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Review

Recent developments in cheese cultures with protective and probiotic functionalities

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Abstract – Microorganisms play essential roles in the manufacture and ripening of cheese, largely contributing to the development of organoleptic properties by their metabolism and varied enzymatic activities, and to microbiological safety through barrier effects of complex microflora and production of several low-molecular-weight antimicrobial compounds. Although extensive research has been done on bacteriocins of cheese bacteria for controlling pathogens in cheese, until now only few applications have emerged. The control of spoilage yeasts and moulds has been traditionally done by chemical additives, but the application of new antifungal protective cultures is very promising, especially for the cheese industry. It has also been recently shown that naturally established cheese microflora can efficiently prevent the growth of pathogenic or spoilage microorganisms. Cheese is also a very suitable but underused carrier for the delivery of probiotic bacteria, conferring health benefits on the host, with specific advantages compared with fermented milks and yoghurts such as high cell viability. This review addresses the latest developments in applications of protective cultures (with bacteriocin and antifungal activities) or microflora with barrier effects, and probiotic cultures for the production of high quality, safe and "healthy" cheese, as well as emphasizing some of the underlying challenges and possible solutions. Furthermore, new safety criteria for food cultures relating to the presence and transferability of antibiotic resistance genes are discussed.

cheese / bacteriocin / antifungal / probiotics / antibiotic resistance / technology

摘要 – 具有保护和移哨功能的干酪发酵剂的研究截展。微生物在干酪的制造和成熟中起着关键性的作用, 微生物的代谢产物和酶的活性赋予干酪的感官品质, 复杂微生物菌群的阻隔效应和产生的一些小分子抗菌化合物是干酪微生物安全的保障。尽管关于细菌素用于控制干酪中病原菌的报道很多, 但到目前为止该技术的实际应用还是非常有限。控制干酪中腐败酵母菌和霉菌的传统方法还是添加化学防腐剂, 因此, 有必要开发一些具有抗真菌作用, 特别是适用于干酪生产的保护性发酵剂。最新的研究结果表明, 天然干酪中形成的微生物菌群可以有效地防止一些病原微生物和腐败微生物的生长。与富含高活力细菌的发酵乳和酸乳相比, 干酪还是一种益生菌很好的载体和传递者,但没引起足够重视, 它有利于人体吸收其中有益健康的成分。本文论述了能产生细菌素和抗真菌的保护性发酵剂或具有阻隔效应的微生物菌群; 能够生产高质量、安全和有益健康干酪的益生菌发酵剂在干酪生产中应用的最新进展以及在工业化生产中存在的问题。此外, 本文还讨论了食品发酵剂中抗生素抗性基因存在和转移的安全标准。

干酪 / 细菌素 / 抗真菌 / 益生菌 / 抗生素的抗性 / 技术

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Résumé – Avancées des connaissances sur les cultures à activité protectrice et probiotique pour des applications fromagères. Les microorganismes jouent un rôle essentiel dans la préparation et l'affinage des fromages en contribuant de façon importante au développement des propriétés organoleptiques par leur métabolisme et la grande variété de leurs activités enzymatiques, et à la sécurité microbiologique du produit à travers les effets barrières des microflores complexes et/ou la production de composés antimicrobiens de faibles poids moléculaires. De nombreux travaux de recherche ont été réalisés sur les bactériocines produites par des bactéries isolées de fromages pour contrôler le développement de microorganismes pathogènes. Cependant, très peu d'applications sont issues de ces travaux. Le développement des levures et moisissures nuisibles à la qualité des fromages est traditionnellement contrôlé par des additifs chimiques mais leur remplacement par des cultures protectrices antifongiques semble prometteur. Des études ont récemment rapporté le potentiel de flores naturellement établies sur des fromages pour le contrôle de flores pathogènes et de dégradation. Les fromages constituent également un vecteur adéquat, mais sous-exploité, pour les bactéries probiotiques conférant un bénéfice santé pour le consommateur. Les fromages permettent notamment de maintenir un meilleur taux de viabilité de ces bactéries comparativement à d'autres matrices telles que les yoghourts et laits fermentés. Cette revue fait le point sur les derniers développements sur les cultures protectrices (productrices de bactériocines, à activité antifongique ou à effets barrières) et probiotiques pour la production de fromages de haute qualité organoleptique et microbiologique, et ayant un effet bénéfique sur la santé du consommateur. Les contraintes technologiques associées à l'utilisation de ces cultures sont également abordées et des solutions proposées. De nouveaux critères pour la sélection de cultures microbiennes destinées à un usage alimentaire et portant sur la présence et le transfert de gènes de résistance aux antibiotiques sont également soulignés.

fromage / bactériocine / antifongique / probiotique / antibiorésistance / technologie

1. INTRODUCTION

The many activities of starter and ripening cultures are central for quality and microbiological safety of cheese. Lactic acid bacteria (LAB) are currently almost always added to cheese milk to produce lactic acid from lactose and contribute to biochemical changes during ripening and develop the characteristics of cheese. Several beneficial health effects related to the consumption of some LAB (called probiotic culture) have also been demonstrated. This potential has been largely exploited in functional dairy foods, mainly yoghurt and fermented milks. Although numerous studies have been devoted to incorporation of probiotic cultures into cheese, few probiotic cheeses are commercially produced.

On the other hand, a large variety of other microorganisms originating from many sources (milk, equipment, ripening rooms, human) and growing in the mass and on the surface of cheese also participate in cheese and greatly contribute to the characteristics and diversity of cheese. This microbiota, which is relatively difficult to control, may also be contaminated by spoilage and/or pathogenic microorganisms and thereby, be a source of undesired contaminants for cheese. In contrast, cultures isolated from cheese or milk have a protective effect against the development of contaminants. The implementation by the cheese industry of such protective cultures could contribute with other classical measures (e.g. milk pasteurization, use of defined starter) to control the development of undesired microorganisms and further improve quality and safety of cheese.

In this review, the latest developments in the application of protective and probiotic cultures for the production of high quality, safe and "healthy" cheese have been reviewed, with underlying challenges and possible solutions.

