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Chapter 10 - Fermentations generating the cheese diversity

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Summary: The cheese making process involves the growth and expressions of a large diversity of species. It starts by the growth of lactic acid bacteria in the curd before or during the curd drainage and it is followed by ripening steps which will determine the cheese properties. In large cheeses, if a phase of ripening is made at high temperature (e.g. 23°C), the development of *Propionibacterium sp.* generates propionic acid and CO₂. These species are responsible of the eyes in Emmental. The development of *Penicillium camemberti* at the cheese surface of soft cheeses will produce the white coverage. If the rind is washed, the moulds cannot grow but the growth of yeasts and corynebacteria will produce cheeses rich in sulfur flavours and with an orange rind (wash rind cheeses). The use of molecular methods permits the description of the microbial ecosystem which appears now more complex than 15 years ago but it is still difficult to understand the interactions between the different species and the curd and also of the interactions between the species at the cheese surface. This emerging knowledge will definitely help to control the sensory properties of the cheese produced. This chapter is proposing to light up how the milk and the cheese technology select families of fungi and bacteria that will give the cheese diversity.

1 Principles of coagulation, chemical barriers to microbial growth

There are 3 principles of milk coagulation each one leading to gels with different structure properties and consequently allowing different levels of draining, different microbial growth and finally different cheese characteristics. These different systems of coagulation are usually combined in cheese making processes. The three principles of coagulation are the following:

- by acidification, usually due to the production of lactic acid by the lactic acid bacteria (LAB),
- by the action of protease such as the chymosin present in the rennet (extract of the calf abomasum), the one produced by moulds like *Mucor pusillus* or *Mucor miehi* or plant proteases such as the one contained in the fig sap
- by the action of the temperature associated to both of the previous systems like in mozzarella and other Italian “pasta filata” type.

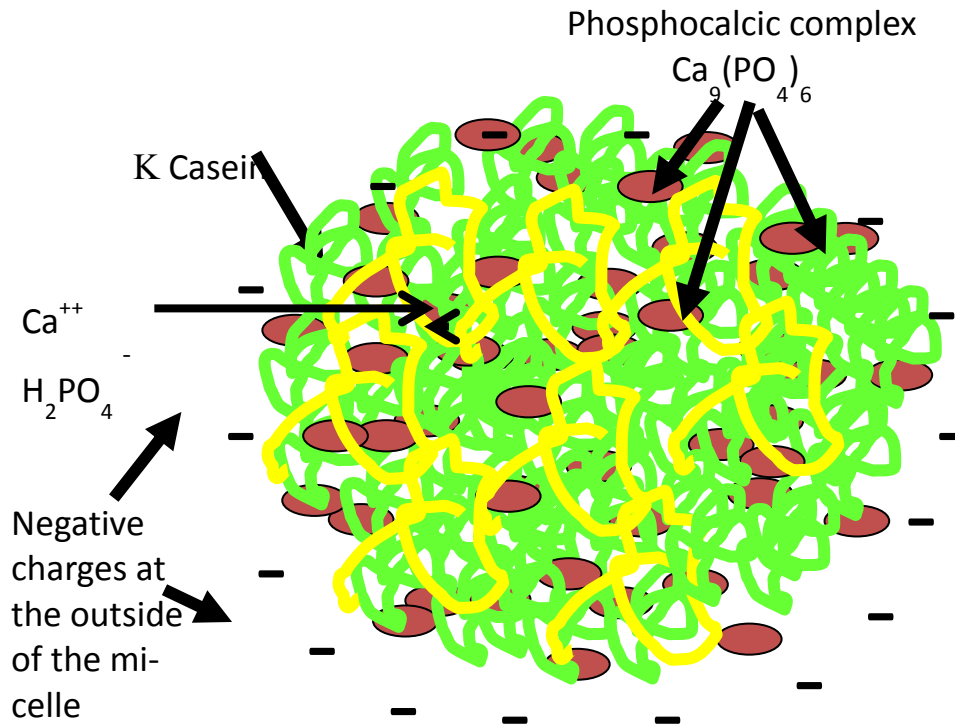


Figure 1: Scheme of the structure of a milk casein micelle. K-casein is represented in yellow. This amphiphilic casein is at the interface between the hydrophobic α and β caseins (in green) and the whey and permits the dispersion of the micelles in the milk. The negative charges of the caseins are grouped outside the micelles and are also contributing to the stability of the dispersion of the micelles in the milk.

