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Figure

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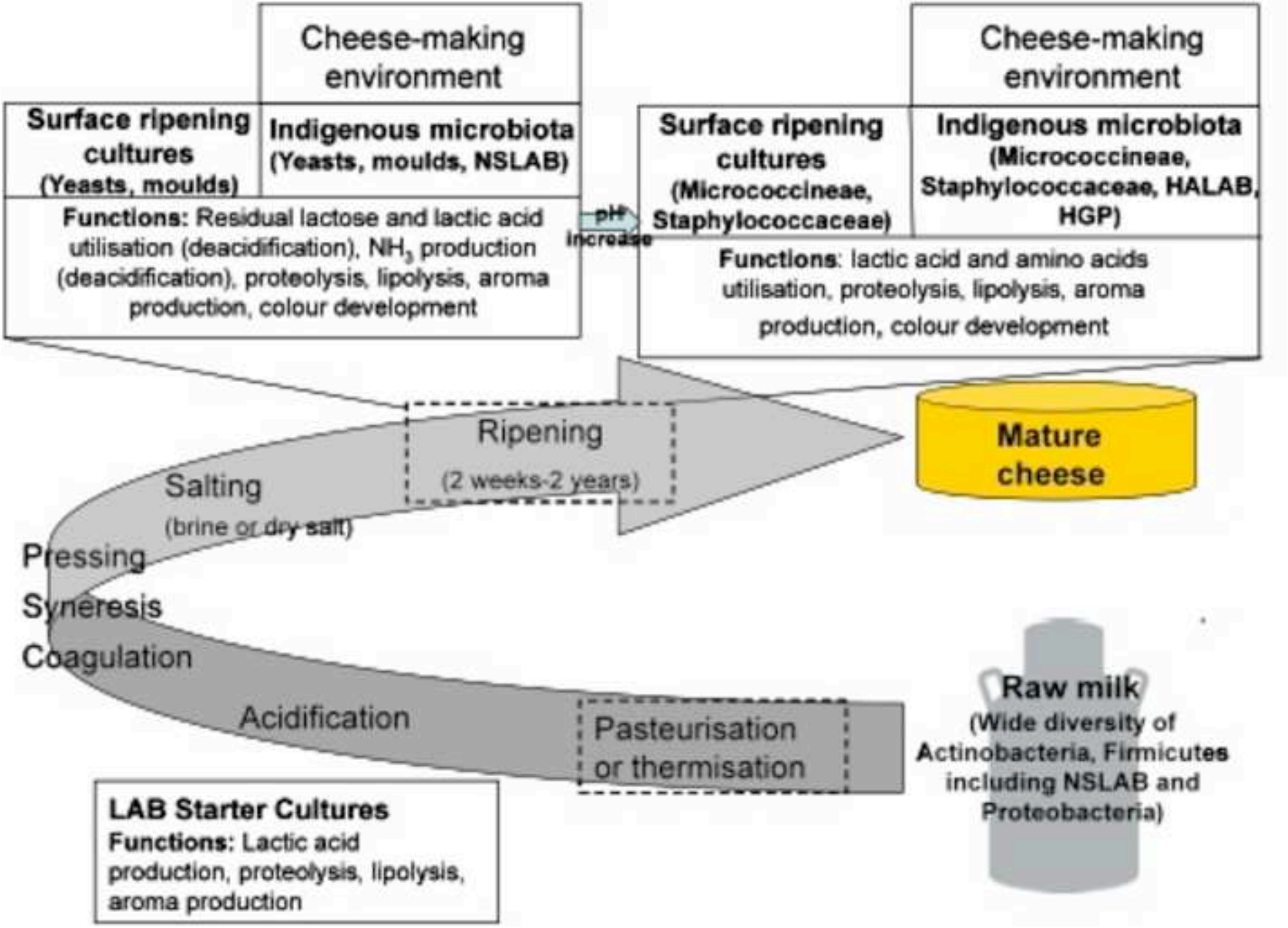
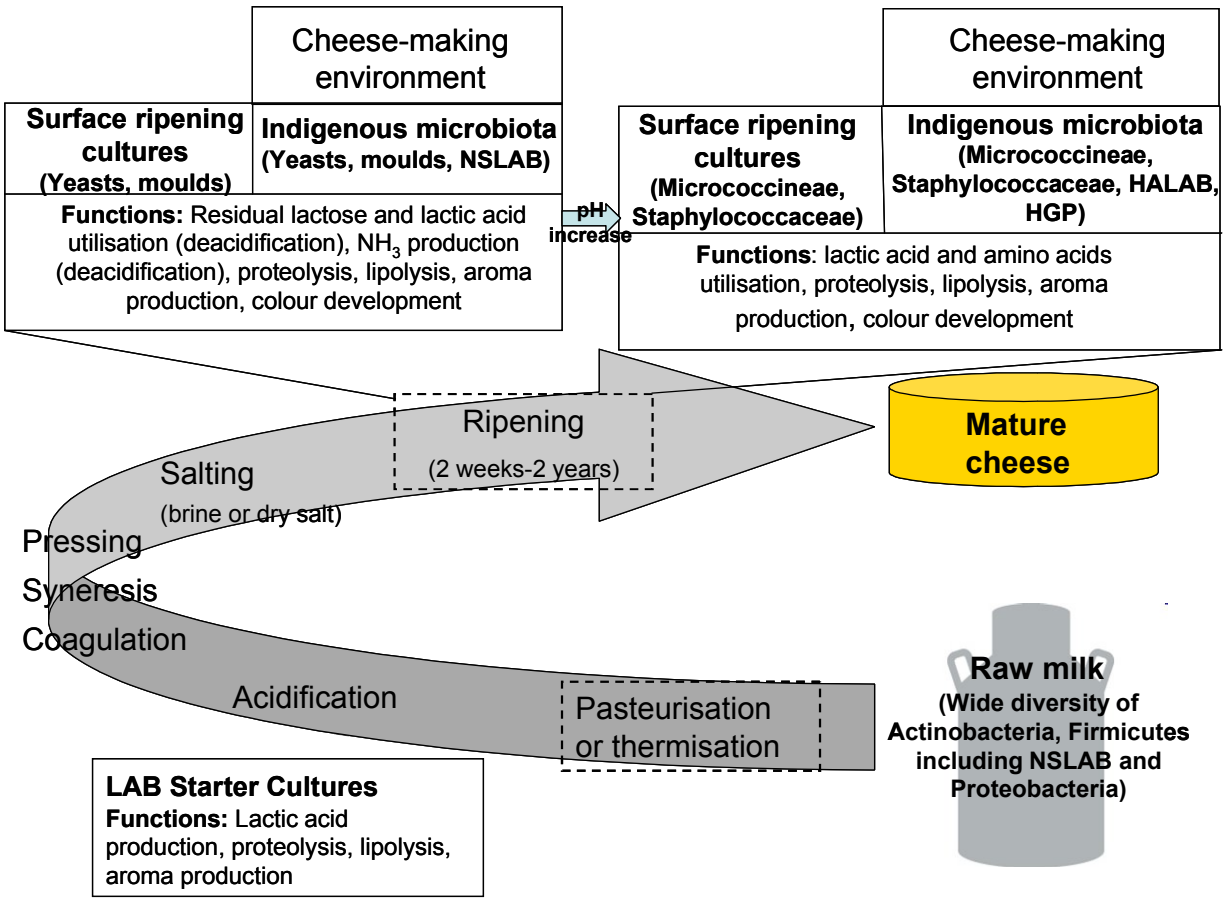