2. PROTECTIVE CULTURES IN CHEESE

The implementation in the nineties of the Hazard Analysis Critical Control Point

plan and other preventive measures in the cheese industry have greatly contributed to the reduction of the incidence of foodborne diseases related to cheese consumption. For example, during the period 1993–1996, the number of dairy products highly contaminated by *Listeria monocytogenes* (i.e. $\geqslant 100$ cfu·g^{-1}) in France decreased by 41% [64]. However, a recent study on retail cheeses in the UK has shown that 5 and 4% of cheeses made from unpasteurized (including thermized milk) and pasteurized milk, respectively, were of unsatisfactory or borderline quality according to EC recommendations 2004/24 and 2005/175 for the presence of *Salmonella*, *Staphylococcus aureus*, *Escherichia coli* and *L. monocytogenes* [82]. A cheese with microbiological borderline quality can also be rapidly considered unsatisfactory if stored under inappropriate conditions at home. Yeasts and moulds are also common, sometimes major, spoilage organisms of food products, especially fermented milk products and cheeses. These organisms, which cause severe economic losses and significantly reduce the shelf life of products, may constitute a health hazard due to the production of mycotoxins [47,48,129]. Therefore, there is a real need to enhance the control of microbiological safety of cheese from milk to the consumer, and this can be achieved by microbial cultures with antimicrobial features and protective effects in cheese.

2.1. Bacteriocins and bacteriocinogenic strains

In the last two decades, several studies have demonstrated the potential of bacteriocins to control growth of pathogenic microorganisms in food products [26]. Bacteriocins are peptides with antimicrobial activity produced by a wide diversity of bacteria. To date, most of the identified Gram-positive bacteriocins are produced by LAB, among which there are generally recognized as safe (GRAS) organisms used for milk fermentations [66]. Moreover, bacteriocins can be rapidly degraded by proteases in the gastrointestinal tract and therefore they should not interfere with human gut microbiota [12]. Finally, bacteriocins produced in situ by food-grade bacteria do not have to be indicated on the product label. Most research on bacteriocins in cheese have targeted the control of *L. monocytogenes*, or other listeria species used as model for this pathogen, and clostridia spores responsible for late blowing defects in semi-hard and hard cheeses.

2.1.1. The "classical" bacteriocins for cheese

Nisin, a class I bacteriocin (< 5kDa peptides containing unusual lanthionine amino acids) produced by lactococci is the best documented bacteriocin of LAB and remains a model for the development of other bacteriocins. To date, nisin, as a food additive, is the only bacteriocin that has received regulatory approval from the US Food and Drug Administration and European Union (listed as E234) and can be labeled as a "natural preservative". Its broad activity spectrum includes listeria, enterococci, staphylococci, streptococci, clostridia, *Campylobacter jejuni*, *Helicobacter pylori* and antibiotic-resistant strains of *Neisseria gonorrhoeae* [94,128]. However, the stability of nisin is highly dependent on environmental conditions, in particular pH, and this limits its use to acidic foods [116]. In addition, nisin can be degraded by proteolytic enzymes in cheese, leading to a significant activity decrease during ripening. For example, Benech et al. [8] showed that only 12% of the initial activity produced by a nisinogenic starter remained in Cheddar cheese after 6 months of ripening.

However, encapsulation of purified nisin into liposomes allowed the retention of 90% of initial nisin activity and thereby, resulted in an improved control of *Listeria innocua* populations in artificially contaminated cheese during ripening [8]. However, until now only few applications of nisin in the cheese industry have emerged, as summarized in recent reviews [21, 55, 130].

In contrast to nisin, lacticin 3147, a two-component broad-spectrum antimicrobial peptide produced by *Lactococcus lactis* subsp. *lactis* DPC3147 showed high stability over a wide range of pH [117]. No decrease in in situ produced lacticin activity was detected in Cheddar cheese over 6 months of ripening [119]. However, the application of the lacticin 3147 producer strain on surfaces of smear-ripened cheese as a pretreatment for controlling *L. monocytogenes* contamination was not effective [102].

Pediocin AcH (also known as pediocin PA-1 or SJ-1) is another broad-spectrum anti-listerial bacteriocin that belongs to class II (non-modified heat-stable peptides < 10 kDa). It is produced mainly by *Pediococcus acidilactici* [39], a minor population in many cheeses. However, *Lactobacillus plantarum* WHE (commercially available as ALC 01, anti-listerial culture, Danisco, Germany) isolated from Munster cheese was also shown to produce pediocin [40]. Production of pediocin AcH by *P. acidilactici* H was considerably reduced when final medium pH exceeded 5.0. In contrast, bacteriocin production by *Lb. plantarum* WHE 92 was not affected for pH values up to 6.0, which is a clear advantage for cheese application [40]. Spraying a cell suspension of *Lb. plantarum* WHE 92 on surfaces of Munster cheese at the beginning of ripening inhibited growth of *L. monocytogenes* for 21 days [41]. However, Loessner et al. [83] stressed the risk of development of resistance when pediocin AcH or the producer strain is used to control *L. monocytogenes* on cheese surfaces.

2.1.2. Newly developed bacteriocins for cheese applications

In recent years, several other bacteriocins with potential use in cheese have been reported. *Streptococcus* and *Enterococcus*, two genera with particular relevance in cheese manufacture, also produce a large diversity of bacteriocins [99].

Thermophilin, produced by some strains of *Streptococcus thermophilus*, is a class II bacteriocin with inhibitory activity against *Streptococcus*, *Enterococcus*, *Lactococcus*, *Bacillus* and *Listeria* [51]. *Streptococcus macedonicus* is a relatively new species first isolated from Kasseri, a Greek hard-cooked cheese [137]. Macedocin, produced by *Str. macedonicus* ACA-DC 198, belongs to the lantibiotic bacteriocins (Class I) and inhibits several LAB as well as *Clostridium tyrobutyricum* [59]. *C. tyrobutyricum* is responsible for large defects in semi-hard and hard cheeses, such as Swiss-type or Gouda, due to high production of butyric and acetic acid, CO_2 and H_2 from fermentation of lactate, leading to significant flavor defects and late cheese blowing [6]. Germination of *Clostridia* spores can be inhibited by additives such as nitrate, lysozyme or nisin, but their uses can be restricted by local regulations [6]. Therefore, the use of the macedocin producer strain as adjunct culture could be a relevant alternative for controlling *Clostridia* development. Unlike nisinogenic lactococci, *Str. macedonicus* is not inhibited by the cooking step of the manufacture process, and macedocin is stable over a wide range of pH and upon prolonged fermentation [138]. Recently, Anastasiou et al. [4] evaluated the performance of *Str. macedonicus* ACA-DC 198 used as sole starter or adjunct culture in the production of Kasseri cheese from pasteurized milk.

