lb. helveticus* attenuated by spray drying, freeze drying or freezing. Although each type of attenuated culture enhanced flavour development, a sweet nutty flavour that was uncharacteristic of Cheddar was prevalent. Pronounced and unpleasant off-flavours were found in cheeses made with freeze dried or frozen attenuated cultures. The addition of freeze shocked or heat shocked lactobacilli to low fat Ras cheese produced a cheese of superior quality (Kebary *et al.*, 1996). Use of attenuated cultures produced variable results, but generally led to flavour improvements. However, these cultures are not widely available for commercial sale.

Fat mimetics

Water dispersable fat replacers (fat mimetics) were used in low fat cheese products initially. Fat mimetics consist of microparticulated whey proteins or carbohydrate based materials. They mimic the properties of fat by entrapping water and giving a sense of creaminess. However, they cannot fully replace the non-polar functional properties of fat such as its flavour carrying capacity.

Cheddar cheese manufactured from whey protein based mimetics such as Simplese (Lucey and Gorry, 1993) or Dairy Lo (Fenelon and Guinee, 1997) have higher levels of moisture and milk solids, and have a softer texture than control cheese (Fenelon and Guinee, 1997; Lucey and Gorry, 1993). The addition of the mimetic did not influence the formation of soluble nitrogen during ripening (Fenelon and Guinee, 1997). However, the starter culture is capable of metabolising carbohydrate replacers that lead to off-flavours.

Rheological measurements (dynamic oscillatory and creep/recovery) for viscoelastic properties of low and full fat Cheddar cheese (Ma *et al.*, 1997) indicate that different types of mimetics provide different effects in improving the protein matrix of low fat cheese. Addition of protein-based (ALACOPALSTM) or whey-based (Dairy Lo) mimetics does not give sufficient improvement in the protein matrix of the low fat cheese to increase consumer acceptance. However, a carbohydrate-based mimetic (Novagel, NC-2000) improved the rheological properties, although it did not completely simulate the protein matrix of full fat cheese.

When low fat cows' milk was used to make Mexican Manchego cheese with Dairy Lo fat replacer, the cheese lost the compact and dense protein matrix characteristic of low fat cheese and exhibited hardness, springiness, cohesiveness and chewiness similar to a full fat control cheese (Lobato-Calleros *et al.*, 2001).

The removal of fat to produce low fat Mozzarella results in a cheese that is low in moisture, giving the cheese poor melting and stretching properties. A study of a variety of fat mimetics for their suitability in making low fat (<6%) Mozzarella cheese found that the largest increase in moisture content was obtained with the fat mimetic that produced the most serum pockets (McMahon *et al.*, 1996). Using a standard cheese process, addition of the carbohydrate-based fat replacers increased moisture levels in cheese by 1.3% and 4.3%, respectively, as compared with control cheeses. However, addition of whey-based fat replacers increased the moisture level by 2.2% and 2.3% as compared with the control cheese. Addition of carbohydrate fat replacers increases melt but addition of whey replacers decreases melt (McMahon *et al.*, 1996).

Improvements in the texture of a low-fat brined cheese made from bovine milk were obtained on addition of two commercial fat mimetics, the micro-particulated protein-based fat replacer (Simplese D-100) and a combination system (Novagel NC-200) containing a blend of microcrystalline cellulose with water-soluble polysaccharides (Romeih *et al.*, 2002). The textural attributes of the product made with Novagel more closely resembled those of the full fat cheese. Although rheological measurements showed an improvement in the texture when the fat replacers were included, sensory evaluation did not detect

this improvement. Overall, the full fat variant was perceived as being more elastic and less salty and had higher flavour and odour intensity scores than all the low fat variants. Although Simplese had a marked improving effect on cheese appearance, the product was rated as harder cheese than its full fat counterpart. The extent of proteolysis and lipolysis in the low fat white-brined cheeses made with the fat replacers increased significantly as compared with full fat cheeses. The kinetics of α_{s1} - and β -casein degradation were not affected by the presence of the fat mimetics. Use of fat replacers is not widely practised, but they can be used with specific applications with careful selection of cultures.

23.3 Effects of fat reduction on flavour, texture and functionality

As fat contributes to the physical characteristics of cheese (opacity and texture) and is a precursor for many lipid-soluble flavour compounds, it has the potential to modify the perception and volatility of flavour compounds in addition to texture and mouthfeel. Low fat cheeses are considered to be less acceptable to consumers than their full fat counterparts in part due to texture and flavour defects. Textural defects include increased firmness, rubberiness, hardness, dryness and graininess (Olson and Johnson, 1990).

The lack of flavour in low fat cheese is in part due to the lack of precursors from the fat, the lack of fat as a solvent for flavour compounds, and differences in the physical structure of reduced or low fat cheese that provides non-optimal conditions for enzymic reactions that are essential for the formation of flavour compounds (Urbach, 1997). The flavour defects are low intensity of typical cheese taste and aroma, bitterness, astringency and unclean flavours (Banks *et al.*, 1989, 1992; Lee *et al.*, 1992). Flavour development in cheese results from a combination of microbial and biochemical activities that lead to the formation of a heterogeneous mixture of volatile and non-volatile flavour compounds and amino acids that increase beyond the concentration that are present in casein (Fox and Wallace, 1997). Alteration of the balance in fat, protein and moisture in production of low fat cheese results in deficiencies not only in milk fat-derived flavour compounds but also in compounds generated from interaction of degradation products of lipolysis and proteolysis. Sensory perception of aroma and flavour compounds is also dependent on the rate of release of flavour compounds during mastication and this will be influenced by the fat content of the cheese (Delahunty *et al.*, 1996).

23.3.1 Flavour compounds in low fat cheese

Long lists of chemical flavour components in cheese are reviewed in this book and Urbach (1997). Fatty acids in cheese originate from lipolysis of the milk fat and bacterial metabolism of amino acids. Several studies have identified deficiencies in butanoic and hexanoic acids in low and reduced fat cheeses

(Banks *et al.*, 1989; Dimos *et al.*, 1996), whereas in full fat Cheddar optimum levels of these compounds are critical to intensity of Cheddar flavour (Barlow *et al.*, 1989). In a comparison of volatiles from full and reduced fat Cheddar cheese, Dimos *et al.* (1996) found that the concentration of methanethiol in cheese is correlated with flavour, which suggests that the lack of flavour in reduced fat Cheddar is due to the lack of methanethiol. Weimer *et al.* (1997) reviewed mechanisms for production of sulphur-containing compounds, a subject discussed in Chapter 10. Use of specific culture combinations provides additional volatile sulphur compounds. For example, slurries made for Cheddar cheese were improved with the addition of starter cultures containing *B. linens* and also enzymes isolated from *B. linens* (Dias and Weimer, 1999).

Deficiency in milk fat-derived flavour compounds, including short- to medium-chain carboxylic acids, methyl ketones and γ - and σ -lactones, is associated with poor flavour development in a 50% fat-reduced Cheddar (Wijesundera and Watkins, 2000). Production of short-chain carboxylic acids in Cheddar cheese is derived from milk fat and bacterial metabolism. Starter cultures and flavour adjunct bacteria utilise amino acids during cheese ripening for the production of these compounds (Ganesan and Weimer, 2004; Ganesan *et al.*, 2004a,b). Keto acid metabolism is also important in these mechanisms, which in full fat Cheddar cheese improves the aroma (Yvon *et al.* 1999).

Factors influencing the development of bitterness in cheese were reviewed by McSweeney (1997). Bitter peptides are formed by the action of various proteinases on the caseins. Bitterness occurs in cheese when these peptides accumulate to an excessive concentration, as a result of either overproduction or inadequate degradation by microbial peptidases. Although bitter peptides can originate from α_{s1} - or β -casein, it is the action of chymosin and/or the lactococcal cell envelope proteinase on the hydrophobic C-terminal region of β -casein which is mainly associated with production of bitter peptides (McSweeney, 1997). Broadbent *et al.* (1998) reviewed the specificities of the known proteinases and their role in bitterness in Cheddar cheese.

Reduced partitioning of hydrophobic bitter peptides in the fat phase of low fat cheeses may be a causative factor in increasing the susceptibility of low fat cheeses to bitter off-flavours (McSweeney, 1997), but this is controversial and other explanations are as likely. Development of bitterness is minimised by increasing salt-in-moisture (Banks *et al.*, 1993; Mistry and Kasperson, 1998) but this also inhibits proteolysis and increases the firmness of the cheese (Mistry and Kasperson, 1998). While it is generally accepted that bitterness in cheese results from the accumulation of an abnormally high concentration of hydrophobic peptides, other compounds such as some amino acids, amides, long-chain ketones, and some monoglycerides may also contribute (McSweeney, 1997).

23.3.2 Proteolysis, texture and functionality

Fat significantly impacts the microstructure, texture and functionality of Cheddar cheese (Guinee *et al.*, 2000). Fracture stress and firmness increase as

the fat level decreases in the range 300–71.5 g kg⁻¹ in full fat, reduced fat, half fat and low fat Cheddar cheese. Reduction in fat content increases the apparent viscosity and melt time and decreases the flowability of the baked cheese. Changes in the rheology and functionality are attributed to the decrease in the quantity of free oil released on cooking and the increases in the content of intact paracasein and the volume fraction of the casein matrix associated with the reduction in fat content.

The effects of varying fat content in Cheddar cheese from 6.3 to 32.5 g 100 g⁻¹ on changes in pH, primary proteolysis and texture were monitored over 225 days of ripening (Fenelon and Guinee, 2000). Reduction in the fat content resulted in significant increases in pH, moisture and protein contents and decreases in the concentration of moisture in non-fat substances. The increase in pH as the fat content increased was attributed to the concomitant decrease in the lactate-to-protein ratio. The concentration of intact casein decreased in all cheeses during ripening, but the rate of decrease was not affected by the fat content. However, for a given concentration of casein, α_{s1} -casein was degraded more slowly and β -casein more rapidly, as the fat content was reduced. The slower degradation of α_{s1} -casein with decreased fat content coincided with a decrease in the ratio of residual chymosin activity to protein in the cheese. At most ripening times reduction in the fat content resulted in significant increases in the concentration of intact casein, fracture stress, fracture strain and cheese firmness.