Figure

Figure 1. Schematic overview of the microbial succession and the functions of the different microbial groups involved during cheese-making. Abbreviations: LAB, lactic acid bacteria; NSLAB, non-starter LAB; HALAB, halophilic and alkaliphilic LAB; HGP, moderately halophilic Gamma-Proteobacteria. , Non-obligatory processing step.

Figure 1.



Microbial interactions in cheese: implications for cheese quality and safety

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Summary

The cheese microbiota, whose community structure evolves through a succession of different microbial groups, plays a central role in cheese-making. The subtleties of cheese character, as well as cheese shelf-life and safety, are largely determined by the composition and evolution of this microbiota. Adjunct and surface-ripening cultures marketed today for smear cheeses are inadequate for adequately mimicking the real diversity encountered in cheese microbiota. The interactions between bacteria and fungi within these communities determine their structure and function. Yeasts play a key role in the establishment of ripening bacteria. The understanding of these interactions offers to enhance cheese flavor formation and to control and/or prevent the growth of pathogens and spoilage microorganisms in cheese.

Introduction

There are over 1,000 varieties of cheeses produced on artisanal and industrial scales. The associated microbiota contributes to the biopreservation and the development of organoleptic properties of cheeses. Therefore, unravelling the microbial diversity and the functioning of these ecosystems is an extraordinary challenge to more effectively control the quality and safety of cheeses. A higher degree of microbiological safety in cheeses is yet to be achieved [1]. A recent study [1], revealed that in 2% of marketed cheeses made from raw, thermised and pasteurized cheeses, counts of *Escherichia coli, Staphylococcus aureus*, and/or *Listeria monocytogenes* were above those allowed by the European Commission Recommendations 2004/24/EC and 2005/175/EC.

This review summarises our current knowledge of microbial biodiversity and interactions within these cheese communities. Central to the discussion is the recent progress made in the understanding of ecological interactions within the different components of the cheese microbiota. We end with a discussion on how these interactions may improve cheese safety and quality and the new challenges facing researchers, cheese-makers and cheese cultures manufacturers.

Cheese-making and microbial diversity

Two reviews entirely devoted to culture-independent methods available for the description of the bacterial and fungal communities in cheeses have recently been published [2, 3]. Most

cheeses harbour a complex microbiota that is characterised by a succession of microbial groups during milk fermentation, curd maturation and storage (Figure 1).

At the beginning of the cheese-making process, lactic acid bacteria (LAB) starter cultures (i.e. *Lactococcus lactis, Streptococcus thermophilus*) grow rapidly and produce acid in milk [4]. During the first days of ripening, yeasts and/or moulds (i.e. *Debaryomyces hansenii, Geotrichum candidum, Penicillium camemberti*) colonize the surfaces of cheese and utilize lactate [5]. This leads to the deacidification of the cheese surface, enabling the establishment of a bacterial community which is less acid-tolerant (i.e. *Arthobacter arilaitensis, Brevibacterium aurantiacum, Brevibacterium linens, Corynebacterium casei*) [6, 7, 8, 9, 10, 11, 12]. Studies of traditional smear cheeses have also revealed the presence of moderately halophilic δ-Proteobacteria i.e. *Halomonas* spp., bacteria from the *Enterobacteriaceae* family (i.e. *Hafnia alvei*), and halophilic and alkaliphic LAB i.e. *Marinilactibacillus psychrotolerans* or *Alkalibacterium olivapovliticus*. The roles of these organisms in cheese ripening have not yet been determined [13, 14].

Understanding the ripening process through the study of microbial interactions

Cheese has a highly heterogeneous physicochemical composition that offers the possibility for the simultaneous occupation of multiple niches by "specialised" strains, i.e. through the utilisation of different carbon sources. Coexisting yeasts and bacteria often reach high population densities, typically between 8 and 10 logs colony-forming unit (CFU) per gram of cheese at the time of consumption. While the inventory and the succession of yeasts, moulds and bacteria have been well described, functional features of these different populations are still not understood in detail. Today, most of the interactions identified in cheeses are related to lactic acid bacteria because of a knowledge of their physiology and the recent publications of genome sequences of several species of LAB [15]. The mechanisms of interactions between LAB and other microorganisms have been extensively reviewed (Table 1 and [15]) and will not be considered further in this review.

Yeast, moulds and bacteria have been selected as cheese surface ripening cultures by the industry because of their interesting technological properties such as aroma or pigmentation.

Recent investigations revealed, however, that these commercial ripening cultures do not establish well on cheese surfaces. For example, *B. linens,* the mostly commonly used bacterium in ripening cultures, was not found to establish successfully in various European cheeses [7, 16]. Indeed, this bacterium did not compete well with the indigenous microflora present in the cheese-making equipment and environment [17].