- Coagulation by lactic acid

The production of lactic acid by LAB has 2 main effects:

- The caseins which are grouped in micelles stabilized by the casein κ (Figure 1) are negatively charged at the surface. These charges provoke repulsion between the micelles and disperse them in the milk. The growth of the lactic acid bacteria during the milk fermentation is associated to an increase of the lactic acid concentration raising the proton concentration shown by the drop in pH. The negative charges of the caseins are neutralized by these protons and the micelles are joining together to build a fragile gel, like in yogurts.
- The acidification has another effect. The calcium and phosphate fixed to the caseins are released because of a displacement from their corresponding salts by the H^+ concentration. The release of these minerals will reduce the possibility of ionic bounds. Mainly hydrogen bounds, Van der Waals or hydrophobic bounds are possible, all with low energies, are binding together

the caseins. The resultant hydrophobic gel will be fragile and should be drained with caution.

In terms of composition, where this principle is the major system of coagulation, cheeses have a lower calcium and phosphate content, they stay more humid and soft, and only small cheeses can be made. The acidification stops the growth of the lactic acid bacteria and so, some lactose is remaining in the curd, at the end of the draining.

- Coagulation by the rennet

The second principle of coagulation is based on the action of the chymosin on the kappa casein. The micelles become less polar and join together to form a gel. In that case the calcium and phosphate remain inside the micelles and the ionic bonds between the caseins permit to make firmer gel with more mechanical strength. That permits to get firm gels after draining, to apply pressure without losing significant amount of caseins in the whey. It is also possible to cook the curd in the whey like in Emmental or Comté. This principle permits to get cheeses with big size. It is used to make Holland type cheese (Gouda, ...), Cheddar, Italian type hard cheeses (Parmigiano Reggiano, Grana Padano, ...), Comté, Cantal, Emmental, etc.

Almost every cheese is made with at least a small amount of rennet.

- Coagulation including heat treatment

The temperature increase the speed of destabilization of the micelles by the two previous principles but it gives a curd that is resistant to stretching. It gives a family of cheese called pasta filata and mainly made in Italy like Mozzarella, Burrata for fresh cheeses and Provolone or Caciocavallo for ripened cheeses. The stretching has for effect to give an orientation of the casein fibers and of the fat along these fibers (Rowney et al. 2004).

2 Lactic acid bacteria in cheeses

2.1. Mesophilic lactic acid bacteria involved in cheese making processes

In almost all cheeses, lactic acid bacteria are present in large concentration (over 10^9 CFU/mL at the beginning of the ripening) but the diversity of these lactic acid bacteria depends on the milk treatment and on the technology used. In soft cheeses the mesophilic LAB are the more common. It includes *Lactococcus lactis* ssp *lactis* or ssp *cremoris*, in the ssp *lactis* the biovar diacetylactis is producing diacetyl and though is interesting for flavour productions especially in non ripened soft cheeses like "fromage frais", "cottage cheese", "quark" or "chakka". These homolactic bacteria could be associated with *Leuconostoc* sp. like *Le. cremoris* or *Le. mesenteroides* species that are also producing flavours. These species are taxonomically close to the hetero fermentative *Lactobacillus* species that are common and important in pressed cheeses. In this genus the species *Lb plantarum*, *Lb casei* and *Lb rhamnosus* are particularly common.