Textural properties of 13% fat Cheddar cheese were greatly enhanced by using a mixture of calf rennet and a protease extracted from the plant *Cynara cardunculus* (Banks *et al.*, 1998). Sensory analysis indicated that the firmness and mouth coating properties of the low fat cheese were identical to those of full fat Cheddar. However, intense bitter off-flavours developed in the cheese.

The maturation time required to have sufficient melt in Mozzarella cheese increases with reduction of fat content of cheese (Tunick *et al.*, 1993). Studies on Cheddar found no association between the hydrolysis of α_{s1} -casein and melt (Bogenrief and Olson, 1995), but degradation of β -casein correlated with increased melting of Cheddar cheese (Bogenrief and Olson, 1995). Dave *et al.* (2003) suggested that meltability is influenced by continued hydrolysis of α_{s1} -casein and β -casein into small peptides rather than the initial hydrolysis of intact proteins. These studies suggest that degradation patterns of cheese proteins, particularly α_{s1} -casein, may vary and thereby play an important role in functionality.

Starter culture activities have been used to influence functional properties of Mozzarella cheese. Proteinase positive (Pr^t_P) strains of starter culture provide increased melt over time in Mozzarella cheese compared with proteinase-deficient (Pr^t_d) strains (Oberg *et al.*, 1991). Exopolysaccharide-producing cultures of *Streptococcus thermophilus* MR-1C improve the moisture level and meltability of low fat Mozzarella (Broadbent *et al.*, 2001).

The pH and calcium concentration significantly affect the type and extent of proteolysis in Mozzarella cheese manufacture during 70 days of storage at 4°C (Feeney *et al.*, 2002). For cheeses with a similar pH, reducing the calcium-to-

casein ratio from 29 to 22 mg g⁻¹ of protein results in marked increases in moisture content and proteolysis. Increasing the pH of directly acidified cheeses of similar moisture content from 5.5 to 5.9, while maintaining the calcium-to-casein ratio at 29 mg g⁻¹, results in a decrease in primary proteolysis but has no effect on secondary proteolysis. Homogenisation of the milk causes hardness of Mozzarella cheese to increase (Tunick *et al.*, 1993) and the meltability to decrease (Jana and Upadhyay, 1992; Tunick *et al.*, 1993; Rudan *et al.*, 1998). Homogenisation of the cream, rather than milk, improves the cheese making performance by reducing the amount of curd shattering and fines, and fat loss. The physical changes in cheese structure due to the reduction in fat particle size significantly increase the whiteness of unmelted cheese (Tunick *et al.*, 1993; Rudan *et al.*, 1998). This is expected to improve consumer acceptance of a reduced fat Mozzarella cheese (Rudan *et al.*, 1998). However, problems with melt and excessive browning during pizza baking were not overcome by homogenisation (Rudan *et al.*, 1998), which limits consumer acceptance for use on pizza. Difficulties in melting low or reduced fat shredded Mozzarella are overcome by lightly coating the cheese shred surface with a small amount of oil to prevent surface dehydration (Rudan and Barbano, 1998a,b). A barrier to block moisture loss using a thin hydrophobic surface coating on the shreds produces excellent melting and browning of fat-free and low-fat mozzarella cheese during pizza baking (McMahon and Oberg, 1998; Rudan and Barbano, 1998a,b).

Low and reduced fat cheeses such as Cheddar and Mozzarella are plagued by physical and textural changes due to the reduction of fat trapped in the curd. These are addressed by modifying the make procedure to soften the protein matrix by changing the pH and moisture content of the ripening curd. These changes lead to differences in proteolysis, salt-in-moisture, and conditions for enzymic reactions that are progressing during ripening. Hence, this collection of changes modifies the ripening environment for the starter cultures. As such, different starter cultures produce acid more slowly and have different proteolytic characteristics compared to full fat cheese starter cultures. Additionally, flavour adjunct bacteria are used in an attempt to provide the missing flavour compounds from the starter culture metabolism. Use of these cultures bring new challenges to the biochemistry of cheese and tend to intensify the off-flavours unless they are strategically coupled to specific starter cultures that work in cooperation to promote beneficial flavour production. Therefore, production of high quality reduced fat cheeses needs attention to all the parameters of cheese making, much like making a new variety of cheese rather than mimicking an existing variety.

23.4 Future trends

Cheeses with reduced fat content are likely to maintain a segment in the market. However, this product is not growing in popularity due to problems of texture, flavour and functionality. This market also depends on consumer desire to

maintain a low fat diet, but most consumers are not willing to sacrifice flavour in their diet, especially in Europe. Therefore, the future for reduced fat cheeses is to produce new varieties for which the consumer has no expectations of flavour or texture. Alternatively, use of smaller fat reductions in current varieties is also of interest, so as to maintain the expected properties. Use of specific starter cultures and flavour adjunct bacteria will likely continue for this application. To make this approach successful, knowledge of the fundamental biochemical and bacterial processes is required. This will be guided by genomics and metabolomics.

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Modelling Gouda ripening to predict flavour development

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24.1 Introduction

24.1.1 Background and rationale

In contrast with many other dairy products, cheese is a biologically and biochemically dynamic product throughout the entire product life. Biochemical events taking place during cheese manufacturing and ripening need to be balanced and synchronised in order to produce cheese with the desired quality (Fox, 1993). Disturbances in these balances or de-synchronisation of the processes can lead to changes in cheese quality often caused by the production of off-flavours. In a cheese production process, variations in cheese quality parameters can, for instance, be induced by variations in milk composition or variations in manufacturing and ripening conditions. Considering the significant variations in cheese quality frequently observed within a single production batch, it is clear that even relatively small variations or inhomogeneities in manufacturing or ripening conditions cause noticeable variation in cheese quality parameters to the consumer.

The complex nature of the process-product interactions during cheese manufacturing and ripening makes it a very difficult process to control. For an optimal cheese quality, cheese producers largely depend on the experience and expertise of production managers and operators. It is, however, difficult to quantify the required changes to processing or ripening conditions to optimise cheese quality based on experience and expertise, especially when there are changes in the process (e.g. variation in the milk supply, replacement of a processing unit, etc.) or the product. One way to overcome this problem is by doing empirical or trial-and-error experiments. However, considering the

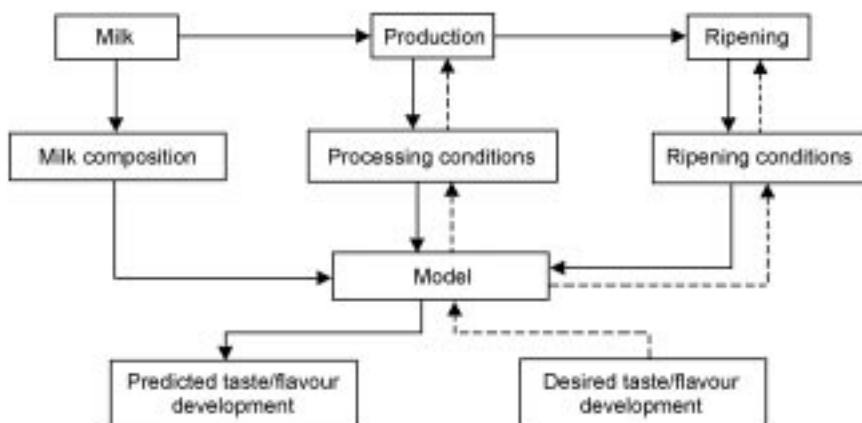


Fig. 24.1 Schematic of the Gouda model.

substantial costs and time involved in cheese manufacturing and ripening, this can be a very costly and an inefficient approach for the expense of time and money.

Another way of tackling this problem is by using models that predict cheese quality parameters as a function of variations in milk composition, manufacturing and ripening conditions (Fig. 24.1). Such a model makes it possible to optimise cheese quality before empirical validation experiments are done. It leads the manufacturer in a cogent direction for improvement, thereby, saving time and money for quick production of high quality cheeses. Another advantage of modelling is that it enhances the accessibility of knowledge, preventing knowledge being available only at a limited number of experts. Models can serve as knowledge reservoirs, capable of combining knowledge and information from various sources. Provided that models are implemented in appropriate software architecture, they can also easily be extended with new information or combined with additional models as new situations or product requirements arise.

24.1.2 Outline

Nowadays it is quite common to use models for process and product optimisation purposes. In literature a wide range of examples can be found related to the production of various cheese types. Many papers focus on individual processing steps and specific product parameters, such as salt and moisture content during brining and ripening (Geurts *et al.*, 1974, 1980; Payne and Morison, 1999), pH and acidity during processing (Paquet *et al.*, 2000), syneresis of cheese curd (Tijskens and De Baerdemaeker, 2004), and water activity during salting and ripening (Saurel *et al.*, 2004). More recently, there is a trend towards modelling quality aspects of cheese, such as sensory attributes (e.g. Trihaas *et al.*, 2005; Pripp *et al.*, 2006) or texture parameters (e.g. Joshi *et*

al., 2004; Blazquez *et al.*, 2006). All these papers focus on relations between certain compositional parameters of the cheese and sensory evaluations or texture parameters. However, in practice, the effects of processing and ripening conditions on these compositional parameters are often not known. Therefore, it is often difficult to make the direct connection between processing conditions and cheese quality aspects based on these models.

In this chapter we present a model developed in a recent research project, which can predict several flavour-related sensorial attributes of Gouda cheese as a function of manufacturing and ripening conditions. Section 24.2 is devoted to the modelling approach that was used to develop this model. Special attention is paid to a procedure of structuring and integrating expert knowledge that resulted in a blueprint for the complete cheese making model. The complete model consists of several sub-models each dedicated to different sub-elements in the cheese production process. Different sub-models require different modelling techniques. These techniques are described in Section 24.2 and several examples of sub-models using different modelling techniques are presented. In Section 24.2 the dataset used for model validation is also described and, finally, it is explained how the various sub-models are integrated to form the complete model. Section 24.3 describes how the model can be used in practice to improve flavour aspects of cheese that focuses on defects. Section 24.4 is devoted to future developments and opportunities, focusing on how the current model can be extended with contemporary and future knowledge and what that means for the use of the model in practice.

