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The Microbiology of Cheese and Dairy Products is a Critical Step in Ensuring Health, Quality and Typicity

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Abstract

Cheese and dairy products require a rigorous observation of the procedures, strictly linked to the different indigenous components, such as the initial raw materials, the process chain, the ripening temperature, the water activity (a_w), the pH and the contamination of the environment and operators. Microorganisms are key agents in the transformation of milk and in the subsequent phases which confer typicity and stability to cheese and dairy products. Contamination by pathogenic microorganisms may occur, compromising the safety of the final products. Meanwhile, beneficial microorganisms present in cheese and dairy products can produce antimicrobial compounds, thus avoiding spoilage of the products, ensuring their safety for human consumption. This mini review reports a description of the microorganisms involved in the fermentation of milk and in the subsequent processes concerning cheeses and derivatives, highlighting the aspects that microorganisms play in terms of quality, typicality and safety. New aspects emerged from this study, suggesting possible insights and future research. These include cultural approaches on the one hand, which allow for the isolation and characterization of new microbial strains that confer peculiarities in terms of quality and typicality to cheeses and dairy products, and the isolation of lactic acid bacteria that produce bacteriocins as important tools for combating microbial pathogens. On the other hand, investigations on cheese and dairy products using metagenomic approaches with DNA extraction followed by amplification and sequencing of microbial genes, allow the description and monitoring of the entire microbiota involved in the transformation processes of cheese and dairy products. Therefore, the combination of cultural dairy microbiology and metagenomic approaches can lead to improving the characteristics of cheeses and dairy products, while maintaining respect for traditions.

Introduction

Dairy products include milk-based food, such as cheese, butter, yogurt, kefir, ice cream, and condensed and powdered milk. Among dairy products, cheese is the most varied and widespread. Dairy products present differences in terms of raw materials, process chain, ripening temperature, water activity (a_w), pH, environment and contamination by operators, and all these aspects interact closely with microbial communities of dairy products [1]. Various microorganisms from the environment, including bacteria, yeasts, molds, viruses and bacteriophages, can enter the dairy supply chain in several stages and can affect the entire dairy supply chain [1]. Microorganisms are the key agents in the critical stages of optimizing the overall quality and safety, flavor, appearance and typicality of cheese and dairy products. The term cheese describes dairy products with the definition of “a ripened or unripened, soft, semi-hard, hard, or extra-hard, dehydrated milk-derived product in which the whey protein/casein ratio does not exceed that of milk” [2]. Cheeses comprehend a great variety of forms, sizes, textures, aromas, and tastes. Production of cheese follows general procedures that include milk acidification; milk coagulation by adding different agents, to be chosen from animal rennet, microbial or vegetable coagulants; cutting the curdled milk originated by coagulation into small pieces; whey drainage; washing; heating to temperatures between 30 °C and 55 °C; pressing and adding salt at concentrations between 1% and 5% NaCl; shaping; aging with maturation and dehydration of the product (Figure 1). Treatments with natural compounds such as immersion in oil, wine or brine, or the addition of ash, flour, spices or vegetable dyes to the packaging of the final product, can also be included [3-5]. When milk is subjected to an acidic coagulation via fermentation of lactic acid bacteria colonizing milk or via addition of acid, it is obtained the production of soft cheeses as Cottage, Quark, Queso Blanco and Afuega'l Pitu (Figure 2).

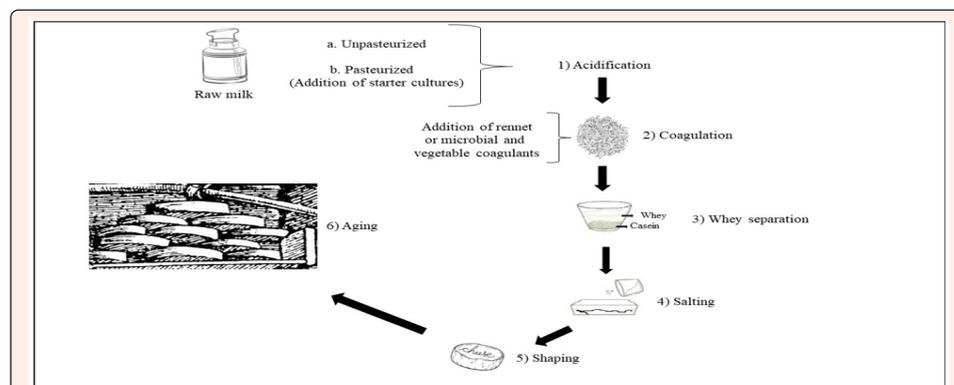
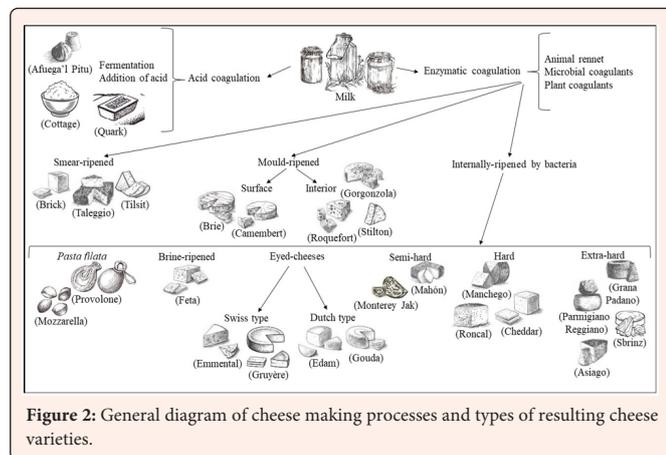