After some changes in the process (i.e. time to reach a pH of 5.2 in the drained curd and temperature/duration of ripening), no major effects were observed on microbiological, physicochemical or sensorial characteristics of cheeses made with *Str. thermophilus* ACA-DC 198 alone or mixed with a commercial starter culture compared with control cheese with only commercial starter [4]. Furthermore, *Str. macedonicus* ACA-DC 198 was used as starter or adjunct culture and macedocin was detected until the end of cheese ripening (90 days) [4]. However, the efficiency of macedocin inhibiting *Clostridia* in situ has not yet been tested in cheese.

Enterococci are used as starter or non-starter lactic acid bacteria (NSLAB) in many traditional Mediterranean cheeses, where they greatly contribute to the development of organoleptic properties during ripening through proteolysis, lipolysis and breakdown of citrate into aroma compounds [60]. Although some members of the *Enterococcus* genus are also pathogenic and involved in the transfer of antibiotic resistance and/or virulence factors, others are considered safe and even used as probiotics [52]. Many enterococci can produce bacteriocins belonging exclusively to the heat-stable, non-lantibiotic class II, with the exception of cytolysin, a two-peptide lantibiotic (Class I) associated with virulence of some *Enterococcus faecalis* strains due to its hemolytic activity [99]. Nuñez et al. [101] have investigated the inhibition of two *L. monocytogenes* strains by enterocin 4, produced in situ by *E. faecalis* INIA 4 during the manufacture and ripening of Manchego cheese. The population of *L. monocytogenes* Ohio, initially at 10^5 cells per mL in raw milk, decreased to less than 10^2 cells per gram of curd 8 h after the start of manufacture when *E. faecalis* INIA 4 was used as sole starter or adjunct culture. From ripening days 2 to 60, *L. monocytogenes* Ohio was only recovered in cheeses manufactured with *E. faecalis* INIA 4, with or without commercial culture, after an enrichment procedure of 25 g of cheese sample [101]. In contrast, the population of *L. monocytogenes* Ohio in control cheeses remained stable at 3.9×10^4 cells per gram. However, *E. faecalis* INIA 4 as sole starter failed to inhibit growth of *L. monocytogenes* Scott A under the same conditions.

Bactericidal synergism between heat pretreatment (65 °C, 5 min) and the enterocin AS-48 produced by *E. faecalis* A-48-32 was shown on inhibition of *S. aureus* in skimmed milk [97]. Compared with acidified skimmed milk a lower efficacy was measured in cheese when *E. faecalis* A-48-32 and *Enterococcus faecium* UJA32-81, both enterocin AS-38 producers, were used as starter or adjunct culture, to inhibit growth of *S. aureus* [97]. This result was tentatively explained by a lower production of enterocin AS-38 in cheese and/or difference in bacteriocin activity due to cheese matrix and temperature conditions (i.e. 28 °C for acidified skimmed milk and 4 °C for cheese ripening) [97]. Another important factor, pH, may have also influenced bacteriocin activity but was not reported in the study.

To conclude this section, the relevance of bacteriocinogenic strains as "protective cultures" varies among the different studies. Indeed, the efficacy of a bacteriocin producer or bacteriocin with high potential in simple experimental set-up conditions (e.g. synthetic medium, controlled temperature and pH) to control growth of pathogens can be very different when used in cheese due to several factors: competition with the starter culture, complex interactions between the bacteriocin and cheese matrix, presence of inhibitors (e.g. proteases), and/or non-optimal conditions (e.g. pH, temperature) for growth and bacteriocin synthesis. In addition, cheese composition may also greatly differ from core to surface in terms of microflora diversity, environmental conditions, particularly salt

and water contents, and pH. Thus, bacteriocin production and efficiency can greatly vary within cheese. Bacteriocinogenic strains used as starter or adjunct culture must also show good growth in milk and/or cheese since bacteriocin production is usually directly correlated with final biomass.

Safety considerations using bacteriocins or their producer strains is an important issue since cross-resistances between different classes of bacteriocins and/or antibiotics have already been reported [74, 98]. Moreover, some bacteriocinogenic strains also belong to genera or species (e.g. enterococci) which include pathogenic strains. Applications of such strains at high levels in cheese should be carefully assessed for the risk to benefit ratio. Solutions proposed to overcome these major drawbacks will be discussed below (Sect. 5). Finally, the balance of the microflora of cheese, a key priority for proper ripening and final quality, can be affected by bacteriocins or bacteriocinogenic strains; this problem should be carefully assessed for each cheese type as discussed below.

2.1.3. Impact of bacteriocins and bacteriocinogenic strains on cheese ripening

The microflora of cheese, through its diverse metabolic activities and release of enzymes, plays an important role in the development of typical texture, flavor and appearance of ripened cheeses. Only GRAS bacteria for specific food uses can be incorporated in dairy products. For in situ bacteriocin production in cheese, the strains must also be able to grow at high levels and/or be metabolically active in the cheese environment. Therefore, only bacteriocins from LAB, generally isolated from cheese, have been used. Bacteriocins, most of them with a narrow, and some like nisin and pediocin with a broader activity spectrum, are active on bacteria closely related to the producer strain or confined to the same ecological niche [75]. Thus, the presence of bacteriocin, produced in situ or added, can disrupt the natural balance and dynamics of the cheese microflora, including starter cultures.