24.2 Modelling approach

24.2.1 'Pre-modelling' phase: structuring and integrating expert knowledge

The multitude and complexity of the biochemical and physical processes taking place in cheese make it extremely difficult to cover all the details of these processes and their interactions with process variables in a single model. Moreover, it is likely that not all these aspects are equally relevant with respect to specific sensorial aspects. Before starting the actual modelling work, it is therefore important to first evaluate the cheese production process as a whole and identify essential aspects of the process with respect to specific cheese attributes.

Figure 24.2 represents the starting point of the evaluation process to build the models. The cheese production process is split into manufacturing and ripening. The manufacturing process starts with milk and ends with the unripened cheese (after brining) and the ripening process encompasses cheese ripening through storage. The milk composition and the processing condition during manufacturing (e.g. pasteurisation temperature, amount of rennet, scalding temperature, brining temperature, etc.) affect the composition of the cheese before ripening. During ripening the cheese is subject to ripening conditions (e.g. temperature, humidity).

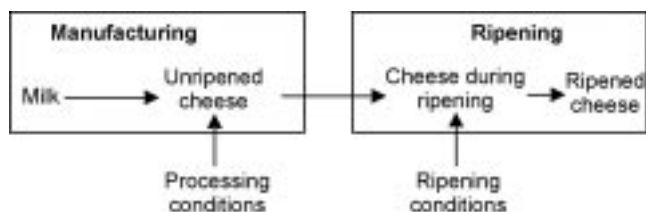


Fig. 24.2 Basic scheme for the development of a knowledge diagram.

First, with the help of several cheese experts, the relevant attributes of the ripened cheese and the main compositional parameters of the cheese before and during ripening that affect these attributes were identified. Next, the effects of relevant processing and ripening conditions on these compositional parameters were identified, described qualitatively based on expert knowledge and merged with information in the literature. The information gathered in this process served as a blueprint for the model to be developed.

The next step was finding the required information to quantify the qualitative relations in the blueprint (i.e. to create the sub-models). The required information comprises experimental data and, if available, details with respect to the physical or biochemical processes involved. Based on this information the most suitable modelling technique for the sub-model can be identified. In principle, data is the most essential ingredient: in case the physical or biochemical mechanisms are not known, purely data-driven black-box modelling approaches can be used. In the next section the various modelling techniques used in the model will be described. Even though data is essential, it is important that the process of generating the model blueprint is not biased by the availability of data. This might lead to missing links in the setup of the model.

24.2.2 Modelling techniques

A wide range of techniques exists to create predictive models, varying from fully deterministic to completely data-driven. Deterministic models are completely based on *a priori* knowledge about the system and are often referred to as white-box models. Purely data-driven models, also called black-box models, require no *a priori* knowledge. However, in practice these extreme cases are rarely encountered, almost all modelling applications being somewhere between black-box and white-box models.

24.2.3 White-box modelling

White-box models explicitly use *a priori* knowledge about the underlying chemical or physical mechanisms, which are translated into a mathematical model. Fully deterministic models based purely on *a priori* knowledge are not common. Underlying chemical or physical principles normally form the fundamental ‘backbone’ of a white-box model. This backbone alone, however,

is normally not able to fully capture the complexity of a real system. To overcome this problem, phenomenological or empirical parameters are used within the model. These parameters need to be determined by fitting the model to experimental data as a method of validation. A typical example of a white-box model is the Arrhenius equation, which is commonly used to model chemical reactions or the inactivation kinetics of microorganisms (De Jong *et al.*, 2002a,b). The sub-model for protein breakdown presented in this chapter (Section 24.4.2) is also an example. The Arrhenius equation has its roots in statistical mechanics. The activation energy and the pre-exponential factor, however, are parameters that need to be determined using empirical or experimental methods.

The main advantage of a white-box modelling approach is that it is based on fundamental physical and biochemical relations. This limits the amount of required validation data and makes it possible to extrapolate the model outside the region for which it was validated. However, the required *a priori* knowledge is not always available or can be difficult to assess, which makes the development of a white-box model very time consuming.

24.2.4 Black-box modelling

Black-box approaches require no *a priori* knowledge, making this approach suitable for modelling parameters such as sensory evaluations for which mathematical models are not available. The main advantage of black-box models is that they are easy to develop, provided the required data is available. The disadvantages are that considerable amounts of data are required (compared to white-box models) and that extrapolating the models is not possible.

For the black-box sub-models in the model presented in this chapter, neural networks were used. Without going into too much detail, we will therefore describe the principles of neural networks. Neural networks are information processing concepts inspired by the way biological nervous systems process information. Figure 24.3 schematically shows an example of a neural network. The input parameters are connected to output parameters through so-called neurons, which can be divided over several hidden layers. For a neural network sub-model for sensory parameters the output value is a sensory parameter (e.g. bitterness) and the input values will be relevant composition parameters of the cheese during ripening (e.g. moisture/salt content, protein breakdown parameters). Figure 24.3 also shows how a neuron processes the input data. The input values are first multiplied by weight factors. After summation a nodal function is applied, which is normally a linear, threshold or sigmoid function. The resulting output value of the neuron, after multiplication by new weight factors, serves as input value for the neurons in the next layer. After initialisation (i.e. defining initial values for the weight factors), the network can be 'trained', which basically is a numerical iterative procedure to minimise the difference between the output of the neural network and the real output values (as given in the available dataset). Since neural nets are purely data-driven, the risk of over-

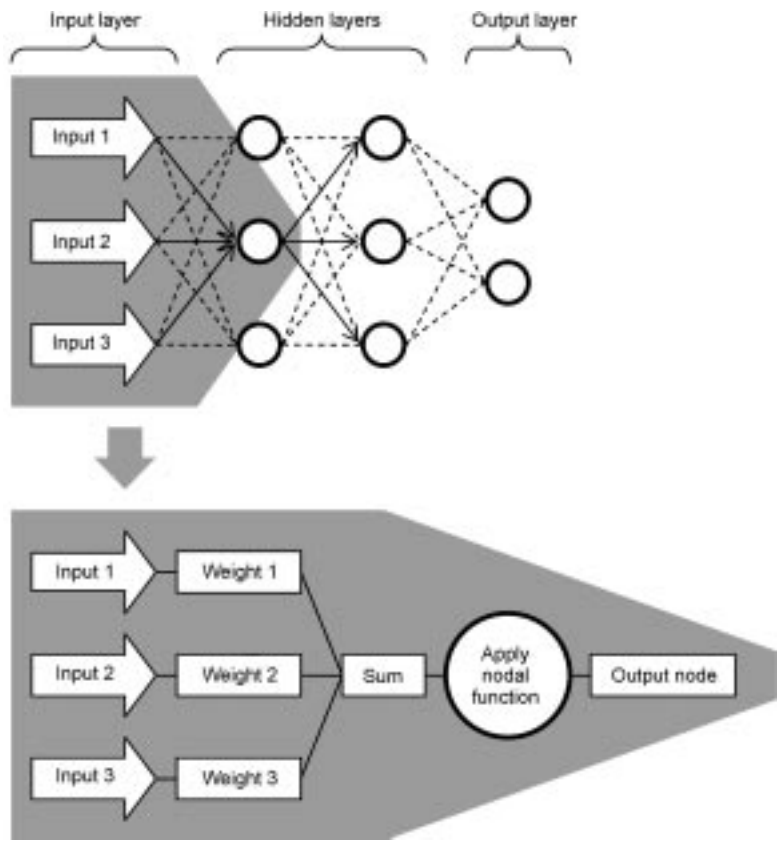


Fig. 24.3 Schematic overview of a neural network.

fitting the model exists. To prevent over-fitting it is essential that not all available data is used as training data. Typically about 10% of the data set is used. During the iterative training process, the model predictions are regularly compared to the test data. When over-fitting occurs the difference between model predictions and test data will increase while the training error is still decreasing (Fig. 24.4). The minimal value of a linear combination of the training and test error is normally used as a convergence criterion. Detailed information about neural networks is described by Gurney (1997) and Haykin (1999).

24.2.5 Hybrid modelling

White-box models have clear advantages over black-box models. Normally less data is required to validate the model and white-box models can be extrapolated. In the ideal situation, a cheese-ripening model would consist only of white-box elements. However, some aspects of the cheese production process, and specifically sensory-related aspects, cannot be captured in white-box models. Because of

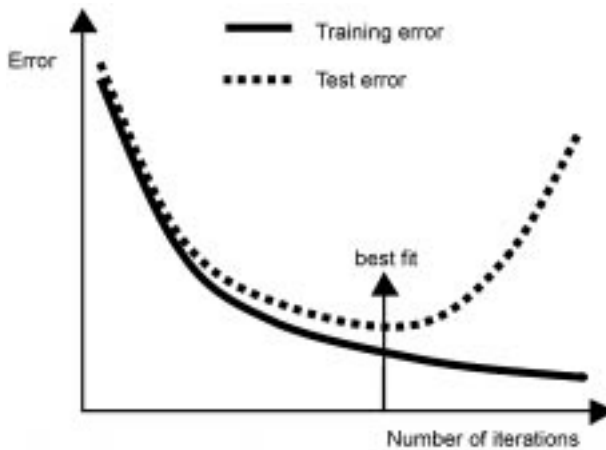


Fig. 24.4 Convergence check for a neural network.

the advantages of white-box approaches, the modelling strategy we used for the model presented in this chapter is to use as many white-box elements as possible. In Section 24.4 some examples of white- and black-box models are presented.

24.3 Validation data

The availability of relevant experimental data is essential for the development of models. An extensive dataset was used for the development of the model presented in this chapter. The major part of the dataset is a result of a research project carried out at NIZO food research in which the effect of various processing conditions on the ripening process was investigated. In this research 386 Gouda cheeses were produced and evaluated. Both manufacturing and ripening conditions were varied (e.g. moisture content before brining, amount of curd washing water, brining conditions, ripening temperature and humidity). Relatively large variations were applied, including variations outside the normal operating standard for commercial Gouda production. The cheeses produced were analysed chemically for moisture and salt content, pH, SN (soluble nitrogen as a percentage of total nitrogen) and AN (amino acid nitrogen as a percentage of total nitrogen), and evaluated by sensory panels at 2, 6, 12, 26, 39 and 52 weeks during ripening. Complementary information and data from various other sources was used for specific variations in processing conditions that were not included in this dataset.