The lactic acid bacteria are all very efficient on the hydrolysis of curd proteins. Some are able to produce flavour compounds from citrate or amino-acids but their flavour capabilities are slower

and of limited impact as compare to the micro-organisms found at the surface of cheese during ripening. This lead to a slow evolution of large cheeses, with a small surface / volume ratio, where lactic acid bacteria are one of the major families of micro-organisms involved.

2.2. Thermophilic lactic acid bacteria involved in cheese making processes.

Thermophilic lactic acid bacteria are mainly represented in yogurt and hard cooked cheeses like swiss type cheeses. *Streptococcus thermophilus* is a species that grows and convert the lactose into lactic acid very quickly in milk at 40-44°C but it is also able to grow at lower temperature consequently this species is now also used in different soft cheese technologies. However *Streptococcus thermophilus* is already inhibited by a pH of 5.6 and it is usually used in combinations with *Lactobacillus* more resistant to low pH such as *Lactobacillus helveticus* and *Lactobacillus delbruecki* subsp. *bulgaricus*.

The lactic acid bacteria have been largely studied. The genomes of many have been completely sequenced. They are particularly well adapted to rich environment. Price et al. (2012) wrote a recent review.

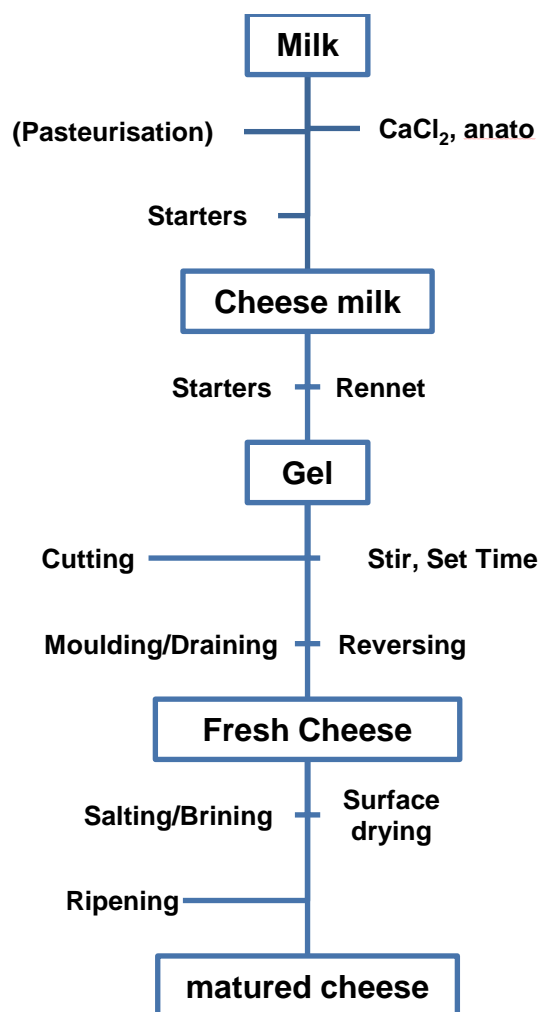


Figure 2: General Technological diagram used to make mould ripened soft cheeses.

3. Diversity of cheese microbial ecosystems in ripening processes

3.1. Mould ripened cheeses

The technology of mould ripened cheeses starts with the production of a curd with a pH between 6.3 and 5.8 at the beginning of moulding. The more acidic it will be at the beginning of moulding, the more chalky will be the curd at the demoulding step and the longer will be the complete ripening. However the one which are the more chalky after draining will be the more creamy at the end of ripening. The technology of stabilized curds permits to keep a quite high pH of the curd in washing the curd with water or using starter sensitive to low pH (such as *St. thermophilus*). In that case, the texture is soft (not chalky) right after the moulding step but this texture will not change much during the ripening.