The acidifying capacity of nisin-tolerant lactococci was not significantly affected by the presence of a nisinogenic strain, whereas a significant decrease was observed for a commercial culture and nisin-sensitive lactococci during growth in milk [8]. Benech et al. [7] studied the textural, physicochemical and sensory attributes of Cheddar cheese made with a mixed culture containing *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* UL719 and two nisin-tolerant lactococci. After 6 months of ripening, rheological properties were not significantly different while proteolysis, lipolysis and formation of hydrophilic and hydrophobic peptides increased in cheese containing the nisin Z producer compared with control cheese [7]. Enzymatic capabilities of *Lc. diacetylactis* UL719 cells or autolysis of a nisin-sensitive subpopulation of the starter may be responsible for proteolysis in cheese containing the bacteriocinogenic strain [7]. In this study, the two nisin-tolerant lactococci were also selected for their high acidifying capacity in milk. However, for cheeses produced using these two strains, in combination or not with *Lc. diacetylactis* UL719, a bitter off-flavor and an acidic taste were observed [7], probably due to a low amino-peptidasic activity of these strains compared with commercial starter cultures. Nevertheless, cheese bitterness could be corrected and flavor improved by incorporating a high proteolytic strain, *Lactobacillus casei* subsp. *casei* L2A, in the mixed lactococci starter containing the nisin Z producer [7]. Similar results were recently reported for Hispanico cheese manufactured using a lacticin 481 producing strain, *Lc. lactis* INIA 639, a non-bacteriocinogenic strain,

Lc. lactis INIA 437, and *Lactobacillus helveticus* with high amino-peptidase activity and sensitive to lacticin 481 [57]. Analysis of volatile compounds also showed an enhanced production of 2-methylpropanal, 2-methylbutanal, ethanol, 1-propanol, ethyl acetate, ethyl butanoate and ethyl hexanoate in lacticin cheeses, which reached higher scores for aroma quality and intensity compared with control cheese made with both starter and adjunct culture [56].

Bacteriocins have been shown to induce lysis of sensitive starter cultures, leading to a fast release of intracellular enzymes which enhance cheese flavor development [86,88,95]. Bacteriocins may also facilitate diffusion in the cells of molecules, such as amino acids, through membrane permeabilization. The increased amino-acid pool may lead to an increased rate of transamination and formation of α-keto acids which can be further degraded enzymatically into flavor compounds [87].

2.2. Protective cultures producing non-proteinaceous low-molecular-weight antimicrobial compounds

Recently, antifungal cultures have gained importance for cheese applications although limited work has been done in this field. The antifungal properties of LAB have been recently reviewed by Schnürer and Magnusson [125]. The assessment of cheese spoilage by yeasts and moulds is complicated because fungal activity during ripening can be either needed or detrimental to product quality, depending on the type of cheese and microorganisms [48]. Spoilage of cheese due to fungal growth is caused by the production of volatile compounds, leading to off-flavors, and also mycotoxin accumulation, which may promote allergies [47, 129]. *Penicillium* spp. and *Aspergillus* spp. are important spoilage moulds in preservative-free hard, semi-hard and semi-soft cheeses, whereas *Candida* spp., *Kluyveromyces marxianus* and *Pichia* spp. are main contaminants in unripened soft cheeses [47,48].

The control of spoilage yeasts and moulds is traditionally done by chemical additives such as sorbic and benzoic acids, which both have a broad activity spectrum. Furthermore, the antibiotic natamycin produced by *Streptomyces natalensis* is effective against fungi and is used as a common preservative on hard cheese surfaces [22, 34]. In contrast, consumers are increasingly demanding high quality, safe and mildly processed foods with long shelf life and low or no addition of chemical preservatives. Furthermore, fungi also showed an increased resistance to antibiotics and chemical preservatives such as sorbic and benzoic acids [18]. Thus, the application of antifungal protective food cultures has a high potential, especially in dairy products such as cheeses or fermented milks.

So far, only a few antifungal protective cultures are commercialized and their applications are still emerging, especially for dairy products but also for other foods and feeds (Tab. I). Two protective cultures are currently marketed by Danisco (Danisco, Copenhagen, Denmark) as the HOLDBAC^{TM} range, combining two strains of *Propionibacterium freudenreichii* subsp. *shermanii* JS and *Lactobacillus rhamnosus* LC705 [133] or *Lactobacillus paracasei* SM20 [91,92]. Both cultures have been successfully tested in yoghurt and on the surface of hard cheese to prevent spoilage by yeasts and moulds [91].

In contrast to antibacterial peptides, only little is known about antifungal mechanisms. So far, research has mainly been directed towards identifying different antifungal metabolites produced in simple in vitro fermentations, but detected antifungal metabolites were not solely responsible for antifungal features of a particular strain or culture. Due

Table I. Antifungal protective cultures on the global market.

Protective culture	Composition	Activity spectrum	Compounds	Recommended application	Reference
HOLDBAC™ YM-B	*Propionibacterium freudenreichii* subsp. *shermanii* JS *Lactobacillus rhamnosus* LC705	Yeasts, moulds *Rhodotorula rubra* *Pichia quliermondii* *Bacillus* spp.	Propionic acid Acetic acid Diacetyl 2-Pyrrolidone-5-caboxylic acid	Yoghurt Sour cream Fresh cheese	Danisco A/S (Denmark) [133]
HOLDBAC™ YM-C	*Propionibacterium freudenreichii* subsp. *shermanii* JS *Lactobacillus paracasei* SM20	Yeasts, moulds *Candida* spp. *Rhodotorula mucilaginosa*	Propionic acid Acetic acid Succinic acid 2-Pyrrolidone-5-caboxylic acid 3-Phenyllactic acid Hydroxphenyllactic acid	Yoghurt Sour cream Fresh cheese	Danisco A/S (Denmark) [91] and unpublished data
Feedtech® Silage F3000	*Lactobacillus plantarum* Milab 393 *Pediococcus acidilactici* *Enterococcus faecium* *Lactococcus lactis*	Yeasts, moulds *Clostridium* spp.	3-Phenyllactic acid Cyclic dipeptides Nisin	Silage	DeLaval (Sweden) [132]

to complex and synergistic interactions between different low-molecular-weight compounds and likely cell-to-cell interactions, the overall mechanisms are difficult to elucidate [125]. Recently, we identified the production of several antimicrobials, including 2-pyrrolidone-5-carboxylic acid, 3-phenyllactic acid, hydroxyphenyllactic acid, and succinic acid besides propionic and acetic acids, during fermentations of *Lb. paracasei* SM20 and *Propionibacterium jensenii* SM11 in whey-based medium (unpublished data). Similarly, *Lb. rhamnosus* LC705 was shown to produce 2-pyrrolidone-5-carboxylic acid [140]. Furthermore, mixtures including acetic, caproic, formic, propionic, butyric, *n*-valeric, and benzoic acids, as well as 3-hydroxy fatty acids, proteinaceous compounds and cyclic dipeptides were described as antifungal compounds of LAB [29, 84, 100, 127, 132]. One common characteristic was that all these compounds had to be present at low concentrations in contrast to high minimal inhibitory concentrations for fungi, confirming the complexity of antifungal mechanisms [132, 140].