24.4 Examples of sub-models

In this section a few examples of sub-models will be explained in more detail. In Sections 24.4.1 and 24.4.2 two white-box sub-models are presented. The first

one predicts salt and moisture diffusion inside the cheese during brining and ripening. The second model predicts protein breakdown during cheese ripening. In the latter model a more phenomenological approach is used. In Section 24.4.3 some black-box examples (neural networks) for sensory attributes are shown. These examples do not cover all the sub-models that are used in the complete model (see Section 24.5). They are meant to give an idea about the difference in model types that can be used.

24.4.1 Salt and moisture diffusion

Brining of Gouda cheese takes place by immersing the cheese loaves in a strong salt solution, typically about 18% NaCl (Walstra *et al.*, 1993). During the brining process cheese loaves absorb salt, concomitantly a considerable amount of moisture is lost. Since the process of salt uptake and moisture loss takes place at the edges of the loaves, steep salt and moisture gradients develop between the surface and centre of the cheese that persist during the rest of the production and remain during ripening. With respect to taste attributes the moisture and salt content in the centre is relevant. Moreover, different brining/drying combinations result in approximately the same moisture and salt content, causing differences in taste. Understanding of the local behaviour of salt/moisture inside the cheese throughout the brining process and during storage is therefore important.

The underlying physical mechanism for the local changes in salt and moisture content is diffusion. Well established white-box modelling approaches are available for diffusion processes, which can also be used as a basis for modelling salt and moisture diffusion in cheese. Geurts *et al.* (1974, 1980) used a Fickian approach with a modified diffusion coefficient to include the effect of the cheese matrix. The basic assumption in Fick's theory is that the diffusion flux of a certain component is proportional to its concentration gradients (Bird *et al.*, 1960). Because of this assumption Fick's law is valid only for dilute binary solutions. In non-dilute, multi-component systems diffusion of a certain component depends on the friction forces exerted by all other components in the system. This is the basis of the Maxwell–Stefan diffusion model (Wesselingh and Krishna, 2000). Since cheese is a multi-component system and local salt concentrations in the cheese are rather high, especially near the rind, the Maxwell–Stefan diffusion model is more suitable than the Fickian approach (Payne and Morison, 1999).

The cheese is approximated as a porous matrix containing a mixture of water and salt (initially only water). The general Maxwell–Stefan transport equation for a solution in a porous matrix is given by Equation 24.1 (Wesselingh and Krishna, 2000):

$$-\nabla\mu_i - V_{m,i}\nabla p - z_i F \nabla \Phi = \sum_{j=1}^n (x_j \zeta_{ij} (\vec{u}_i - \vec{u}_j)) + \zeta_{iM} \vec{u}_i \quad 24.1$$

where subscripts i and j indicate the components in the solution (1 = salt, 2 = water), μ is the chemical potential, V_m is the molar volume, p is pressure, z is the ion valence, F is Faraday's constant, Φ is the electrical potential, x is the mole fraction, ζ is the friction coefficient and $\vec{u}$ is velocity.

The left side of Equation 24.1 comprises the driving forces as a result of chemical potential gradients, pressure gradients and gradients in the electric potential, respectively. NaCl is ionic in solution at the pH of cheese. However, perturbations in ion concentration gradients causing electrical potential differences will immediately disappear. NaCl can therefore be modelled as one, uncharged component and the third term on the left-hand side can be omitted. Furthermore, it is assumed that the cheese matrix has no resistance against the small deformations taking place during brining (shrinking). The second term on the left side of Equation 24.1 can therefore also be omitted, which leaves only the chemical potential gradient as the driving force for diffusion. The chemical potential μ of component i can be written as Equation 24.2 (Kestin, 1979):

$$\mu_i = \mu_{i0} + RT \ln(a_i) = \mu_{i0} + RT \ln(\gamma_i x_i) \quad 24.2$$

where R is the universal gas constant, T is the temperature and a is the activity, which is defined as the molar concentration x multiplied by the activity coefficient γ . The activity of the salt solution is described by the Debye-Hückel relations (Robinson and Stokes, 1965).

The right side of Equation 24.1 defines the friction force exerted on component i by all other components in the system. The force due to friction with other components in the solution is assumed to be proportional to the molar fraction (x_j), the friction coefficient (ζ_{ij}) and the velocity difference ($\Delta\vec{u}$) between the components. The second term on the right side represents the friction force of component i within the cheese matrix, which is written as a friction coefficient (ζ_{iM}) multiplied by the velocity of component i .

The molar flux of component i is defined in Equation 24.3:

$$\vec{N}_i = \epsilon \vec{u}_i c_i = \epsilon \vec{u}_i x_i c_{\text{tot}} \quad 24.3$$

where ϵ is the porosity of the cheese matrix, $\vec{u}_i$ is the velocity of component i , c_i is the molar concentration of component i , x_i is the molar concentration of component i , and c_{tot} is the total molar concentration. The tortuosity of the cheese matrix can be taken into account by multiplying spatial coordinates in the transport equation with a tortuosity coefficient τ . Taking into account the simplifications mentioned above, combining the Maxwell-Stefan Equation 24.1 with Equation 24.3 now yields Equation 24.4:

$$-\nabla \mu_i \cdot x_i = \frac{\tau}{\epsilon c_{\text{tot}}} \left\{ \sum_{j=1}^n [\zeta_{i,j} (x_j \vec{N}_i - \vec{N}_j x_j)] + x_i \zeta_{i,M} \right\} \quad 24.4$$

where μ_i is the chemical potential of component i , x_i is the molar concentration of component i , $\vec{N}_i$ is the molar flux of component i , ϵ is the porosity of the cheese matrix, τ is the tortuosity coefficient, c_{tot} is the total molar concentration,

$\zeta_{i,j}$ is the friction coefficient between salt and water, and $\zeta_{i,M}$ is the friction coefficient between the cheese matrix and component i (salt or water).

The local mass balance equation for component i in the cheese can be written as Equation 24.5:

$$\frac{\partial \epsilon c_i}{\partial t} + \nabla \cdot \vec{N}_i = 0 \quad 24.5$$

where ∂ is the partial differential operator, here with respect to time t .

The relations for the molar fluxes $\vec{N}_i$ can be derived by combining Equations 24.2 and 24.4. Summation of Equation 24.5 over all components results in the following relation for total mass conservation defined in Equation 6:

$$\frac{\partial \epsilon c_i}{\partial t} + \nabla \cdot \sum_i \vec{N}_i = 0 \quad 24.6$$

The salt and moisture profiles as a function of time can now be computed by the mass balance equations solving on a spatially discretised grid representing the cheese, with the appropriate initial and boundary conditions. For the brining process the initial condition is $x_1 = 0$ (initially there is only water present in the cheese matrix) and the boundary condition is $x_1 = x_{1p}$, where x_{1p} is the molar salt concentration in the brine.

Figures 24.5 and 24.6 show model predictions and experimental results for a brining process (Geurts *et al.*, 1974). Due to the experimental design, which is

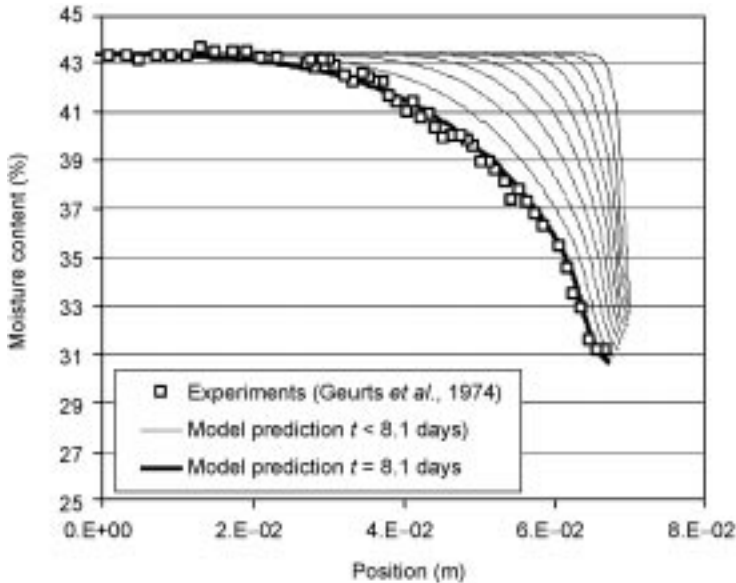


Fig. 24.5 Experimental results (squares) from Geurts *et al.* (1974) and model prediction for the moisture profile in the cheese during brining: for $t > 8.1$ days (black line) and for $t < 8.1$ days (grey lines: 0.048, 0.12, 0.24, 0.43, 0.73, 1.21, 1.79, 3.17 and 5.08 days, respectively).

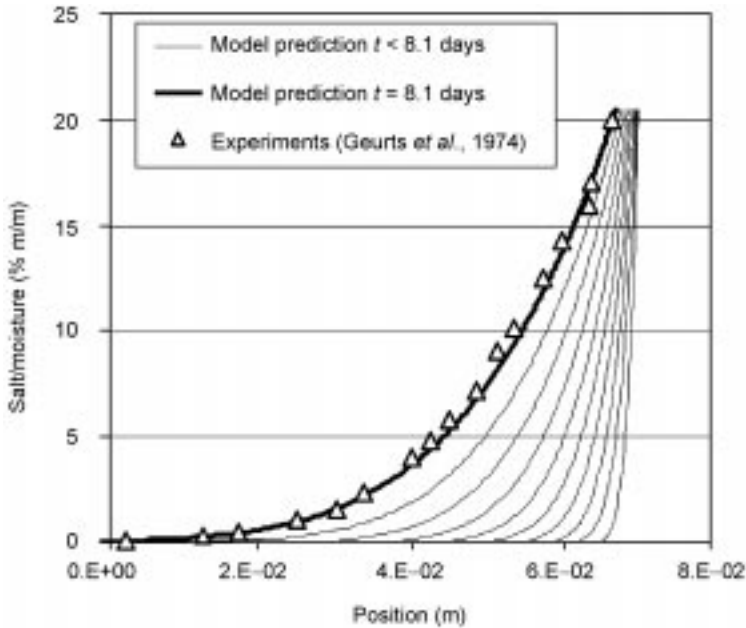


Fig. 24.6 Experimental results (triangles) from Geurts *et al.* (1974) and model prediction for the salt profile (% salt-in-moisture) in the cheese during brining: for $t = 8.1$ days (black line) and for $t < 8.1$ days (grey lines: 0.048, 0.12, 0.24, 0.43, 0.73, 1.21, 1.79, 3.17 and 5.08 days, respectively).

described in detail in Geurts *et al.* (1974), a one-dimensional modelling approach can be used. The results show that there is a good match between the model predictions and the experimental results. The model prediction after 8.1 days of brining follows the experimental results within experimental error. For the Fickian diffusion models this is typically not the case, especially near the cheese rind (Geurts *et al.*, 1974; Payne and Morison, 1999). Due to the volumetric differences in moisture and salt diffusion fluxes, the cheese shrinks during brining. This effect can also be observed in the results (Figs 24.5 and 24.6). Similar results were obtained by Payne and Morison (1999), even though their formulation of the Maxwell–Stefan model is slightly different.