Spores of *P. camemberti* (10^4 spores /ml) are usually added in the milk with very low level of *Geotrichum candidum* (20 to 100 spores/ml). *Brevibacterium linens* is often added to give more flavours but it seems that the typical flavours of several mould ripened cheeses is related to the occurrence of Gram negative bacteria. For example, in camemberts made from pasteurized milks *Hafnia alvei* is frequently added to improve the flavour typicality of the mould ripened cheeses.

The development of the yeasts and mould, by consumption of the lactate and production of ammonia, for the first 10 days of ripening, leads to a raise in pH (Fig. 3) at the surface but also inside the cheese. This step is a major one in the ripening process. The desacidification changes the texture and permit to the ripening bacteria to grow. As shown in figure 3, the pH rise is quicker at the surface than inside the cheese. At the cheese surface, the lactic acid available is quickly consumed by the fungi. This surface consumption is only related to the metabolic activity of the fungi, itself mainly driven by the temperature level. However, the change in pH inside the cheese is related to the transfer of lactate from the center of the cheese onto the surface driven by the concentration gradient between low lactate concentration at the surface, consequence of the fungal consumption of this solute, and the high concentration of lactate inside the cheese. This mass transfer should be equilibrated with the mould activity. Any increase in temperature of the ripening chambers will activate the mould physiology quicker than the mass transfer. If the transfer is too slow as compare to the biological activity at the surface, *P. camemberti* can exhaust the lactate at the surface and starts to attack the proteins and lipids which could lead to the production of styrene (with a strong plastic odor) and 1-octene 3-ol with a mushroomy flavour. An important part of the control of the quality of soft cheese ripening is in the control in the equilibrium between the mass transfer of the mould nutrients from inside the cheese to the surface and the biological activity of moulds and yeasts at the surface.

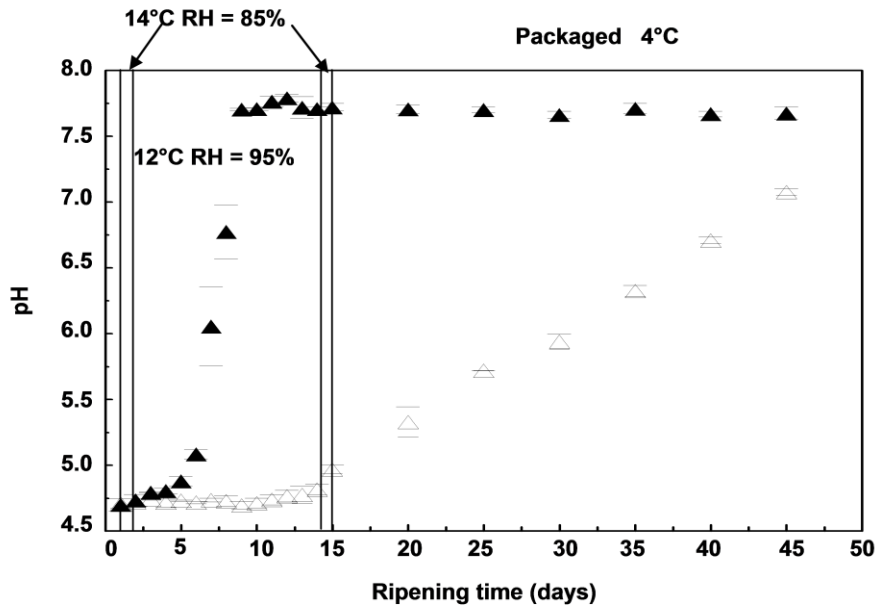


Figure 3: Changes in cheese pH versus ripening time (45 days). Solid symbols = pH of cheese rind and open symbols = core pH. Each evolution represents the mean of two trials and ' _ ' Std deviation (From Leclercq-Perlat et al. 2004a).