These findings underline the fact that attention must be paid to the ecological adaptation of strains used for cheese inoculation, to their colonization capabilities or their ability to resist and/or adapt to disturbance (environmental conditions, contamination by a pathogen etc.) and to the interactions occurring between microorganisms. Indeed, the addition of microorganisms that express a specific function in pure culture, does not lead necessarily to a similar phenotype in microbial association. This reveals the limits of a strategy based on the addition of single strains to complex cheese surface microbiota.

Until recently, all the studies on the growth behaviour of microorganisms from cheese surfaces have been done on mixed cultures with only two microorganisms. An alternative strategy has been recently proposed to identify interactions within a microbial ecosystem in model smear cheeses [18]. The three-step strategy consisted of i) using a model ecosystem of nine species that was previously shown to have great similarities with the odor profile of a Livarot cheese, ii) describing the growth dynamics of each member of this community using the generalized Lotka-Volterra (GLV) model as a preliminary approach to represent inter- and intra-species interactions, and iii) omitting specific strains from this community to evaluate the consequences of these omissions on the development of the rest of the community. GLV modelling succeeded in representing yeast-bacterium interactions but did not succeed to represent bacterium-bacterium interactions because growth of most of the bacteria was highly correlated [18]. Nevertheless, these studies confirmed key role of yeast in bacterial development. These studies also demonstrated that commensalism between yeast and bacteria (Table 1). Indeed, the consumption of lactate and the production of alkaline metabolites such as ammonia from amino acid deamination by moulds and yeast, in particular, *D. hansenii* and *G. candidum,* leads to the deacidification of the cheese surface. This deacidification enables the outgrowth of less acid–tolerant, aerobic bacteria such as *Arthrobacter* spp, *B. linens*, *C. casei*, micrococci and staphylococci.

Surface pH is not the only factor that influences bacterial development. The bacterial development and distribution of different species might be modified, depending on the yeast present in the ecosystem [18]. It appeared that the growth of some bacterial species such as *A. arilaitensis* and *H. alvei* relied on a deacidification function, whereas the growth of others such as *B. aurantiacum* and *Leucobacter* sp. relied on a specific yeast species [18]. Each one of the yeast species developed specific interactions with the other strains showing consequently a low redundancy between their functions. It is worth noting that only one inoculum level of yeasts and bacteria was tested in [18] and growth dynamics of this community may have changed with different inoculum levels.

Moreover, apart from inhibition e.g. amensalism through the production of antagonistic metabolites such as organic acids and/or bacteriocins (Table 1), growth suppression may result from non-specific competition between species for nutrients. This mechanism of inhibition has been described as the "Jameson effect" [19]. It can be described as a race between species to use the resources of the environment to maximise their growth and population numbers. When those resources are depleted, the race is over, and the growth of each species in the population stops. The Jameson effect is often observed in foods, particularly those in which lactic acid bacteria dominate the population due to a growth rate advantage. This mechanism was also identified in the inhibition of *Listeria monocytogenes* by the natural biofilm microbiota on wooden shelves used in the ripening of a smear cheese [20]. Indeed, the growth of *L. monocytogenes* stopped as soon as the biofilm microbiota entered in stationary phase. This effect was observed for two different inoculum levels of *L. monocytogenes* at two different times of inoculation in the biofilm [20].

It is also noteworthy that in dairy fermentations, nitrogen is limiting and organisms initially compete for the free amino acids and small peptides available in milk [15]. In the later stages of ripening, they compete for the peptides released by the actions of proteolytic enzymes. Thus, the ability to utilize amino acids efficiently is of primary importance in determining growth rate and population dynamics [15]. Moreover, micronutrients such as iron may be limiting for strains of smear cheeses. Consequently, siderophore-deficient isolates (*Brevibacterium, Microbacterium* spp., etc.) compete for iron through the use of specialised molecular systems for harvesting iron, including siderophores produced by microorganisms (*Arthrobacter, Brevibacterium, Corynebacterium* spp., etc.) [21]. This knowledge can be used to stimulate the growth of auxotrophic strains assigned to *Brevibacterium* sp., known to be a weak competitor in cheese manufacture.