By applying the appropriate initial and boundary conditions, one can apply the same model to predict the time-dependent salt and moisture profiles during ripening. The initial conditions are the profiles obtained at the end of the brining process. The boundary conditions are such that there is no salt flux and a moisture flux, which depends on the local air conditions (temperature, relative humidity and air velocity). It is important to realise that even though this model is a white-box model, it still requires validation data to fit model parameters. Use with other brining conditions or under ripening conditions requires additional validation experiments. Further detailed experimental data for salt and moisture profiles at various brining and ripening conditions were not available in

the dataset we used. This data set only contains average salt and moisture contents of the cheeses excluding the rind were available. In the complete model, we therefore used an approximate version of the model presented here that can be validated using the average internal moisture contents available in the dataset.

24.4.2 Protein hydrolysis

Protein degradation is essential with respect to flavour development in cheese (Exterkate *et al.*, 1995). Protein hydrolysis is a complex process that involves a complex sequence of enzymic reactions, each with a specific kinetic reaction for multiple substrates. From a fundamental point of view this complicates modelling, since kinetic data for every step in the process is not available. However, despite the complexity of the process, it is still possible to create a predictive model for protein degradation using a phenomenological approach with a physical ‘backbone’ based on the Arrhenius equation. In this section we present such a model for global protein hydrolysis parameters SN and AN.

The characteristics of the hydrolysis of α_{s1} -casein (Fig. 24.7), which dominates the initial primary proteolysis (Exterkate *et al.*, 1995), are used as the basis for the protein hydrolysis model. In this sequence, α_{s1} -casein is first hydrolysed into α_{s1} CN(f1-23) and α_{s1} CN(f24-199). After this initial step, the α_{s1} CN(f24-199) peptide degrades further into smaller peptides and amino acids. This process can be written as a sequence of reactions defined by Equations 24.7 and 24.8:



where k_1 and k_2 are the reaction rate constants, A represents α_{s1} -casein, B and C are α_{s1} CN(f24-199) and α_{s1} CN(f1-23), respectively, and nD represents the hydrolysis products of α_{s1} CN(f24-199). The subsequent kinetic hydrolysis steps, in terms of mass fractions, can be expressed as Equations 24.9–24.12:

$$\frac{dm_A}{dt} = -k_1 m_A \quad 24.9$$

$$\frac{dm_B}{dt} = \alpha k_1 m_A - k_2 m_B \quad 24.10$$

$$\frac{dm_C}{dt} = (1 - \alpha) k_1 m_A \quad 24.11$$

$$\frac{dm_D}{dt} = k_2 m_B \quad 24.12$$

where t is time, m_i is the mass fraction of component i , and α is the ratio of the molar masses of component B (i.e., α_{s1} CN(f24-199)) and A (i.e., α_{s1} CN(f1-23)). Since the initial cleavage takes place at bond 23–24, the parameter α is

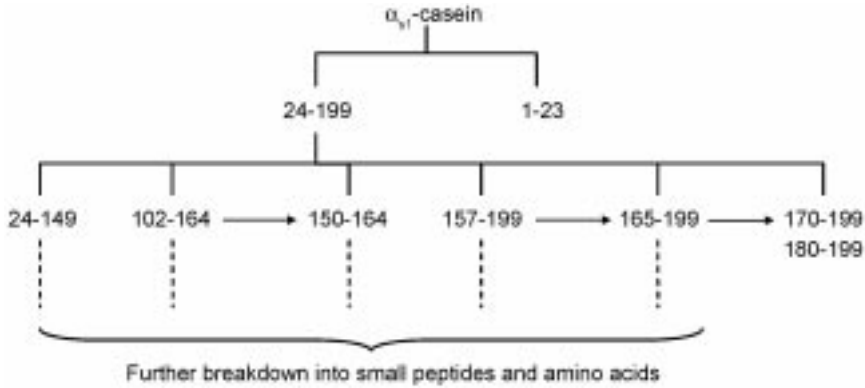


Fig. 24.7 Sequence of proteolysis of α_{s1} -casein (Exterkate *et al.*, 1995).

estimated as 0.88. This value is used for the SN model. For AN, α is treated as a phenomenological fit parameter. Using the identity $m_A + m_B + m_C + m_D = 1$, the set of rate equations can be solved analytically to describe the fate of specific peptides (Equations 24.13–24.16):

$$m_A = m_{A,0} \exp(-k_1 t) \quad 24.13$$

$$m_B = \frac{\alpha k_1 m_{A,0}}{k_2 - k_1} \cdot \exp(-k_1 t) + \left(m_{B,0} - \frac{\alpha k_1 m_{A,0}}{k_2 - k_1} \right) \cdot \exp(-k_2 t) \quad 24.14$$

$$m_C = m_{C,0} + (1 - \alpha) m_{A,0} (1 - \exp(-k_1 t)) \quad 24.15$$

$$m_D = 1 - m_A - m_B - m_C \quad 24.16$$

The reaction rate constants are defined by the Arrhenius relation (Equation 24.17):

$$k_i = k_{0,i} \exp \left[-\frac{E_a}{R} \left(\frac{1}{T} - \frac{1}{T_{\text{ref}}} \right) \right] \quad 24.17$$

where E_a is the activation energy, R is the universal gas constant, T is the temperature and T_{ref} is a reference temperature. The effect of temperature on protein hydrolysis is described by the Arrhenius relation. The effect of all other parameters that influence the degradation process (e.g. the amount of active rennet, the starter, moisture and salt content and pH) can be included in the more empirical pre-exponential factor $k_{0,i}$. Within limited operational windows it is possible to write $k_{0,i}$ as a multiplication of ‘influence factors’, each representing the effect of a different parameter.

In the dataset described in Section 24.3, casein hydrolysis results are available for a wide range of ripening temperatures. These data were used to determine the model parameters, activation energies and pre-exponential factors, in the protein degradation model. Figure 24.8 shows typical model predictions and experimental data for SN and AN for 13°C and 17°C.

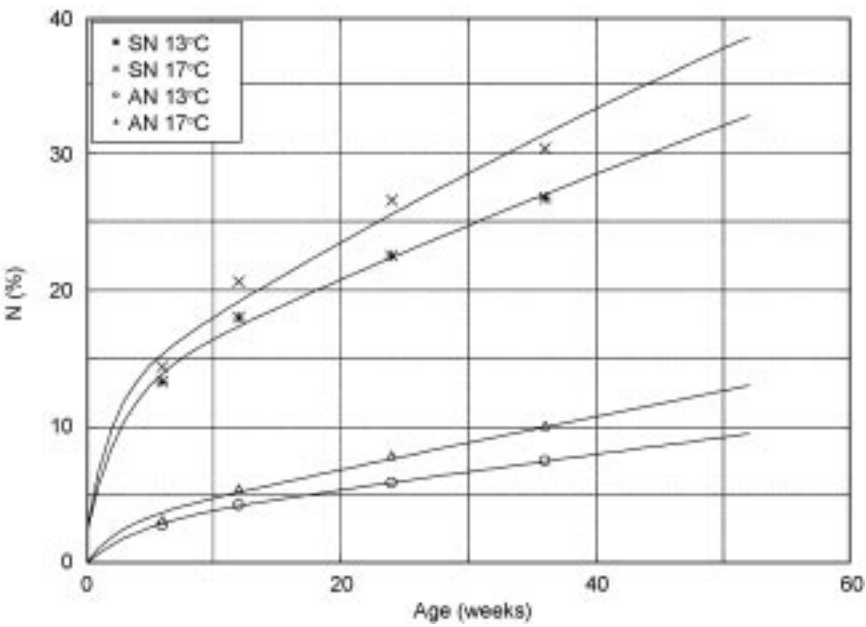


Fig. 24.8 Effect of ripening temperature on SN and AN: experimental values and model predictions (N represents SN or AN).

Visser (1977) investigated the effect of the amount of rennet added to the cheese milk on protein hydrolysis in detail. He carried out experiments using a wide range of rennet dosages without using a starter culture. Not the amount of rennet itself, but rather the amount of active rennet remaining in the cheese after production influences the protein degradation, as one would expect. The amount of active rennet is not only related to the rennet dosage, but it is also a function of various processing parameters (pasteurisation intensity, cooking temperature, etc.) that affect the enzyme kinetics and activity. The effect of rennet is included in the model by multiplying the pre-exponential factors $k_{0,i}$ with a linear term $f_{AR,i} = 1 + \beta(AR_{rel} - 1)$, where AR_{rel} is the relative amount of active rennet (equal to 1 at reference conditions) and β_i is a fit parameter. Figure 24.9 shows the result of the fit procedure. The results for no added rennet (0n) were not included in the fit procedure, since this is too far outside the normal operational window.