3.2. Washed rind or smear cheeses

The microbial ecosystem of washed rind cheeses is probably the ecosystem that has been studied the more in the recent years. It has been checked in Munster, Livarot, Tilsit, Limburger, Taleggio, Gubbeen, Reblochon etc. In this variety of cheese where some are made from quite lactic curd (Epoisses) and where the curd of others are more rennet curds and even pressed curd (Reblochon), the common treatment is the wash of the surface with brushes or cloth regularly during ripening. This mechanical treatment, often completed with the addition of salt onto the surface and of ripening starters (such as *B. linens*), will prevent the development of moulds. It leads to the formation of a smear which is a quite complex ecosystem made of yeasts and ripening bacteria. In Livarot for example, up to 80 different types of bacteria (Gram + and Gram -) and yeasts have been evidenced through molecular methods (Mounier et al. 2008).

The yeasts (*Kluyveromyces lactis*, *Debaryomyces hansenii*, *Geotrichum candidum*, *Yarrowia lipolytica*,...) are selectively growing just after the beginning of the ripening due to the curd acidity. Their metabolisms lead to the consumption of the lactic acid and to the production of ammonia from the curd amino acids and consequently to a raise in pH, as in mould ripened cheese, but slower. This increase in pH will be favorable to the growth of different bacteria, mainly coryneform bacteria (*Arthrobacter arilaitensis*, *Corynebacterium casei*, *Microbacterium gubbeenense*, *Brevibacterium linens*, ...) but also some Staphylococci (e.g. : *St. equorum*, *St. xylosus*, *St. saprophyticus*) or Gram negative bacteria such as *Proteus vulgaris* (Larpin-Laborde et al. 2011). It has recently been evidenced (Monnet et al. 2012) from the total DNA sequence of several of these strains that iron limitation in the curd is a significant limitation growth factor that probably lead to the selection of micro-

organisms adapted to low iron environment.

This microflora will generate the typical yellow to brown colour of this cheese type, the typical strong garlic, sulfury flavours and a texture that could be chalky on young cheeses but that will move to very creamy textures (see §4.1).

3.3. Blue Cheeses

In the blue veined cheeses, a main objective is to create a curd with openings in which *Penicillium roquefortii* will be able to grow. To open the curd, diverse strategies have been developed like the use of a large proportion of heterofermentative lactic acid bacteria, such as *Leuconostoc sp.*, producing bubbles of CO₂ in the curd. The stirring of the curd before and during draining is also a mean used to obtain a coating of the curd grains that will produce opening in the cheese during moulding. After moulding the cheese is pierced with holes (40/cheese for a typical Roquefort), this piercing will create some kinds of chimney by which the air will fill up the inside openings and lead to the development of *P. roquefortii*. These types of cheeses are usually rather salted (up to 6.6% of dry matter) (Prieto et al. 1999), considering the moisture level of these cheeses, it leads to a ratio salt/water which is about 14% which is selective for the growth of secondary flora in the Blue cheese ecosystem. Recent studies shows that *Yarrowia lipolytica* is a common yeast in the secondary flora of blue cheese and is an important yeast for the flavour of this type of cheese (Gkatzionis et al. 2013)

3.4. Pressed cheeses

In this family, depending on the coagulation, on the surface treatment and on the geometry of the cheese, a large diversity of cheeses is obtained. The curd is mainly made from the rennet activity and the lactic acid bacteria are growing late during the moulding. When the cheeses are small or of a limited thickness, like in Reblochon or Saint Nectaire, the ripening is rather quick. The fungal surface microflora made of moulds, if the surface is not washed or only made of yeasts if washed, have strong activities and are able to change the texture and to develop a significant lipolysis and proteolysis. The activities of the bacteria inside and outside are probably less important for the texture. However the bacterial flora is important for the flavour in these cheeses.

A constant characteristic of the bigger cheeses like Gouda, Cantal or Cheddar is the much lower level of lipolysis happening during ripening. The ripening is mainly the fact of the activity of lactic acid bacteria and of yeasts in anaerobiosis and so with a much slower metabolism than in the previous category. In these cheeses the subtle flavours are related to the proteolysis and mainly to amino acid catabolism.