Figure 1: Procedures for cheese preparation comprehending six steps: 1) acidification, 2) coagulation, 3) whey separation, 4) salting, 5) shaping, and 6) aging.

In the case that milk achieves coagulation via enzymatic treatment, by adding animal rennet or microbial coagulants or plant coagulants to pasteurized or unpasteurized milk, different cheeses can be obtained via three distinct processes: smear-ripening, mould-ripening and internally-ripening by bacteria. Smear-ripened cheeses develop a viscous, red-orange smear on their surfaces during ripening. For this reason, they are also called red-smear cheeses or bacterial surface-ripened cheeses. Typical smear-ripened cheeses include Tilsit, Limburger, Romadour, Chaumes, Taleggio, Brick (Figure 2). Mould-ripened cheeses are divided in surface mould-ripened, soft and creamy with a white skin cheeses including Brie, Camembert and Carré de l'Est; or in interior mould-ripened, as in the case of Roquefort, a classic blue cheese made from ewe's milk, and other blue-veined cheeses as Stilton, Cabrales and Gorgonzola (Figure 2). Cheeses internally-ripened by bacteria comprehend many different kinds, evidencing diverse aging and compactness. *Pasta filata*, 'spun paste', cheeses undergoing a single processing with the spinning of the curd dipped in hot water or brine until a semi-flowing plastic consistency is obtained, as in the case of Mozzarella, Provolone, Scamorza and Caciocavallo, mainly from Southern Italy. Brine-ripened cheese refers to cheese that is matured in brine, giving the cheese good stability and inhibiting growth of undesired bacteria, even in warmer climates. Brine-ripened cheeses are mainly produced in countries bordering the Mediterranean Sea and in the Balkan countries and comprehend Beyaz peynir, Sirene, Domiati, Feta and Halloumi cheeses. Eyed-cheeses can be hard-cooked Swiss-type cheese such as Emmental and Gruyere, heating to around 52 °C to remove moisture and harden the curd or Dutch-type semi-cooked cheeses such as Gouda, Edam and Maasdam. Semi-hard cheeses comprehending Mahón from the island of Menorca (Spain), Monterey Jack, from California (USA), semi-hard cheese and Caerphilly, a crumbly white cheese originating from the town of Caerphilly, Wales (UK), with all three of these cheeses from cow's milk. Hard cheeses, with Manchego originating from milk of the sheeps in the La Mancha region of central Spain; Roncal, a Spanish cheese from the Roncal Valley, north Navarra in the Basque territory near the border of France; Cheddar, with its origin in Leicestershire county, in the East Midlands (UK); Ras cheese, the main traditional hard cheese in Egypt. Extra-hard cheeses include Grana Padano, originating in the Po river Valley in northern Italy; Parmigiano Reggiano with a defined geographical area comprehending the territories of the Provinces of Bologna to the left of the Reno River, Mantua to the right of the River Po, Modena, Parma and Reggio in the Emilia Region (Italy); Sbrinz, produced primarily in the Central Swiss cantons of Obwalden, Nidwalden, Lucerne and Zug (Switzerland); Asiago, produced around the alpine area of the Asiago plateau in the regions of Veneto and Trentino-Alto Adige (Italy) (Figure 2).