A similar approach can be used to include other parameters in the pre-exponential factors. The effects of moisture, salt content and pH could not be distinguished. This does not necessarily mean that the effects are always small, however. It might also be caused by relatively small variations in the available dataset. For example, the moisture content was important in this case. The drying rates did not deviate much from the reference case. It is known, however, that moisture content has an effect on protein hydrolysis due to a strong decrease in moisture content in the rind protein, in which hydrolysis stops locally, whereas it

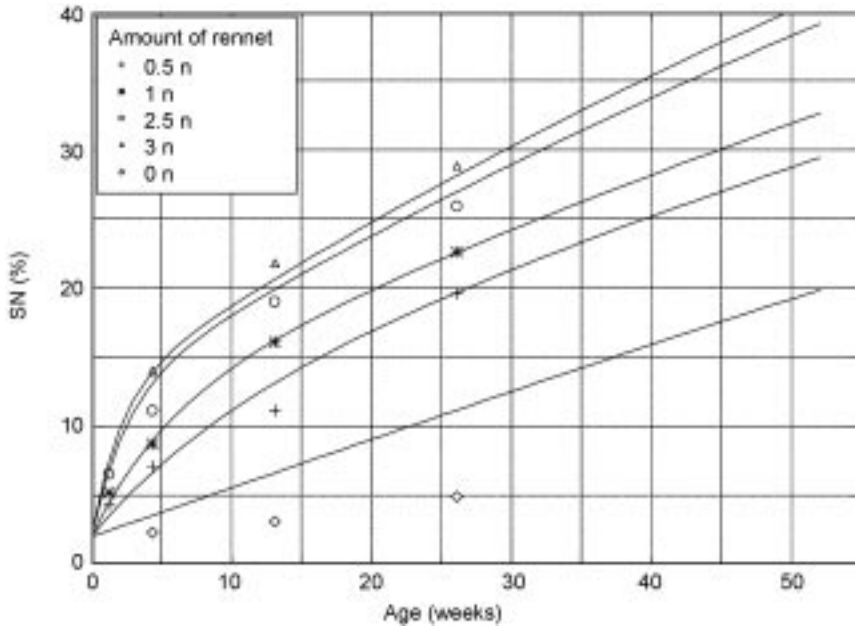


Fig. 24.9 Effect of amount of rennet on SN: 0.5, 1, 2.5 and 3 times the normal amount of rennet (0.5n, 1n, 2.5n and 3n, respectively) and no rennet (0n). Experimental values (Visser, 1977) and model predictions.

continues in the centre of the cheese at higher moisture contents. The effect is probably non-linear – a pre-exponential factor that is more or less constant for higher moisture contents and which rapidly decreases below specific moisture content values. Local protein breakdown data could be used to model this effect, which would make it applicable for a wide range of moisture contents.

24.4.3 Sensory parameters

As mentioned, to model sensory attributes it is not possible to use a white-box approach. Therefore, a black-box modelling (neural networks) approach is used to correlate sensory evaluation to relevant compositional parameters of the cheese during ripening.

A large part of the data set that was used to develop the model presented in this chapter was produced in which the effect of various processing parameters on the ripening process was investigated. The sensory panels performing the sensory evaluations of the cheese were asked to estimate the age of the cheese using standard Gouda as a reference. Earlier research showed that the estimated age (EA) depends on the moisture content and AN. Therefore, a multiple linear regression model (MLR) was created using the moisture content and AN as input variables. Within the framework of the research presented in this chapter, a neural network was trained using the approach described in Section 24.2.2

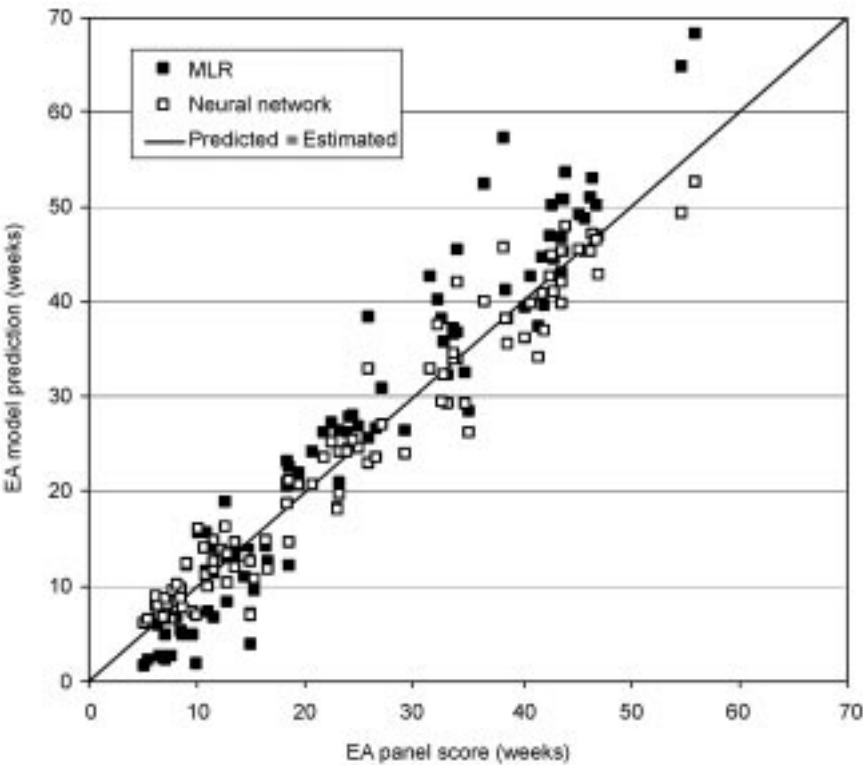


Fig. 24.10 Estimated age (EA), panel scores versus neural network prediction for the multiple linear regression model (MLR, black squares) and the neural network model (white squares).

(Gurney, 1997). The dataset used for the MLR model was also used to train the neural network and, as in the MLR model, the moisture content and AN were used as input variables (Fig. 24.10). The neural network model performs better, due to its ability to handle non-linear effects. The standard deviation for the MLR model is 2.3 weeks, whereas for the neural network model the variation is 1.8 weeks. Moreover, the skewness is reduced at higher values of EA because the MLR model tends to overestimate the EA, which is not the case for the neural network model.

The same approach can be used for other sensory parameters, using the appropriate compositional parameters of the cheese during ripening as input parameters. Figure 24.11 shows a typical result for the average intensity (AI) of the bitter defect of the cheese as a function of SN concentration predicted by the neural network model. In this example the other input parameters were kept constant: EA = 15 weeks, moisture content = 40.5% and AN = 5.5%. The moisture content refers to that of a sector sample from which 1.5 cm of the rind was removed. These results show a transition from non-bitter to bitter as the SN concentration increases. Figures 24.12 shows results for varying moisture

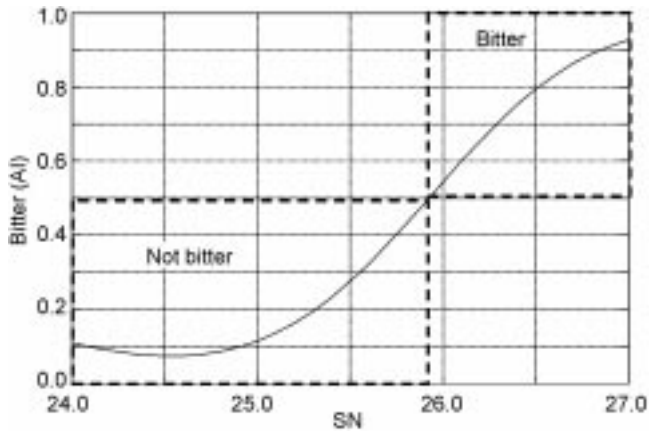


Fig. 24.11 Model prediction for bitter as a function of SN. Other input variables are constant: EA = 15 weeks, moisture content = 40.5% (of a sector sample excluding 1.5 cm of rind) and AN = 5.5%.

content and AN, respectively, to link these two variables. In all cases the transition from non-bitter to bitter is observed, but the curve shifts position, depending on the moisture content, to impact the bitter perception. Similar results are observed for increasing bitterness and decreasing AN. Both observations are confirmed by expert knowledge about the system and are well known in the literature (Stadhouders and Hup, 1975; Stadhouders *et al.*, 1983).

24.5 Hybrid modelling: integration of sub-models

The complete model consists of sub-models based on the models described in the previous sections and some additional sub-models.

- A sub-model that computes the active amount of rennet in the cheese as a function of the rennet dosage, the acid number of the cheese milk, the pasteurisation intensity and the cooking temperature.
- A sub-model that predicts the pH of the cheese during the production process. The pH during manufacturing is mainly determined by the amount of starter culture added. During storage the pH increases. This could not be described in a model, due to the inaccuracy of the pH measurements during ripening and the lack of data with respect to the buffering capacity (see also discussion in Section 24.6.2).
- A sub-model which predicts the moisture content before brining as a function of CaCl_2 dosage, the amount of acid/caustic added, the amount of water added, the amount of whey cream, the renneting and cooking temperature, the duration of pressing and the pH after 4 hours.
- A sub-model for the salt and moisture content and their distributions during brining and storage. During brining the salt and moisture profiles are

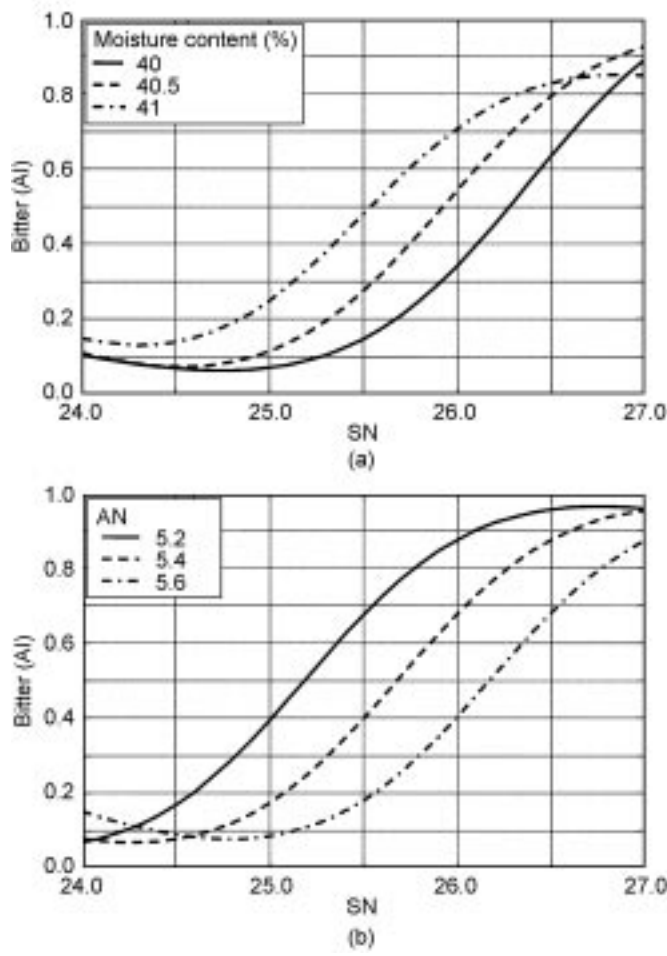


Fig. 24.12 (a) Model prediction for bitter as a function of SN for different moisture contents (of a sector sample excluding 1.5 cm of rind). EA = 15 weeks and AN = 5.5%. (b) Model prediction for bitter as a function of SN for different AN fractions. EA = 15 weeks and moisture content = 40.5% (of a sector sample excluding 1.5 cm of rind).

determined by moisture content before brining and by the brining conditions (e.g., duration, strength and temperature). During storage the ripening temperature, relative humidity and air velocity are the driving factors for changes in the salt and moisture profiles. As explained in Section 24.4.1, we used an approximate version of the model described in that section.