The application of antifungal protective cultures is very promising, especially for the cheese industry. Several strains have been recently developed for cheese applications but further research is required to elucidate their mechanisms, reduce costs and for implementation by the industry. High effectiveness of protective cultures is often related to high inoculation levels, which can affect food quality and increase costs compared with traditional chemical preservation [91]. Clearly, careful optimization of antifungal systems and their applications are required.

2.3. Barrier effects of complex cheese microbiota

Cheese surface consortia are known to be complex ecosystems, exhibiting high species and strain diversity of bacteria and eukaryotes. Recently developed molecular tools such as pulsed field gel electrophoresis (PFGE), which is a culture-dependent method, temporal temperature gel electrophoresis (TTGE), denaturing gradient gel electrophoresis (DGGE), single strand conformation polymorphism (SSCP), and terminal restriction fragment length polymorphism- (TRFLP-) or length heterogeneity- (LH-) PCR have been successfully used to explore diversity and dynamics of cheese microflora [30, 35, 49, 80, 103, 113, 114]. Throughout cheese manufacturing and ripening, the development of the microflora is influenced by negative interactions between the different microbial populations, such as competition for nutrients, as well as stimulating interactions, such as pH increase and production of growth factors [96]. Both bacterial and eukaryotic populations evolve, with some species disappearing and others becoming predominant.

Naturally established cheese microflora were found to be more or less permissive for the growth of pathogenic or spoilage microorganisms [13, 85, 93, 124]. The inhibitory activity was related to biodiversity and/or dynamics of the microflora because no antimicrobial activity was detected with pure cultures of the isolated strains.

Different hypotheses can be formulated to explain the antimicrobial activity of complex consortia. Certain factors associated with their metabolism (e.g. organic acids and H_2O_2 production, nutrient depletion) together with cheese manufacture parameters (e.g. temperature, salt concentration) might exert synergic actions, inhibiting pathogenic or spoilage microorganisms. It is also well known that bacteriocin synthesis can be regulated by quorum sensing systems [62]. In this case, signaling molecules produced by one population of cheese consortia are believed to trigger bacteriocin production by another

population, thus explaining the absence of anti-microbial activity of pure cultures.

The selection of complex surface microflora to control the growth of spoilage and pathogenic microorganisms in cheese appears to be a promising avenue because no modification of process is required. However, further studies are needed to understand better mechanisms contributing to antimicrobial activity. The controlled propagation-production of such microflora for large-scale applications is a technological challenge, as presented below.

3. PROBIOTIC CULTURES IN CHEESE

Probiotics are defined by the FAO/WHO [45] as "live microorganisms which when administered in adequate amounts confer a health benefit on the host". The beneficial effects in treatment and prevention of various diseases or gut disorders such as inflammatory bowel disease or lactose intolerance are greatly debated in the literature [106]. Probiotic microorganisms include several genera of bacteria and yeasts [68], and among those, strains of enterococci, lactobacilli and propionibacteria are important for manufacturing some cheeses [52, 90, 108], and well adapted to the cheese environment. On the other hand, bifidobacteria and *Lactobacillus acidophilus* are most used in functional dairy foods containing probiotics, especially milk, yoghurt, ice cream and desserts [20]. However, conditions encountered in these products are very different from those of their natural habitat, the gastrointestinal tract of humans and animals. Thus, product composition can have deleterious effects on their viability, which is one of the most important prerequisites for beneficial health effects [106]. Due to its limited acidity, low oxygen level, high lipid content and low storage temperature, cheese is a suitable carrier for delivering live probiotic bacteria [15].

3.1. Characteristics of probiotic bifidobacteria and lactobacilli related to their viability in cheeses

Viability of probiotics is a key parameter for efficacy of probiotic products. Although the amount of cells required to produce beneficial health effects is not known, some authors have suggested a daily intake of at least 10^8–10^9 viable cells as the minimum intake to provide a therapeutic effect [118]. A minimum level of 10^6 cfu of probiotic bacteria per gram of product at the time of consumption is generally accepted and selected to provide bacterial concentrations that are technologically attainable and cost-effective [79, 123]. According to this criterion, most cheeses seem to be suitable carriers for probiotic bacteria, as indicated by the high stability of viable cell counts reported for many cheeses during storage (Tab. II).

Probiotic bifidobacteria and lactobacilli of gut origin are generally classified as strictly anaerobic or microaerophilic but their resistance to oxygen is strain- and species-dependent [134]. They usually show poor resistance under prolonged acidic conditions, with lactobacilli being less affected than bifidobacteria [20]. These characteristics explain the higher survival of probiotic cultures in cheese compared with other dairy products, in particular yoghurts and fermented milks [72, 126]. Indeed, yoghurt has an initial pH of about 4.1–4.4 that decreases to 3.8–4.2 during refrigerated storage due to lactic acid production by *Lactobacillus delbrueckii* subsp. *bulgaricus*. On the other hand, the curd of soft cheeses has a pH below 5.0 after production (about 4.5 for Camembert cheese) but it increases rapidly during ripening. In 35-day

ripened Camembert cheese, pH in the core and the rind reached high values, around 6.8 and 7.5, respectively [2]. The pH of hard and semi-hard cheeses, generally above 5.0 after production, remained relatively constant throughout the ripening period [23]. Furthermore, the cheese core can be considered as an anaerobic environment with very low redox potential (E_h) of about –250 mV [9]. The redox potential of Camembert cheese during ripening has been shown to increase from +330 to +360 mV on the surface and decrease from –300 to –360 mV in the core [2]. In contrast, E_h is positive in yoghurt and increases with storage time above +120 mV [32,33]. Other parameters have been reported to influence probiotic viability in cheese, such as temperature and duration of ripening, presence of NSLAB with antagonistic activities, milk pasteurization and salt content [63, 104, 105, 112, 141].