responsible of cheese deterioration, opportunistic and pathogenic organisms, which develop and change during production and maturation [7,8]. From the systematic point of view, cheese bacteria as lactic acid bacteria, enterococci and staphylococci belong to the phylum Firmicutes; bifidobacteria, propionibacteria and corynebacteria to the phylum Actinobacteria; enterobacteria to the phylum Proteobacteria [9,10]. Archaea have rarely been found in the cheese microbiota, with few members belonging to the genera *Thermocodium* and *Sulfurisphaera* of the phylum Crenarchaeota, and to the genus *Methanohalobium* of the phylum Euryarchaeota [11,12]. Within eukaryotes, yeasts are present in cheese with the genera *Geotrichum*, *Debaryomyces*, *Kluyveromyces*, *Candida*, *Yarrowia*, filamentous fungi in cheese include members of the species *Penicillium camemberti*, *P. roqueforti* and others *Penicillium* spp., which are abundant in mould-ripened cheese varieties [13-15]. The species of filamentous fungi *Fusarium domesticum*, *Scopulariopsis flava* and *Sc. casei* were also detected, even if in low quantities, on the surface of most cheeses [13,16,17]. Except *P. roqueforti*, all these mold species are highlighted only in the cheese, suggesting a possible domestication of fungi to this particular habitat. *P. camemberti* likely derives from the wild ancestor *Penicillium commune* through adaptation processes, as reduction of reproductivity, reduction of mycotoxins production, reduction of pigmentation and a shift from earthy to cheesy in the volatile compounds production [18]. Among bacteria, the phenomenon of adaptation via domestication was detected in *Lc. lactis* ssp. *lactis* and in *Lc. lactis* ssp. *cremoris* strains [19,20]. These, and the domesticated strains of other lactic acid bacteria species found only in milk and dairy products, seem to have emerged recently due to the selective pressure imposed by the dairy technologies. Over the centuries, strains of lactic acid bacteria, yeasts, and molds have evolved to their domesticated roles, leading to genome decay, loss of pathways, acquisition of genomic elements, and beneficial mutations that provide an advantage in their nutrient-rich food environments [21]. In Figure 3, some morphologies of microorganisms present in cheese have been reported.

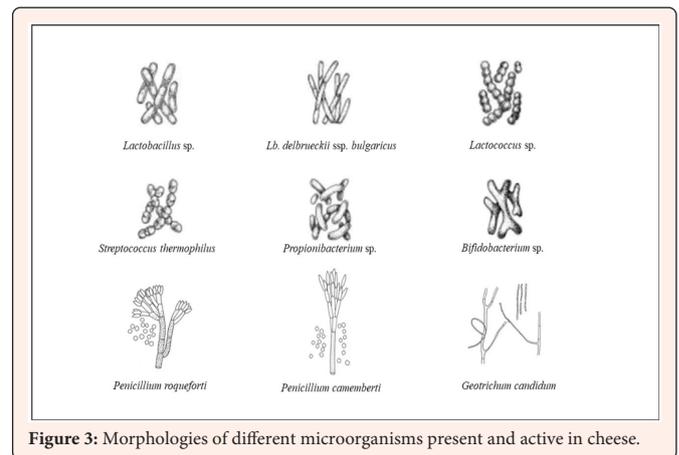


Figure 3: Morphologies of different microorganisms present and active in cheese.

Lactic Acid Bacteria

Lactic acid bacteria are the most important microorganisms in milk fermentation, converting lactose to lactic acid, which results in an increased acidity that makes growth conditions of microorganisms other than lactic acid bacteria increasingly unfavourable. Lactic acid bacteria are Gram-positive bacteria with a low Guanine+Cytosine (G+C) content in their DNA. They are acid tolerant bacteria, non-motile, non-spore forming and have rod- or cocci-shaped morphologies [22]. The most common lactic acid bacteria present in milk include the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Pediococcus* and *Weissella* [23,24]. Lactic acid bacteria need carbohydrates to produce the energy necessary for growth. In milk and dairy products, lactose is the main natural carbohydrate. Lactose is made up of glucose and galactose. The fermentation of lactose is called glycolysis or glycolytic pathway. Obligatory homo-fermentative lactic acid bacteria are able to ferment lactose into pyruvic acid, which is then reduced to lactic acid by the reducing power previously produced in the form of NADH. Thus, in obligatory homo-fermentative lactic acid bacteria, lactic acid is obtained as the sole product, with a chemical pathway with 1 mole of glucose giving 2 lactic acid moles and 2 ATP moles, via this process called homo-lactic fermentation [25]. Obligatory homo-fermentative lactic acid bacteria include strains of the species *Lactobacillus acidophilus*, *Lb. amylophilus*, *Lb. bulgaricus* and *Lb. helveticus* [25]. Homo-lactic fermentation should theoretically produce 2 moles of lactic acid per mole of glucose consumed with a theoretical yield of 1 g of product per g of substrate. In reality, a part of the carbon source is used by the bacteria themselves for the production of biomass and therefore the experimental yields are