- A sub-model for protein hydrolysis (as described in Section 24.4.2), which predicts the formation of SN and AN as a function of the ripening temperature, the active rennet concentration and the type of starter used.
- Finally, a sub-model for the sensory attributes as a function of relevant compositional parameters of the cheese during ripening. The sensory

attributes included in the current model are the estimated age and the defects bitter, flat, salty and acid.

All these sub-models are implemented and integrated into a single software platform. Within this platform the models can be used individually, but they can also be connected (e.g., the output of one sub-model can be used as input for another one).

24.6 Improving the flavour of cheese by modelling

24.6.1 Examples of integrated, hybrid model: off-flavours/defects

In the previous section some examples of sub-models and results of individual use of these models were shown, such as the results of the neural network for bitterness as a function of its input parameters (estimated age, moisture content, SN and AN) in Section 24.4.3. In practice, however, using the neural network model for bitterness as an individual model is not very efficient. First of all, in the examples in Section 24.4.3, the input parameters were treated as independent variables (i.e., one parameter was varied, while the others were kept constant). In reality, the input parameters are not independent. The EA, for instance, depends on the moisture content and AN. Moreover, in practical situations, values for SN, AN and moisture content are not specifically known. Individual use of the model would require these parameters to be measured in real time. In contrast with the individual sub-models, the complete model (consisting of the integrated sub-models) allows evaluations of the sensorial parameters as a function of the processing conditions.

To test this hypothesis, three sub-models were used to estimate cheese age as a function of the actual age of the cheese, with ripening temperatures ranging from 1° to 19°C (Fig. 24.13). As expected, the results show an increase of EA with increasing ripening temperature. The protein hydrolysis model was used to predict AN as a function of the ripening temperature, as well. The salt/moisture profile sub-model was used to predict the moisture content in the centre of the cheese as a function of the ripening temperature. The model predictions for AN and the moisture content served as input values for the neural network model to estimate age. Use of the EA model individually requires input values for AN and the moisture content, whereas in the coupled system the ripening temperature can be used directly. In a similar way the complete model can be used to predict the salt perception as a function of the brining time (Fig. 24.14) or the flat defect as a function of the ripening temperature (Fig. 24.15). As expected, there was a transition from non-salty to salty for increasing brining duration between 105 and 110 hours (Fig. 24.14). The flat defect is observed at lower ripening temperatures for young cheese (8 weeks) (Fig. 24.15). This effect is mainly caused by the relatively low rates at which the AN increases at lower ripening temperatures.

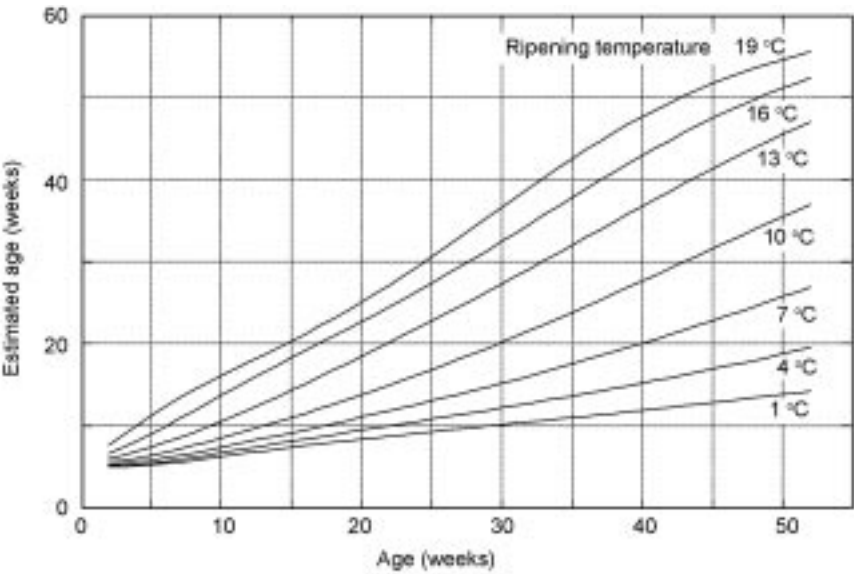


Fig. 24.13 Estimated age (EA) as a function of the actual age of the cheese for different ripening temperatures ranging from 1° to 19°C.

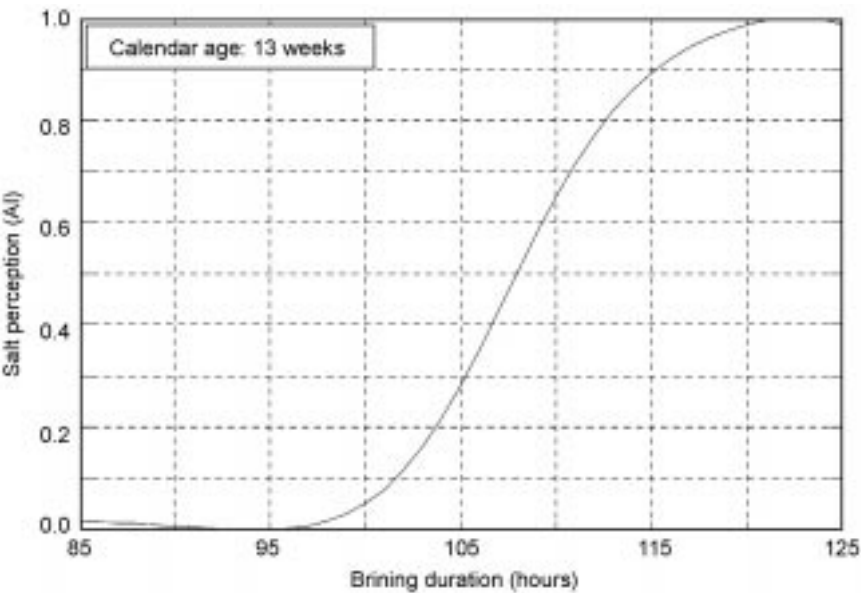


Fig. 24.14 Salt perception as a function of the brining duration for a 13-week-old cheese.

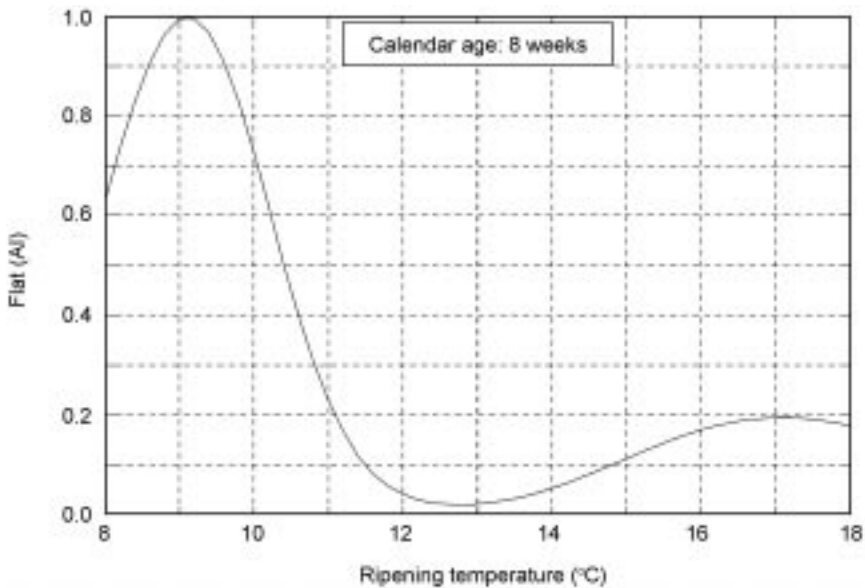


Fig. 24.15 The defect flat as a function of the ripening temperature for an eight-week-old cheese.

24.6.2 Practical use of the model: possibilities and limitations of the current model

In general, models (white-box and black-box) can be applied within the range for which they were validated. The model presented in this paper covers a large part of the operational window used in commercial Gouda production. Moreover, for some parameters extreme variations were used (e.g. ripening temperature). The model can therefore, at least in part, be used outside the normal operational window. Furthermore, as explained in Section 24.2.1, white-box models can be extrapolated beyond the model parameters. For example, the salt/moisture diffusion model can also be applied to Gouda cheeses with other size dimensions. However, considering the fact that the sub-systems under consideration are complex, which can never be fully covered by a white-box sub-model, it is always advisable to validate these models when extrapolating. This normally only requires a limited amount of experimental data. Black-box sub-models cannot be extrapolated. In this case the model needs to be revalidated using additional data.