Several bifidobacteria strains have been successfully incorporated into cheeses with no major changes in the process (Tab. II). This is different from yoghurt, where the incorporation of bifidobacteria is generally limited to *Bifidobacterium animalis*, a bacteria isolated from animals, due to its high acid and oxygen tolerance compared with human bifidobacteria [20, 72]. Because the health benefits of animal bifidobacteria have not been researched to the same extent as human species [72], cheese is a promising carrier for enlarging the range of bifidobacteria added to functional foods. Furthermore, cheese is also a suitable protective carrier against harsh stomach conditions. Vinderola et al. [139] have reported high survival for *Bifidobacterium bifidum, Lb. acidophilus* and *Lb. casei* added to Argentinian Fresco cheese and subjected to a pH of 3.0 for 3 h at 37 °C. In an in vitro test, Cheddar cheese containing a probiotic strain of *E. faecium* gave greater protection to the strain exposed to porcine gastric juice at pH 2.0 than yoghurt [58]. Cellular response of *P. freudenreichii* to technological stresses during Swiss-type cheese manufacture has also been reported to increase resistance of cells to acidic conditions [71].

However, although cheese is likely one of the best carriers for probiotics, the addition of high numbers of viable and metabolically active cells can affect product quality, especially organoleptic properties, as discussed below.

3.2. Effects of probiotic bifidobacteria and lactobacilli on cheese quality

The gross chemical composition of cheese (i.e. salt, protein, fat and moisture) and pH are generally not influenced by added probiotic bacteria [10, 11, 19, 28, 31, 37, 61, 104, 105, 141]. However, in Cheddar cheese containing *Bifidobacterium lactis* Bb-12, Mc Brearty et al. [89] measured a higher moisture level of 40%, exceeding the legal limit, compared with control cheese containing about 38% moisture. This effect, leading to low body/texture scores in sensory analysis, was explained by a rapid acidification during cheese manufacture with the starter added together with *B. lactis* Bb-12 [89].

Incorporation of probiotic cultures in cheese does not generally affect primary proteolysis which in many cheeses results from activity of the coagulant agent (except for high cook cheeses) and, to a lesser extent, of plasmin and subsequently, residual coagulant and enzymes from the starter microflora [131]. However, changes in secondary proteolysis and increases in free amino acid content have often been reported when probiotics were added to cheese [11, 61, 89, 104, 105]. Peptides and amino acids directly contribute to cheese flavor (such as sweet, bitter or malty) and can be precursors for the synthesis of other

Table II. Variability of probiotic cultures in cheese experiments.

Cheese Type	Ripening / Storage conditions	Strains	Cell counts (log cfu·g^{-1})		Changes in process or remarks	Ref.
			Start[1]	End[1]		
Cheddar	6–7 °C, 24 wks	*B. bifidum*	6.0	7.0	Addition of bifidobacteria prior to mixing and packing into hoops Commercial culture or immobilized freeze-dried culture	[37]
	4 °C, 12 wks	*B. infantis* ATCC 27920G	6.7	6.59	Cheese milk standardized with cream fermented with *B. infantis* ATCC 27920G	[31]
	8 °C, 28 wks	*B. lactis* Bb12 *B. longum* BB536	8.9 6.4	8.2 5.2	Addition of bifidobacteria with starter culture	[89]
	9–10 °C, 32 wks	*B. lactis* B94 *B. lactis* Bb12 *Bifidobacterium* sp. DR10 *Lb. acidophilus* L10 *Lb. acidophilus* La5 *Lb. paracasei* L26 *Lb. casei* Lc1 *Lb. rhamnosus* DR20	8.0 8.0 8.7 7.6 8.3 8.4 7.8 8.2	7.6 8.2 8.7 3.7 3.6 7.4 7.2 8.0	Addition of mixed probiotic cultures with starter culture B94, Bb12 and DR10 were mixed together L10 and La5 were mixed together L26, Lc1 and DR20 were mixed together	[112]
	4 °C, 24 wks	*Lb. acidophilus* 4962 *Lb. casei* 279 *B. longum* 1941 *Lb. acidophilus* L10 *Lb. paracasei* L26 *B. lactis* B94	8.3 8.5 8.0 8.4 8.5 7.5	8.3 8.6 8.3 8.5 8.5 7.5	Addition of mixed probiotic cultures with starter cultures LA4962, LC279 and BL1941 were mixed together L10, L26 and B94 were mixed together	[104, 105]
White-brined cheese	4 °C, 90 days	*B. bifidum* Bb02 *Lb. acidophilus* La5	9.0 9.0	7.0 7.0	Addition of probiotic cultures with starter culture	[141]

Table II. Continued.

Cheese Type	Ripening / Storage conditions	Strains	Cell counts (log cfu·g^{-1})		Changes in process or remarks	Ref.
			Start[1]	End[1]		
Semi-hard cheese	12 °C, 4 wks	*Lb. acidophilus* *Lb. paracasei*	6.9 7.39	7.8 9.1	Addition of lactobacilli with starter culture	[10, 11]
Semi-hard goat's milk cheese	6 °C, 70 days	*B. lactis* *Lb. acidophilus*	8.3–8.6 7.8–8.5	6.9–8.0 6.8–7.5	Probiotic bacteria used as starter cultures Modification of process: addition of milk protein hydrolyzate, amount of starter inoculum, temperature of milk and curd, curd cutting, concentration of salt, ripening time	[63]
Canestrato Pugliese Hard cheese	12 °C, 86 days	*B. bifidum* Bb02 *B. longum* Bb46	7.1 7.0	6.0 5.0	Addition of bifidobacteria with starter culture Modification in the process: heating of the curd	[28]
Minas Fresh cheese	5°C, 21 days	*Lb. acidophilus* La5	6.3	6.7	Addition of lactobacilli with starter culture	[19]
Crescenza Soft, rindless cheese	4 °C, 14 days	*B. bifidum* *B. longum* *B. infantis* Mixed culture FC Mixed culture IC	6.0[2] 6.0[2] 6.0[2] 6.0[2] 6.0[2]	8.0 7.1 5.2 5.0 5.2	Addition of bifidobacteria with starter culture Bifidobacteria were tested in mixed culture or individually Mixed culture was immobilized or not	[61]
Argentinian Fresco cheese, Soft cheese	4 °C, 60 days	*B. longum* (2 strains) *B. bifidum* (2 strains) *Bifidobacterium* sp (1 strain) *Lb. acidophilus* (2 strains) *Lb. casei* (2 strains)	6.4–7.6 7.7–8.7 7.0–7.6 6.0–8.7 7.0–8.4	5.9–7.3 6.9–8.6 6.6–7.6 5.6–8.6 7.0–8.7	Addition of probiotic culture with starter culture Probiotic cultures were mixed in different combinations	[139]

[1] Cell concentrations at the beginning and the end of the ripening period.