Microorganisms in Cheese and Dairy Products Transformation

Microorganisms are responsible for the fermentation of milk and the numerous biochemical reactions that occur during production and maturation, which give rise to the characteristic textures and flavors of the cheese [5]. Microorganisms play essential roles in the transformation of milk, in the production and maturation of cheeses, making an important contribution to the development of organoleptic properties through their metabolism and various enzymatic activities. Microorganisms also contribute to the microbiological safety of cheese through the barrier effects of complex microflora and the production of numerous low molecular weight antimicrobial compounds [6]. From the microbiological point of view, cheese and dairy products, both fermented in a natural way and with the addition of starters and/or adjunct cultures, contain a complex mixture of microbial communities, including those relevant for carrying out the processes, or

generally lower [25]. Hetero-lactic fermentation is the process that is characterized by the formation of co-products such as CO₂, ethanol and/or acetic acid in addition to lactic acid as the final product during the fermentation-phosphoketolase pathway. The first step of glucose degradation, which is called pentose phosphate pathway, leads to glyceraldehyde 3-phosphate, acetyl-phosphate and CO₂. Glyceraldehyde 3-phosphate enters the glycolysis through which it is transformed into lactic acid, while acetyl-phosphate is converted into acetic acid and/or ethanol. In the hetero-fermentative pathway, glucose can give lactic acid and CO₂ and ethanol and ATP; or glucose can give lactic acid and CO₂ and acetic acid and 2 ATP and 2 NADH. Microorganisms that use only this metabolic pathway for the consumption of carbohydrates are called obligatory hetero-fermentative, among which are *Lactobacillus brevis*, *Lb. fermentum* and *Lb. reuteri* [26,27].

Lactic acid bacteria are characterized by their safe metabolic activity while growing in milk and dairy substrates, producing organic acids and other metabolites. They are commonly accepted as Generally Recognised as Safe (GRAS) for human consumption [28]. Lactic acid bacteria are associated with production of many beneficial compounds such as organic acids, polyols, exopolysaccharides and antimicrobial compounds due to their metabolic activities [27]. In milk, the main function of lactic acid bacteria is to produce lactic acid and to acidify milk. Moreover, lactic acid bacteria contribute to the flavour, texture and nutritional value of fermented milk, through production of aroma components, and used as adjunct cultures. Other actions of lactic acid bacteria include production or degradation of exopolysaccharides, lipids and proteins, production of nutritional components such as vitamins, and used as functional cultures, and promoting therapeutic effects and used as probiotics. In addition, they contribute to the inhibition of spoilage and pathogenic microorganisms and thus, used as bioprotective cultures [6,29]. Lactic bacteria are involved in the development of aroma in cheeses and dairy products by virtue of three metabolic pathways they possess: glycolysis for fermentation of sugars; lipolysis responsible of fats degradation; and proteolysis for degradation of proteins [30]. Lactate is the main product obtained in milk from the metabolism of the sugar lactose, and during this process, a fraction of the intermediate pyruvate can alternatively be converted to diacetyl, acetoin, acetaldehyde or acetic acid, important for typical yogurt flavours [31]. Degradation of proteins by the activities of rennet enzymes and the cell-envelope proteinase and peptidases yields small peptides and free amino acids, the latter of which can be further converted to various alcohols, aldehydes, acids, esters and sulphur compounds for specific flavour development in dairy products [32].

From a metabolic point of view, lactic bacteria are fastidious microorganisms and are unable to synthesize many amino acids, vitamins and nucleic acid bases. Lactic acid bacteria require 6 to 14 different amino acids, depending on which species a strain belongs to. Free amino acids in milk are limited and amino acids are present as protein components, so the growth of lactic bacteria requires hydrolysis of milk proteins [33]. The hydrolysis of peptides into free amino acids and the subsequent utilization of these amino acids is a central metabolic activity in lactic acid bacteria. Proteolysis by lactic bacteria is therefore the key process influencing the rate of flavor and texture development in most cheese varieties. The breakdown of milk proteins into peptides is catalyzed by proteolytic enzymes present in lactic acid bacteria and the peptides are then further hydrolyzed by exopeptidases and endopeptidases into small peptides and amino acids [34]. In milk, the breakdown of fat releases free fatty acids and glycerol, monoacylglycerides or diacylglycerides. Some free fatty acids are peculiar aromatic compounds in some cheeses, such as in the case of goat cheeses. Free fatty acids can react with free alcohols or sulphhydryl groups to form esters and thioesters, respectively, or act as precursors to numerous other beneficial compounds, such as lactones [35]. Esterase activity was found in various lactobacilli [36], and this is an important feature as the esters contribute to the characteristic flavor of the Swiss type [37] and White-brined cheese [38].