3.5. Hard cooked cheeses

In this family are included most of swiss type cheeses like Emmental, gruyere and Comté but also the hard cheeses from Italy such as Parmiggiano Reggiano or Grana family cheeses. The swiss cheeses have even a lower level of lipolysis than the pressed cheeses, when lipolysis is over 2%, taints are observed. After moulding and salting, the cheeses are kept at a temperature of 12 °C for at least 2 weeks in order to permit the salt to diffuse homogeneously inside the cheese. The target level of salt is around 1.7% W/W of the humid cheese. After this period, depending on the opening desired, the transfer of the cheese in a warm cellar can be applied for 3 weeks to a month (Temperatures up to 33°C). This warm room ripening will increase the speed of the propionic fermentation happening in

these cheeses. The high level of propionic fermentation not only produces propionic acid but also CO₂. *Propionibacterium* like *P. freundenreichii* or *P. acidilactici* are able to produce large amount of CO₂ and are responsible of the large eyes present in Emmental. After this period the cheese is cooled down to 12°C until the cut of the wheel and should be maintained at 4°C after cutting. For blind cheeses (without eyes) like Beaufort or Comté, cheese ripening proceeds at low temperature (8-12°C) from the beginning to the end and, in that case, propionic fermentation is slow. The CO₂ diffuses from inside the cheese to outside at a quicker speed than the CO₂ is produced and so the CO₂ concentration never exceeds the saturation in the cheese curd and no openings are formed. Dominant flavours for swiss cheeses will be fruity flavours and nutty flavours. They are developed from amino acid catabolisms. In terms of texture the proteolysis will permit, after a long time (more than 6 months) to develop a crumbly texture and permit the precipitation of crystals that are easily perceived when tasted and that are the witness of long ripening.

At the opposite, in the hard cheeses from Italy, that are often made from lamb rennet, the lipolysis level is rather high and due to the lipase activities present in this type of rennet. This lipolytic activity will be responsible of the piquant of these cheeses, probably due to the level of free fatty acids in the cheese.

4 Functionalities developed by the microbial ecosystems important for the cheese sensory perception.

A large number of interactions between micro-organisms are at the origin of the diversity of sensorial characteristics perceived in cheese, for a recent review see the work of Irlinger and Mounier (2009).

4.1. Texture

Several parameters have an impact on the texture. The major one is probably the pH of the curd. Drained acidic curds have a chalky texture like in a young traditional camembert cheese. But as the pH is rising during the ripening, in soft cheese the texture gets more and more creamy with the age. In mould ripened cheeses the strong proteolytic and lipolytic activities present at the surface will be a lever on the change in texture. These activities emphasize the changes in texture.

In the big cheeses the texture is changing slowly. It is mainly related to the proteolysis. Progressively the texture will be less elastic and will become crumbly with long aging. Long aging can lead to the formation of crystals that are perceived when the cheeses are tasted.

4.2. Colour

In mold surface ripened cheeses the white colour of the mycelium of *Penicillium camemberti* will mainly determine the cheese colour. However the development of *Brevibacterium linens* may lead to the occurrence of yellow to orange spots corresponding to the growth of colonies of *B. linens*. When the surface of these mould ripened cheeses become brownish, it can be related to the surface humidity of the cheese that provokes the cell lysis of the mycelium. This aspect is a characterized defect.

In washed rind cheeses, the colour is related to the development of the yeasts and bacteria

mixture at the cheese surface. The interaction between yeasts and bacteria on the cheese colour has been shown to be related to the strains used. For example, the association of *Debaryomyces hansenii* with *B. linens* will give a colour that will depend on the *D. hansenii* strain used (Leclercq-Perlat et al. 2004b). *Geotrichum candidum* will usually decrease the orange intensity of *B. linens* colour. Apart from *B. linens*, several other bacteria species will contribute to the cheese colour (e.g. *Arthrobacter arilaitensis*).