[2] For Crescenza cheese, starting concentrations correspond to log of cfu per mL of milk used for cheese-making.

flavors or volatile aroma, resulting in off-flavors [5, 128].

During ripening, lipolysis also plays an important role in the development of cheese characteristics. The addition of probiotic cultures does not seem to affect the free fatty acid profile of cheese, likely due to a higher lipolytic activity of starters and some NSLAB compared with probiotic cultures [28, 61, 63].

Most cheeses containing probiotic lactobacilli and bifidobacteria have high acetic acid content due to heterofermentation [28, 31, 61, 63, 89, 104, 105]. Bifidobacteria produce mainly acetic and lactic acid, in a molar ratio 2:3, from lactose fermentation via the fructose-6-phosphate shunt pathway. Some lactobacilli can also produce acetic acid, but to a lesser extent compared with bifidobacteria [36]. Acetic acid contributes to the typical flavor of different cheeses, but excessive concentrations can also result in off-flavors [53, 120].

Lactose maldigestion can be alleviated by β-galactosidase activities of bifidobacteria [67]. A complete lactose hydrolysis was observed in Crescenza, Canestrato Pugliese and Cheddar-like cheeses to which bifidobacteria were added, eventually leading to a small galactose accumulation [28, 31, 61].

As already reported by several authors [15, 118], cheese is a promising food matrix for probiotics. However, only a few probiotic cheeses have been successfully developed for the market compared with yoghurts or fermented milks. Furthermore, strain selection and possible process adjustments should be carefully evaluated to maximize probiotic cell viability during cheese manufacture and storage, as well as to limit possible changes in organoleptic properties. Finally, dairy propionibacteria which have been recently proposed as probiotics [107] could be further researched for applications as probiotic and ripening cultures, especially for Swiss-type cheeses.

4. ANTIBIOTIC RESISTANCE

Until recently, safety criteria for food cultures were mainly based on a long history of safe use [54, 121]. These criteria were often considered sufficient for use of any strains belonging to a particular “safe” genus or species in cheese technology. However, it is now increasingly accepted that strains of almost any bacterial species can acquire plasmids or transposons containing antibiotic (AB) resistance genes or virulence factors and therefore, safety considerations have to be placed on particular strains. Increasing concerns over the AB resistance situation in food-related bacteria are partly due to excessive use of antibiotics in both human and veterinary medicine and agriculture [1, 81, 109] and to the high transferability potential of acquired AB resistance genes [3, 110]. During the last decade, the hypothesis that non-pathogenic microorganisms in fermented food, e.g. LAB in cheese, can also function as reservoirs for transferable AB resistance determinants has been verified [122, 135]. As a consequence, horizontal gene transfer (HGT) of such determinants to other bacteria can occur in the cheese matrix and surface and once cheese is consumed, between cheese organisms and commensal microbiota in the gastrointestinal tract. HGT by conjugation between the cheese isolate *Lb. plantarum* harboring the plasmid pLFE1 with the erythromycin resistance gene *erm*(B) and *E. faecalis* was measured at high frequency in gnotobiotic rats even without selective pressure [46].

The presence of AB resistance genes in both isolated LAB from fermented foods, such as cheese, and LAB of human origin is the first evidence for such AB resistance gene transfer, as recently reviewed by Ammor et al. [3]. The best documented examples are tetracycline and erythromycin resistance genes found in enterococci in European cheeses [70, 135]. AB resistance genes were also characterized in

cheese LAB isolates belonging to the genera *Lactobacillus*, *Lactococcus* and *Leuconostoc* [3]. Furthermore, transferability of AB resistance genes derived from plasmids and transposons of cheese LAB isolates was demonstrated in vitro between different species of LAB by conjugation on agar plates [109].

A second indication of HGT is the increasing prevalence of AB-resistant non-*Enterococcus* LAB isolates from cheeses [3,27,50,69,109,136]. Phenotypic analysis of AB resistance among LAB was always limited by different methodological approaches used to determine AB susceptibility-resistance breakpoints of dairy strains and bifidobacteria. The use of the micro-dilution method for determining minimal inhibitory concentrations of antibiotics in non-clinical, non-*Enterococcus* LAB and bifidobacteria was highly recommended [38, 76]. Results for phenotypic resistance are then compared with the breakpoints established for the species [50]. Now available are new molecular screening tools based on microarray hybridization allowing rapid screening of AB resistance genes [111]. With a microarray targeting ninety different AB resistance determinants mainly from Gram-positive bacteria, Kastner et al. [73] showed that among thirty LAB starter strains used for cheese production, none contained acquired AB resistance genes, in contrast to several meat LAB starters and two probiotic cultures: *Lactobacillus reuteri* SD 2112 with the plasmid-encoded tetracycline resistance gene *tet*(W) and the lincosamide resistance gene *lnu*(A); and *Bifidobacterium animalis* subsp. *lactis* DSM 10140 with *tet*(W).

To conclude, AB resistance screening of industrial cultures and novel strains should be done systematically. Strain identification by phenotypic and genotypic methods and the absence of transferable AB resistance genes and virulence factors are important criteria which will be implemented in European food legislation [42, 43]. According to criteria set by the European Food Safety Authority [44], intrinsic AB resistance or AB resistance due to mutation of chromosomal genes represent acceptable risks of dissemination by HGT, but acquired AB resistances are risk factors for public health.

5. TECHNOLOGICAL CHALLENGES AND PROSPECTS FOR INNOVATIVE TECHNOLOGIES

The incorporation of functional cultures in cheese must have no deleterious effects on the cheese manufacturing process and product quality. This implies that metabolic activities (e.g. lactic acid, aroma and flavor productions) of cultures with added functionalities must be similar to those of the original starter culture. On the other hand, a functional culture should not be impaired to provide the desired effect. Technological challenges and possible solutions for application of protective and probiotic cultures in cheese are discussed below.