Starter and Non-Starter Microbial Cultures

Microorganisms can grow in milk left at room temperature with a prevalence of mesophilic lactococci [7]. These microorganisms can be maintained by inoculating fresh milk with part of the previous day's fermented product, and this was at the basis for the production of fermented dairy products for centuries, even before it was known that the bacteria were actually involved. The isolation of lactic acid bacteria from the milk environment began in the second half of the nineteenth century, with the development of starter cultures for the production of fermented dairy products [39]. Starter cultures may contain mixed strain cultures with unknown strains or can be composed of pure and well characterized strain cultures from which isolated bacterial strains can be

obtained and maintained at -80 °C or lyophilized for successive utilization as starter cultures [40]. Starter cultures drive the production of acid during the beginning of the fermenting process, nevertheless, these same bacteria may be involved in cheese ripening by producing enzymes involved in proteolysis and the conversion of amino acids into aromatic compounds [41]. Starter bacteria are able to acidify milk producing sufficient acid and reducing the pH to values <5.3 in about six hours and at temperatures in a range from 30 to 37 °C. Depending on the cheese to be prepared, mesophilic or thermophilic starter cultures can be used. The production of Cheddar, Gouda, Edam, Blue and Camembert, requests the addition of mesophilic cultures, whereas cooked hard cheeses such as Emmental, Gruyere, Parmigiano Reggiano and Grana Padano require the milk to be heated to around 50-55 °C and therefore demand the addition of thermophilic starter cultures. The most frequent used starter bacteria originated from one or more of the species *Lactococcus lactis*, *Streptococcus thermophilus*, *Lactobacillus helveticus* and *Lb. delbrueckii* and the choice of the starter culture depends on the type of cheese to be produced. As an example, the mixed-strain starters for the manufacture for Gouda cheese are composed of acid-forming lactococci, *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*, together with citrate-utilizing strains, *L. lactis* subsp. *lactis* biovar *diacetylactis* and *Leuconostoc* spp. [7]. Industrial starters are mainly based on a single species, namely *Lactococcus lactis* for most cheeses [42,43]. Another genus involved in starter cultures is represented by the genus *Bifidobacterium* [44]. Bifidobacteria are Gram-positive bacteria that show a high G+C content, over 50%, comprehending fermentative microorganisms producing several acids including lactate [22].

Non-starter bacteria cultures are secondary addition to the cheese matrix and influence the aging phase, with interactions with starter bacterial cultures still present. The whole microbiota, composed by starter and non-starter microorganisms, develops the flavor and texture of cheese. The whole microbial population of the secondary addition differs from the starter culture, being a combination of prokaryotic and eukaryotic microorganisms represented by bacteria, yeasts and molds. The choice of the secondary addition depends on the type of cheese to be produced [45]. Non-starter lactic acid bacteria are composed by mesophilic lactobacilli and pediococci. They represent important components of the microbial communities of most cheeses during ripening and are characterized by a scarce adaptability to grow in milk and to contribute to acidification processes during cheese formation [46]. Considering their metabolism, cheeses host the facultatively heterofermentative group of non-starter lactic acid bacteria lactobacilli, also defined facultatively heterofermentative lactobacilli, with the most diffused *Lactobacillus casei*, *Lb. paracasei*, *Lb. plantarum*, *Lb. rhamnosus* and *Lb. curvatus*. Concerning the genus *Pediococcus*, *Pe. acidilactici* and *Pe. pentosaceus* are the most common species which can be found in cheese [45,47]. Nonstarter lactic acid bacteria include strains essential for producing the characteristic flavours of traditional cheeses. Thermophilic lactic acid bacteria, with an optimal growth between 30 °C and 45 °C are best known for their role in yoghurt-type products and as ripening agents in Swiss-type and Italian cheeses [7]. The numbers of non-starter lactic acid bacteria are reported to be higher in Cheddar cheeses made from raw milk than in those from pasteurised milk [7]. Intense flavor are present in raw milk cheeses, suggesting that the indigenous non-starter lactic acid bacteria play an important role in flavour development. Moreover, they have been shown to contribute to the formation of small peptides and amino acids, which are the precursors for the flavour components. During ripening Cheddar cheese, and for most cheeses, up to 20 strains form the non-starter lactic acid bacteria population [45]. Complex dynamics are present among non-starter lactic acid bacteria, with one non-starter lactic acid bacteria strain that can affect the development of another. The outgrowth of a *Lb. rhamnosus* strain, added on purpose as an adjunct in a Cheddar cheese trial, could be retarded by a simultaneously added *Lb. casei* strain. The development of a secondary flora depends also on the properties and composition of the starter used. The presence of a *Leuconostoc* species in the starter appears to affect the development of adventitious non-starter lactic acid bacteria [7]. Adjunct cultures are microorganisms added to cheese for purposes other than acid formation. Selected adjunct non-starter lactic acid bacteria cultures can accelerate ripening, producing desirable flavour. Adjunct cultures may eliminate defects inhibiting growth adventitious non-starter dangerous lactic acid bacteria. In Cheddar cheese, cheese flavour was improved by adding strains isolated from raw milk cheese and able to increase formation of amino acids. Cheese made from milk inoculated with strains of *Lb. plantarum* or *Lb. casei* subsp. *pseudoplantarum* received the best gradings [48]. Table 1 [49-54] refers to starter and non-starter microbial cultures involved in cheese and other dairy products fermentation and successive ripening and flavor development.