4.3. Flavours

Cheese ripening is the main step where flavour formation occurs. Considering the four pathways involved in cheese volatiles biosynthesis (glycolysis and the utilization of citrate, proteolysis and lipolysis), the identification of key aroma components shows that amino acid degradation is a major process for aroma formation in various cheeses (Curioni et al. 2002). In addition, enzymatic hydrolysis of triglycerides to fatty acids and related volatiles (e.g. methylketones) is also essential to a cheese flavour development (Spinnler 2011).

Production of the aroma components relies on the milk-degrading enzymes of each strain, as well as on the complementation of metabolic pathways between strains, which may lead to an enhancement in the quantity and the variety of flavours. So the complexity of the cheese microbiota, and its functional diversity, plays a crucial role in the final flavour of cheeses (Irlinger et al. 2009).

Here, we will describe the main steps leading to key aroma compounds, with a focus on metabolic complementarities between members of the microbial ecosystem.

Considering amino acid degradation, branched-chain amino acids (leucine, isoleucine and valine - BcAAs), methionine and aromatic amino acids (phenylalanine, tyrosine, tryptophan - ArAAs) are the major precursors of cheese aroma compounds (Yvon et al. 2001). Their catabolism proceeds by two different pathways. The first one, initiated by elimination reactions, has been observed for methionine and aromatic amino acids and leads by a single step to phenol, indol and methanethiol, respectively. The second pathway, observed for ArAAs and methionine, is initiated by a transamination reaction and the resulting α -keto acids are then degraded to aldehydes, alcohols, carboxylic acids, hydroxy acids or methanethiol for methionine via 1 or 2 additional steps. If the variety of aromatic compounds is potentially huge, specific pathways will be involved depending on the specific environment (pH, a_w , redox) and the microflora of each cheese. For example, 3-methylbutanal (leucine) was identified as a potent odorant in Camembert, aged Cheddar, together with 2-methylbutanal (isoleucine) in Emmentaler and Gruyere cheese varieties (Smit et al. 2009). Nevertheless, aldehydes are transitory compounds in cheese because they are rapidly reduced to primary alcohols or even oxidized to the corresponding acids. If the primary alcohols (3-methyl-1-butanol and 2-methyl butanol) confer a pleasant aroma of fresh cheese, the corresponding acids are important flavour compounds in many cheeses and are associated with typical "old cheese", "sweaty socks" notes. *Propionibacterium freudenreichii* is by far the main agent of the formation of methylbutanoic acids, key aromatic compounds in Swiss-type cheese, where this bacteria prevails (Thierry et al. 2011).

Concerning methionine catabolism, the γ -elimination (direct or following the transamination step) producing methanethiol seems to be a major pathway especially in *B. linens*, *P. camemberti* and *G. candidum*. Methanethiol can be further chemically oxidized in polysulfides ; dimethyl disulfide

(DMDS), dimethyl trisulfide (DMTS) and even dimethyl quadrisulfide (DMQS). Coryneform bacteria, especially *B. linens*, are considered to be the key agents of sulphur-compound production in smear ripened and mould-ripened cheeses such as munster, livarot or camembert (Landaud et al. 2008). Sulfur compounds are of main importance in the basic flavour of cheeses. For example DMDS is present in all ripened cheeses.