Microbes may also interact with and influence each others' metabolism through physical contact. Thus, in experimental cheese cultures when *Yarrowia lipolytica* was grown in association with *G. candidum,* hyphal formation was inhibited and *G. candidum* grew as spaghetti-like structures instead [18]. Ammonia and proline, produced in great quantities by *Y. lipolytica,* may influence yeast mycelium formation. Ammonia as a signalling molecule has been reported to be important for yeast colony survival and development [22, 23], but the molecular basis of these effects in complex environments such as cheese remains unclear.

Implications of microbial interactions for food safety

Protective cultures

In recent years, great efforts have been devoted to taking advantage of the antagonistic activities of microorganisms in cheese. For example, LAB cultures were used to prevent and control *L. monocytogenes* and spoilage microorganisms including *Clostridium tyrobutyricum*, within the framework of hurdle technology (e.g. milk pasteurisation, use of defined starter and ripening cultures) [24, 25, 26]. Protective cultures are used because they produce antagonistic metabolites such as bacteriocins, peptides and/or low-weight non-proteinaceous compounds (organic acids, fatty acids, H_2O_2, etc.) and differ from starter cultures that are mainly used for their technological functions (acid and aroma compound production). The recent developments of cheese cultures with protective functionalities and the limits of their utilisation (*in situ* production, developed resistance of the target organisms) have been recently reviewed [27] and are shown in Table 1. Finally, it is worth noting that very few commercial protective cultures are marketed today, underlining the difficulty to develop such cultures [27]. The restrictions on using GMOs in the European market also impede the development of more effective protective cultures.

Hurdle effect of the cheese microbiota

Most recent studies focused on the ability of the cheese microbiota to control and/or prevent the growth of *L. monocytogenes* in cheese [28, 9], as well as in the cheese-making environment [20]. The hurdle effect of complex microbiota on *L. monocytogenes* seems to be highly variable [28] and remains unclear at this time. It is probably related to multiple factors such as competitive interactions and/or synergistic effects of antagonistic metabolites produced *in situ,* such as organic acids, volatile compounds and H_2O_2. An innovative tool was recently developed for the screening the antilisterial cheese communities using PCR- Single

Strand Conformation Polymorphism (SSCP) analysis by comparing the biodiversity profiles of distinct cheese microbiota from the same cheese variety and their antilisterial activity [9]. While three of the six members of cheese microbiota limited or prevented the growth of *L. monocytogenes*, none of the isolates from these cheeses produced any inhibitory substances towards *L. monocytogenes* in an agar diffusion assay [9].

Implications of microbial interactions for food quality

Microbial interactions and cheese colour

Cheese colour is an important criterion of acceptance by cheese consumers. This is especially true for red-smear cheeses which are characterised by the red-orange microbial mat on their surfaces. For a long time, it was believed that *B. linens*, because of its ability to produce carotenoids, was the main microorganism responsible for the colour development. The contribution of *B. linens* cannot be ruled out when this bacterium is present in high numbers. However, because of its sub-dominance in many traditional cheeses, it is unlikely that it is responsible for the colour development of the rind [16]. Today, it is believed that cheese colouration results from complex interactions between the components of the cheese surface microbiota [29]. The orange colour was most likely due to the interactions of yellow-pigmented bacteria such as *A. arilaitensis* and *Microbacterium* spp., and other microorganisms such as orange-pigmented staphylococci and micrococci [17, 29], as well as yeast [18]. For example, pigment production in *B. linens* was found to vary as a function of the yeast used for deacidification [30]. Two cheeses with similar bacterial distribution and population had different surface-colour, implying that species-specific pigmentation of bacteria also differed depending on the yeasts present in a complex consortium [18].

Taking advantage of microbial interactions to enhance cheese flavour formation

Four pathways, e.g., glycolysis and the utilisation of citrate, proteolysis and lipolysis, are involved in cheese flavour formation [31, 32]. Production of the flavour compounds 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. Therefore, functional diversity, which is closely related to the complexity of the cheese microbiota, plays a crucial role in the flavour compound multiplicity produced during ripening.