When applying the model in practice to Gouda cheese production, one should also keep in mind that the prediction is limited by the quality of the sensorial data used in the model. In this case, the data were produced specifically by trained sensory panels. These sensory evaluations do not necessarily match the sensory evaluations of the panels of cheese producing companies. Unless the NIZO evaluations can be quantitatively mapped on the sensory evaluations of the cheese producing company involved, the model results with respect to taste

can merely be used as qualitative information. To make sure the predictions of the taste-related sub-models can be interpreted quantitatively, the black-box models need to be revalidated using the company's own production and sensorial data.

Even though the current model was created for standard Gouda cheese, the model is such that it can be extended to other cheese types as well. The connected sub-models structure enables changing, replacing or adding sub-models. Moreover, it is also possible to extend or replace underlying datasets, which facilitates (re-)validating or (re-)calibrating the model or relevant sub-models. For instance, for cheese types other than Gouda the protein breakdown process will be different. Nevertheless, the available model for SN and AN can still be used as a basis, the Arrhenius relation still being the 'backbone' of the model. The model needs to be revalidated by using protein hydrolysis data of other cheese types.

Whereas acceptable correlations were found for the defects bitter, flat and salty, the predictions of the current model for acidity are poor. This is partly due to the inaccuracy of the pH measurements in the available dataset and the lack of data with respect to the buffering capacity of the cheese. Another reason is the complex nature of the acidic taste itself. For instance, acidity is not simply related to the pH; the type of acid and the presence of other acids is also a relevant factor. Further research is required to cover these aspects.

Although the current model focuses on cheese defects or negative flavours, the modelling approach can also be used for positive flavour attributes. Many cheese quality parameters are related to positive flavour attributes. Extending the model for these quality parameters would therefore be very interesting. Whereas the defects considered in the current model could be related to global protein degradation parameters (SN and AN) and some additional compositional parameters of the cheese, modelling positive flavour attributes requires more detailed information with respect to specific flavour components and the pathways leading to these components. Nevertheless, it is expected that similar modelling techniques can be applied. Moreover, there is ample knowledge about these systems. Modelling and knowledge are, therefore, not the limiting factors, but rather availability of data is the main bottleneck.

24.7 Future trends

As mentioned in the previous section, for improving modelling to optimise cheese quality, input of detailed flavour attributes is essential. Flavour measurement of cheese has been based on the sensory evaluation by tasting panels or experienced production personnel. A high degree of objectivity can only be guaranteed when a panel of well-trained employees is available. Even then, the application of sensory evaluation for quality control of products has disadvantages and rapid, objective instrumental analytical methods are desired for determining quality of cheeses. A commonly used method in this respect is the

determination of the dominant compounds, the so-called key-flavour components. This can be done by separation of compounds by gas chromatography (GC) and analytical identification by the human nose (olfactometry). Similarly, for tastants a combination between separation (liquid chromatography, LC) and tasting of fractions (LC-taste) is applied.

Although the determination of most important flavour compounds and their concentration has some flaws, recombination of the key-flavour compounds usually gives a sufficient representation of the cheese studied (see other chapters for these compounds). Since not all products were analysed by GC–olfactometry, not all flavour components may be called key-flavours. The flavour compounds are categorised by the substrate they are most likely derived from. Not only the amounts of the individual components, but also the balance between these components is very important in the ultimate perception by the consumers. The work on controlling the flavour formation in cheese can focus on an overall increase in all important key-flavour components (improved/faster cheese ripening) or an increase in specific flavour molecules. The latter approach may result in a new cheese variety with an appreciated flavour diversification, but may also result in a perceived unbalance of the off-flavours.

Knowing key-flavour components and pathways leading to these components, the ingredients to set up a model are available. If a great level of detail is known about the pathways, it is in principle possible to develop a deterministic kinetic model that includes details of the individual steps in the pathways. Disadvantages of this approach are that the models can get very complex and that kinetic data with respect to individual steps in the pathways are required, which are normally not available and probably difficult to determine. Alternatively, the detailed pathways can be used as a basis for a phenomenological model that captures the essential parts of the pathway related to the specific key-flavour. This approach is similar to the approach used for the protein breakdown model presented in this chapter. Developing such a model also requires empirical data, however, that are not readily available. Measurements of the key-flavour molecules at various processing conditions are required. Moreover, to model the corresponding sensorial attributes, sensorial data also have to be available. An advantage of key-flavour components is that there is a direct link with the corresponding sensorial attributes, which probably leads to acceptable correlations in the black-box sub-models.

The knowledge regarding flavour-forming pathways can also be used for developing superior flavour. An important finding concerning this has been that a large diversity in required enzyme activities occurs among LAB strains. Consequently, starter cultures can be developed by careful selection and combination of strains with desired activities that meet the predictions of the models. Recent technological breakthroughs in the field of automated screening and genomics allow the efficient exploitation of this large biodiversity. In such screening programmes, miniaturised fermentations are carried out in 96-well format using robotics for liquid handling, key-enzyme activity measurement with colorimetric substrates, and analysis of flavour compounds with GC–TOF

or HPLC–MS. Subsequently, strains exhibiting the desired activities are tested in product model systems and pilot products.

An example of the application of strains with selected enzyme activities for improving cheese flavour is the development of so-called debittering strains. A bitter off-flavour may occur in many food fermentations and is often caused by unbalanced proteolysis resulting in the accumulation of certain hydrophobic peptides with a strongly bitter taste. It has been shown that the intracellular aminopeptidase pool, which includes PepN, in *Lactococcus lactis* is capable of degrading a bitter peptide that accumulates in bitter cheese. Extensive screening programmes have helped to identify strains that produce high aminopeptidase activity (Fig. 24.16). Including the aminopeptidase activity in the current bitterness models may lead to a new model to predict the debittering potential of specific starter cultures.

In summary, detailed knowledge regarding key-flavour compounds, the flavour-producing pathways for their formation and the strains possessing the enzymes involved provides the tools to rationally direct flavour formation in cheese. In the current model sensorial parameters are related to global protein breakdown characteristics. Correlations would greatly improve if the global protein breakdown parameters are replaced by specific components such as bitter peptides or key-flavour compounds. Predictive models for the formation of key-flavour compounds as a function of relevant processing conditions can be developed based on the detailed knowledge about the pathways. Because of the complexity of these pathways and the current lack of kinetic data, in the near future models will be phenomenological in nature. Further ahead, however, development of improved high-throughput screening techniques might make it possible to generate the required data to create more deterministic models using complex kinetic modelling techniques such as are used in metabolic pathway modelling. Other recent developments in the areas of texture–taste interactions, flavour release modelling and physiological aspects of flavour perception, are also expected to contribute to improving taste-related models in the future. This will result in more white-box approaches to taste modelling. Deterministic pathway modelling and truly white-box taste models would greatly improve the predictive capabilities of the complete model.

24.8 Sources of further information and advice

Besides neural networks, fuzzy logic is also a suitable black-box modelling technique. Fuzzy logic explicitly employs qualitative expert knowledge, which is implemented by using linguistic programming. For details on fuzzy logic applications see, e.g., Sugeno and Yasukawa (1993) and Klir and Folger (1988).

For future developments and for optimal use of the model in practice, flexibility in the modelling and software environments are required. Further information on open architecture software and modelling environments can be found in Pantelides and Urban (2004) and Braunschweig *et al.* (2000).

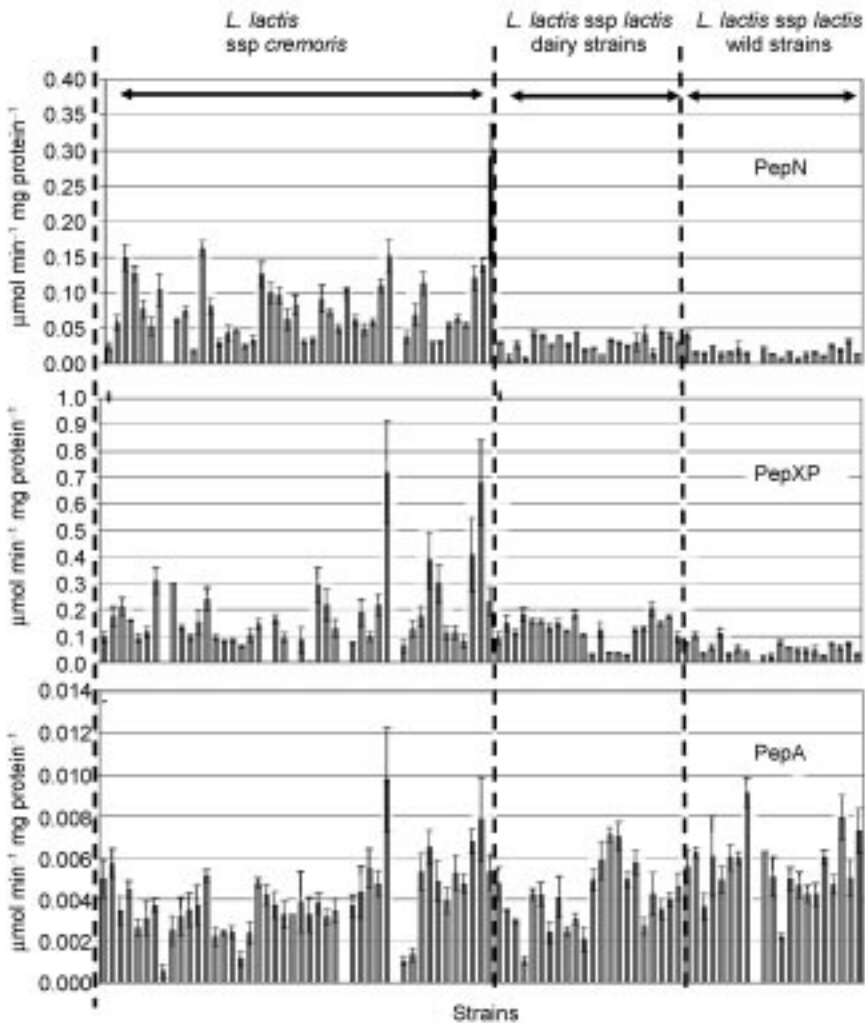


Fig. 24.16 Diversity of aminopeptidase N (PepN), X-Pro dipeptidyl-peptidase (PepXP) and Glutamyl-aminopeptidase (PepA) activity among *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* grown in LM17 medium.

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