Table 1: Starter and non-starter cultures of microorganisms that play a role in dairy products.

| Microorganisms | Dairy Products | Functions/Features | Time of starter Addition | References |
|--|--|---|--------------------------|------------|
| Lactic Acid Bacteria | | | | |
| <i>Lactobacillus acidophilus</i> | fermented milk, acidophilus milk | acidification and flavor development | primary addition | [49] |
| <i>Lactobacillus brevis</i> | kefir | acidification and flavor development | primary addition | [50] |
| <i>Lactobacillus casei</i> | artisanal cheese, fermented milk | flavor development | secondary addition | [51,52] |
| <i>Lactobacillus casei</i> ssp. <i>casei</i> | probiotic fermented milk | acidification and flavor development | primary addition | [49] |
| <i>Lactobacillus delbrueckii</i> ssp. <i>bulgaricus</i> | yoghurt, fermented milk | acidification and flavor development | primary addition | [49] |
| <i>Lactobacillus delbrueckii</i> ssp. <i>lactis</i> | Italian and Swiss cheese types | acidification and flavor development | primary addition | [51,52] |
| <i>Lactobacillus helveticus</i> | semi-hard, hard cheese | flavor development and health benefits | secondary addition | [49] |
| <i>Lactobacillus johnsonii</i> | fermented milk | acidification and flavor development | primary addition | [49] |
| <i>Lactobacillus kefir</i> | kefir | acidification and flavor development | primary addition | [50] |
| <i>Lactobacillus kefirifaciens</i> | kefir | acidification and flavor development | primary addition | [50] |
| <i>Lactobacillus paracasei</i> | artisanal cheese | flavor development | secondary addition | [51,52] |
| <i>Lactobacillus paracasei</i> spp. <i>paracasei</i> | kefir | acidification and flavor development | primary addition | [50] |
| <i>Lactobacillus plantarum</i> | artisanal cheese, kefir | flavor development | secondary addition | [51,52] |
| <i>Lactobacillus rhamnosus</i> | fermented milk | acidification and flavor development | primary addition | [49] |
| <i>Lactococcus lactis</i> ssp. <i>cremoris</i> | most cheeses | acidification and flavor development | primary addition | [51,52] |
| <i>Lactococcus lactis</i> ssp. <i>lactis</i> | most cheeses, butter, buttermilk | acidification and flavor development | primary addition | [51,52] |
| <i>Lactococcus lactis</i> ssp. <i>lactis</i> var. <i>diacetylactis</i> | cheese, butter, buttermilk | diacetyl production | primary addition | [49] |
| <i>Leuconostoc lactis</i> | soft and semi-hard cheese | flavor development and CO ₂ production | secondary addition | [51,52] |
| <i>Leuconostoc mesenteroides</i> ssp. <i>cremoris</i> | soft and semi-hard cheese, butter, buttermilk | flavor development and CO ₂ production | secondary addition | [49] |
| <i>Streptococcus thermophilus</i> | Italian and Swiss cheese types, yoghurt, fermented milk | acidification and flavor development | primary addition | [51,52] |
| Bifidobacteria | | | | |
| <i>Bifidobacterium bifidum</i> | yoghurt, fermented beverages, cottage cheeses, ice cream, desserts, cheese | probiotic activity | primary addition | [53,54] |
| <i>Bifidobacterium longum</i> | yoghurt, fermented beverages, cottage cheeses, ice cream, desserts, cheese | probiotic activity | primary addition | [53,54] |
| Propionibacteria | | | | |
| <i>Propionibacterium freudenreichii</i> | Swiss-type cheese | hole formation, flavor development | secondary ripening | [51,52] |
| Other Bacteria | | | | |
| <i>Brevibacterium linens</i> | smear-ripened cheese | color, flavor development | secondary ripening | [51,52] |
| <i>Corynebacterium casei</i> | smear-ripened cheese | flavor development | secondary ripening | [51,52] |
| Fungi | | | | |
| <i>Debaryomyces hansenii</i> | smear-ripened | aspect, texture, and flavor development | secondary ripening | [51,52] |
| <i>Geotrichum candidum</i> | smear-ripened | aspect, texture, and flavor development | secondary ripening | [51,52] |
| <i>Penicillium camemberti</i> | white moldy cheese | aspect, texture, and flavor development | secondary ripening | [51,52] |
| <i>Penicillium roqueforti</i> | blue-veined cheese | aspect, texture, and flavor development | secondary ripening | [51,52] |