Enhancing flavour formation has two major goals: (i) to shorten ripening time, and (ii) to improve cheese sensory quality. Different approaches have been proposed to achieve these goals. The first one is to select new adjunct cultures that are used in combination with the starter culture or the ripening culture. These adjunct cultures can enhance flavour formation because they produce enzymes that complement metabolic routes leading to aroma compounds [33, 34]. For example, S-methylthioacetate production was enhanced when a yeast *Kluyveromyces lactis*, able to produce esters and hence to accumulate acyl CoA, was cocultivated with *B. linens,* producing methanethiol [33]. 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 [35]. The second approach to enhancing flavour formation is the use of autolytic LAB cultures or, bacteriocinogenic cultures or bacteriocins to promote lysis of LAB, which increase proteolysis and, as a result, flavour formation [36, 37, 38].

Future challenges

The surface-ripening cultures marketed today are far from adequate for dealing with the diversity encountered in cheese microbiota and managing processes to support the growth of desirable microorganisms while preventing the undesirable ones. That is why understanding cheese microbial ecology is crucial to produce a product with a constant level of quality and safety. One solution for developing competitive and complex cultures would be to isolate and identify the desirable and dominant microorganisms within each cheese consortium and to return them to cheese-makers [39, 40, 41].

On the other hand, because cheese microbial communities are much less complex than environmental communities, they offer an ideal system to test new technologies. Indeed, the increasing number of available full genome sequences of cheese-related bacteria and fungi that are of industrial importance opens new routes to study their interactions *in situ* using comparative transcriptomic and proteomic approaches. Such studies pose technical challenges that need to be addressed, i.e., efficient RNA extraction from cheese [42, 43]. The application of metabolomic and metagenomic tools will also undoubtedly provide new insights into the understanding of the functioning of complex microbial communities (ecogenome) from cheese, but this has not yet been done.

Glossary

Clostridium tyrobutyricum is a spoilage organism responsible for flavour defects and late cheese blowing

Commensalism can be defined as a relationship between two species in which one benefits from the other while the other is unaffected [15]

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Table1

Table 1. Inventory of microbial interactions in cheeses

Interaction types	Effect in cheese and / or molecules produced	Microorganisms involved	Microbiological phenomenon observed	References
Commensalism	Cheese-surface deacidification: Lactic acid utilisation Alcaline metabolites (NH_3) production	Yeasts, ripening bacteria	Growth of acid-sensitive bacteria	[18]
Amensalism	Curd acidification Organic acids (i.e. lactate)	LAB, spoilage and pathogenic bacteria	Inhibition of acid-sensitive bacteria	[15, 27, 44, 45]
	Bacteriocins		Lysis of pathogenic and spoilage bacteria	[15, 27]
Competition	Harvest of Iron	Siderophores containing bacteria, auxotrophic bacteria	Reduced colonization capacity of auxotrophic strains	[21]
	Jameson effect	Microbiota of wooden shelves, *Listeria monocytogenes*	Limited colonization of *L. monocytogenes*	[20]
Parasitism	Failure of fermentation	Phage, bacteria	Inactivation of dominant strains	[15]

Abbreviations: LAB, lactic acid bacteria

Table2

Table 2. Recent applications of bacteriocinogenic cultures and bacteriocins against *L. monocytogenes* in fresh or soft cheeses.

Bacteriocin	Source organism	Mode of utilisation	References
Enterocin 416K1	*Enterococcus casseliflavus*	Bacteriocin entrapped in polymeric film	[46]
Cerein 8A	*Bacillus cereus*	Surface application of the bacteriocin	[47]
Enterocin A and B	*Enterococcus faecium*	Adjunct culture in brine and smearing solution	[48]
Lacticin 3147	*Lactococcus lactis*	Smeared on the cheese surface	[26]
Nisin and Pediocin PA-1	Recombinant *Lactococcus lactis*	Starter culture	[49]
Enterocin A	Recombinant *Lactococcus lactis*	Starter culture	[50]