During cheese ripening, free fatty acids containing four or more carbon atoms may originate from lipolysis of milk fat or the breakdown of amino acids, as state above. Shorter ones are directly linked to lactate fermentation, as for example by *P. freudenreichii* resulting in the formation of propionate, acetate (and CO₂), both acids being considered as flavour compounds in Swiss-type cheese (Thierry et al. 2011). However, most of the fatty acids having between 4 and 20 carbon atoms come from the lipolysis of triglycerides by moulds. For example, hexanoic acid is a characteristic flavour component of specific hard cheeses (Grana Padano and Roncal). More significantly, fatty acids are the precursors of important aromatic compounds, in particular methylketones, alcohols, lactones and esters (Spinnler 2011). Indeed, the terminal oxidation of free fatty acids produces methylketones, compounds that are primarily known for their contribution to the aroma of surface-mould ripened and blue-veined cheeses, like sulfur compounds they seem to be present in all ripened cheeses. In surface-ripened cheeses and blue-veined cheeses, the synthesis of methyl ketones is related to the enzymatic activity of *P. roqueforti*, *P. camemberti* or *G. candidum*. Fruity, floral and musty notes are associated with various methylketones such as 2-octanone, 2-nonanone, 2-decanone and 2-undecanone, while Blue cheese notes are attributed to 2-heptanone, key compounds of these cheeses. In anaerobiosis these methyl ketones are usually reduced in secondary alcohols having lower olfactive thresholds and fruity notes. For this reason blue cheeses are kept for a while in anaerobiosis to get rounder flavour notes. Another important odorant is 1-octen-3-one, especially in Camembert or aged Cheddar, and has been commonly associated with mushroom odours. The production of this compound by *P. camemberti* probably derives from the linoleic and linolenic pathways.

Finally, esters are common cheese volatiles whose production is at the cross-road of lactose fermentation, amino acid and lipid catabolism (Liu et al. 2004, Sourabié et al. 2012). Their precursors are activated short- to medium-chain fatty acids (acetyl or acyl CoA) and primary and secondary alcohols. Most esters encountered in cheese are described as having sweet, fruity and floral notes, especially ethyl esters known for their important role in the formation of a fruity character in cheese. The thiols like alcohols can react with acylCoA to produce thioesters. Among them, S-methyl thioesters, are noteworthy. Their very powerful odors are often associated with cheese flavours because of their low perception thresholds. They have been reported in numerous cheeses including camembert and smear-ripened cheeses (Martin et al. 2004).

Different approaches have been proposed to enhance cheese flavour, including selection of adjunct cultures based on metabolic complementation. For example, S-methylthioacetate production was enhanced when a yeast *Kluyveromyces lactis*, able to produce esters and hence to accumulate acyl CoA, was co-cultivated with *B. linens*, producing méthanethiol (Arfi et al. 2006). There has also been a recent interest in studying flavouring capacities of Gram-negative bacteria such as *Proteus vulgaris* or *Psychrobacter sp.* that have been isolated from different traditional cheeses (Deetae et al. 2009).

The recent identification of new key compounds in cheeses, such as thiols (Sourabié et al. 2008),

shows that understanding the flavour of cheese is still a vast and attractive field of research.

Conclusions

Diversity of cheeses is due to the diversity of milks, coagulation methods, curds obtained and resulting microbial ecosystems. The type of curd and its consequence on the possible size of cheeses, will be a determining step that will determine the balance between the ripening micro-organism that will develop at the surface with high metabolic activities and the ripening less active micro-organisms that will develop in anaerobiosis. This balance will determine on the one hand the conservation of the cheese and on the other hand the elaboration of colour and aspect, texture, taste and flavours of the diversity of the cheeses. In soft cheeses the works of the last 10 years have shown how the equilibrium between the microbial activities at the surface and the diffusion of substrates like lactic acid from the cheese center is important. Flavour defects like styrene flavours have been shown to be related with a lack of balance between the diffusion and the *Penicillium* activity.

For more and more cheese micro-organisms the complete genomes are available. These genomes give new insight in the adaptation of the micro-organisms to dairy product and cheese technology. Among them it appears that iron limitation of the milk is a selective substrate on which a strong competition between the strains of the ecosystem is observed (Monnet et al. 2012). The recent access to RNA sequences expressed by the micro-organisms in the cheese ecosystem is expecting to give new interesting results on the role of each species in the elaboration of cheese organoleptic characteristics (Monnet et al., 2016).

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