Quality Features and Flavor Development in Cheese

Sensorial properties confer unique characteristics to cheese and depend on the type of milk used, the type of feed supplied to the animal that providing the milk, the practices adopted in the processing of the milk, the ripening environment, the type and duration of ripening, the quantity of microorganisms involved and the role they play in the forming product [4]. The active agents at the base of cheese flavour development are enzymatic reactions of microbial origin that create a balance between different components [55]. The characteristics of the flavour profile of ripened cheeses are mainly affected by proteolysis of caseins and in some types also by lipolysis. The typical cheese flavour results from further degradation of amino acids, due to the pathways for conversion of amino acids by starter bacteria [56]. That is true in cheeses produced from pasteurised milk and using starter cultures under aseptic conditions. However, cheese ripening is influenced by different factors, including the microflora of the raw milk, coagulant, starter cultures and by adventitious contamination of the cheese by non-starter bacteria [42]. Based on sensory evaluation and chemical analysis of cheeses, various groups of volatile compounds have been identified as being responsible for the final taste and aroma of cheese. These compounds comprise fatty acids, esters, aldehydes, alcohols, ketones, sulphur compounds and various other components [57-60]. The starter lactic acid bacteria, *Lactococcus* and *Streptococcus*, were the dominant microorganisms during the fermentation period, but as the cheese entered the aging period, the relative abundance shifted with detection of non-starter lactic acid bacteria, which were unidentified members of the family *Lactobacillaceae* [61]. Starter lactic acid bacteria communities appeared to be significantly high (10^8 CFU g^{-1}) in the initial state of aging, but the population decreased by two or more logs with further aging (Settanni and Moschetti 2010). On the other hand, non-starter lactic acid bacteria exhibited continuous growth in the population with further aging [62]. During cheese manufacturing, protein degradation occurs during the aging period and contributes to the flavor, texture, and appearance of the final product [63]. The enzymes of non-starter lactic bacteria contribute to the proteolysis process, represented by the hydrolysis of proteins to small peptides and free amino acids, which are the precursors of flavor-forming reactions [63]. In artisanal cheeses, during the aging period, the non-starter lactic acid bacteria *Leuconostoc mesenteroides*, *Lactobacillus helveticus*, *Lactobacillus zaeae*, and *Enterococcus* spp. were detected in low numbers. These non-starter lactic acid bacteria contributed to the fermentation of residual lactose or other sugars and were involved in citrate, peptide, and amino acid production, including formation of aromatic compounds, as well as contributing to the aging process [64]. *Lactobacillus plantarum* was detected during the cheese aging phase and influenced the sensory improvement characteristics [65]. Non-starter lactic acid bacteria mostly originate from raw milk [66]. The non-starter lactic acid bacteria also have been isolated from various locations within cheese production facilities including floors, drains, and equipment surfaces [67]. Cross contamination can be another source of non-starter lactic acid bacteria because some non-starter lactic acid bacteria found in their cheese samples were found in starter lactic acid bacteria of the other types of cheese that were not part of the study but had been produced in the same facility [45,61].