5.1. Challenges related to bacteriocinogenic cultures in cheese

As discussed previously, bacteriocinogenic strains can affect the growth of starter cultures, and milk and curd acidification. Conversely, good growth of the producer strains in the presence of the starter culture is also needed because bacteriocin production is directly related to cell concentration. To overcome this major limitation, bacterial strains for starter cultures must be carefully selected. Bouksaim et al. [14] highlighted the importance of the inoculum ratio of bacteriocinogenic strains and starter cultures for controlled acidification and production in Gouda cheese.

A very small change in the proportion of mixed culture inoculum from 0.4/1.4% to 0.6/1.4% between the nisinogenic strain (*Lc. diacetylactis* UL719) and Flora Danica starter led to a large increase in numbers of UL719 accompanied by changes in nisin Z activity in cheese, i.e. from non-detectable to 256 international units (corresponding to 6.4 μg pure nisin) per gram of cheese, although composition of cheeses was similar to control cheese made with 1.4% Flora Danica only. This study clearly showed the great challenge of controlling bacteriocinogenic strain activity in cheese. The use of bacteriocin-resistant strains has also been proposed by several authors to overcome the deleterious effects of bacteriocin on acidifying capacity of starter cultures [8,95]. However, the selection process can be difficult, particularly in the case of cheese with complex microflora. Furthermore, other technological parameters (e.g. bacteriophage resistance of starter and bacteriocin cultures) are also important for cheese applications.

Technological properties (e.g. ability to grow and acidify in milk) and/or safety status (e.g. bacteriocinogenic strains carrying AB resistance and/or belonging to genus or species which includes pathogenic strains) of bacteriocinogenic strains can also limit their applications in cheese. As a possible solution, the heterologous production of bacteriocin in GRAS strains with relevant technological properties was successfully attempted [16, 115].

Despite extensive research done on bacteriocins and bacteriocinogenic strains, few products are commercially available, reflecting the difficulties of implementation of such protective cultures in cheese manufacturing. Although heterologous production of bacteriocins has good potential to facilitate applications of bacteriocinogenic cultures in cheese, regulatory considerations and general consumer opposition to use of genetically modified organisms in food will likely block this development.

5.2. Innovative technologies for production of cheese cultures

Industrial processes for food culture production, including cheese cultures and probiotics, almost exclusively use conventional batch fermentation with suspended cells cultivated separately. Separately produced cultures are eventually mixed after production to formulate commercial preparations. For bacterial surface-ripened cheeses, cheeses after salting are either inoculated with commercial defined mixed cultures containing different strains of *Brevibacterium linens*, *Debaromyces hansenii* and/or *Geotrichum candidum*, or in some countries are washed with smears from older cheeses (“old-young” smearing method) [17].

However, both approaches have disadvantages, such as limited biodiversity for controlled inoculation and contamination risks by undesirable contaminants for the “old-young” smearing method. The development of technologies enabling a controlled propagation of complex microflora could be very useful for controlling contamination and developing cheese surface flora, leading to high product quality and safety.

Cell immobilization has been used to perform high cell density fermentations for both cell and metabolite productions with several advantages over free-cell fermentations, including: high cell densities, reuse of biocatalysts, improved resistance to contamination and bacteriophage attack, enhancement of plasmid stability, prevention from washing-out during continuous cultures, and the physical and chemical protection of cells [78, 79]. Recently, we developed and validated new continuous fermentation systems with immobilized human fecal microbiota to closely

model intestinal fermentation and stably cultivate complex microbiota [24,25]. Similar approaches could be used to stabilize and propagate complex cheese surface microflora, and eventually develop dynamic fermentor models to study cheese surface ecosystems.

Current data also suggest that cell immobilization combined with continuous culture might also be used to efficiently produce, in a one-step process, functional cultures and probiotic cells with enhanced tolerance to environmental stresses and improved technological and functional characteristics [78, 79]. Recent studies with immobilized lactobacilli and continuous mixed strain cultures have reported significant physiological changes, such as a large increase in cell tolerance to nisin and low pH, and enhanced acidification capacity of cheese cultures [65, 77].

6. CONCLUSION

Despite a great deal of basic and applied research carried out on identification, characterization and development of bacteriocinogenic strains in cheese over the past thirty years or even more, very few applications have reached the market. This situation is largely explained by the difficulty of applying bacteriocinogenic strains in the context of industrial cheese production where needs for robust and reproducible processes are high. Selection of competitive bacteriocinogenic strains in cheese environments and suitable starters with low sensitivity to the bacteriocin is a prerequisite for successful large-scale applications. Development of culture rotation to respond to bacteriophage attacks is also needed. Furthermore, strict process control is required to achieve proper growth of starter, secondary and adjunct cultures and production of uniform, high quality and safe cheese. If this can be achieved, in situ bacteriocin production could be used both to enhance microbiological safety of cheese and to accelerate cheese ripening. One of the most promising applications of protective cultures is on cheese surfaces where post-processing contamination is more likely to occur and no interference with starter cultures is expected. The selection and development of complex natural cheese surface microflora with protective properties against spoilage and pathogenic contaminants is especially attractive but will require development of suitable technologies for controlled propagation of complex cultures. Furthermore, the development of antifungal cultures with protective effects associated with minimal metabolism in cheese is rapidly evolving and similar concepts relying on synergistic mixtures of non-protein low-molecular-weight metabolites could be further developed to target bacterial contaminants.

Cheese is also a very suitable but underused carrier for the delivery of probotics, with specific advantages compared to fermented milks and yoghurts for maintaining high cell viability and with potential to enlarge the range of probiotic strains used in functional dairy products. It is therefore expected that new functional cheeses will be developed on the market, eventually harboring resident cultures with probiotic features not yet characterized and exploited.

Finally, safety of food cultures will become increasingly important with the emergence and dissemination of AB resistance genes. In the near future cheese and probiotic cultures, like any other food cultures, will have to fulfill strict testing to guarantee the absence of acquired AB resistance genes with transfer potential.

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