The yeast *Geotrichum candidum*, formerly *Oidium lactis*, represents a key microorganism in the catabolism of triglycerides and casein and also in cheese organoleptic properties and appearance development. *G. candidum* relates to characteristics associated with carbohydrate, lipid, and amino acid metabolism [68]. First classified as mould, *G. candidum* has been classified as yeast by Barnett and coll. [69]. The yeast *G. candidum* is an important component of the microflora of soft cheeses such as Camembert and semi-fresh goat's and ewe's milk cheese. *G. candidum* starts to grow on the surface of the rind of cheese at the beginning of the cheese ripening process, where it contributes to the development of typical cheese flavours. Many enzymes contribute to the action of *G. candidum* in cheese ripening, with peptidases playing a major role in the breakdown of bitterness and the production of flavour compounds [68]. *G. candidum* is present in raw milk cheeses and it has been detected in cheese regardless of the type of milk used, that can be indifferently represented by milk from cows, ewes and goats. The most likely source of *G. candidum* in pasteurised milk cheeses is the environment of the cheese factory, comprehending air, floors and/or walls, equipment, and workers. Commercial strains of *G. candidum* are available for use as starter cultures for cheese ripening and may be added to the milk, to the brine, or sprayed on the cheese surface [68]. *G. candidum*, as in the French cheese St. Marcellin, can grow forming a white and uniform surface on the surface at temperatures ranging from 5 to 38 °C and at pH 5.0-5.5. *G. candidum* lipases are characterized by a high specificity in the use of unsaturated fatty acids as substrates. Concerning proteinases and peptidases of *G. candidum*, intracellular and extracellular proteolytic activities were highlighted. *G. candidum* contributes to an aroma of mild cheeses such as Brie and traditional Camembert. In addition, *G. candidum* can contribute to a decrease in diacetyl production, the main flavour compound in cheese, by means of its diacetyl-reductase activity. *G. candidum* reduces bitterness through the activity of its aminopeptidases by hydrolysing low molecular weight hydrophobic peptides originating

from the degradation of casein by *Penicillium camemberti*, conferring to the cheese organoleptic properties and appearance that are characteristic of traditional Camembert. *G. candidum* plays an important role in the competition with undesirable microorganisms in mould fermented cheese [68]. The yeast *Debaromyces hansenii* is involved in the metabolism of other amino acids [70]. Yeast *D. hansenii* metabolism evidences a role in the deamination of amino acids present in cheese, to the corresponding keto acids and ammonia (NH₃), with a consequent increase in pH values [47,71]. The different strains of the entire microbial community of cheese are capable of producing enzymes responsible for the degradation of milk. Furthermore, these enzymes may also be responsible for the production of the aromatic compounds in cheese, thus improving its quality and variety of flavor. The microorganisms of cheese therefore represent crucial elements in the development of aromatic compounds that originate during the ripening and maturing phases [72,73].

Pathogenic Microorganisms in Cheese and Dairy Products

Pathogenic bacteria including *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella* spp., *Staphylococcus aureus*, *Campylobacter* spp. can contaminate raw milk, persist in this environment and be transmitted through cheese and dairy products, threatening human health [74]. Other bacterial pathogens such as *Brucella* sp. [75] and *Clostridium botulinum* [76] were detected in dairy products, highlighting the sensitivity of these food products to possible contamination by dangerous microorganisms. In Table 2, examples of pathogenic bacteria contaminating dairy products have been reported [77-83].

Table 2: Examples of outbreaks caused by bacterial pathogens in fermented dairy products. VTEC: Vero-cytotoxin producing *E. coli*.

| Pathogens | Fermented Dairy Products | Outbreaks/ Causes | References |
|--|------------------------------|---|------------|
| <i>Brucella</i> sp. | Pecorino cheese | Brucellosis, one of the most important zoonoses in the Mediterranean and Middle East regions / Originated from raw milk and insufficiently ageing | [75] |
| <i>Clostridium botulinum</i> | Yoghurt | Botulism/Insufficient process of conserve used as a flavour | [76] |
| <i>Escherichia coli</i> O157:H7 | Gouda cheese | Infection is a major public health concern in North America, Europe, and other areas of the world/Raw milk used to make cheese; handling problems including insufficient ageing | [77] |
| <i>Escherichia coli</i> (VTEC) O157:H7 | Yoghurt | Possible improperly cleaned pump | [78] |
| <i>Listeria monocytogenes</i> | Hard cheese | Listeriosis/Postmanufacture contamination | [79] |
| <i>Listeria monocytogenes</i> | Queso fresco | Listeriosis | [80] |
| <i>Salmonella</i> Enteritidis | Pecorino cheese "primo sale" | Salmonellosis | [81] |
| <i>Salmonella</i> sp. | Hard cheese | Salmonellosis/Cheese made from raw milk | [82] |
| <i>Staphylococcus aureus</i> | Sheep milk cheese | Foodborne outbreaks caused by staphylococcal toxins/Raw milk used in production | [83] |

L. monocytogenes can be life-threatening, with outbreaks associated with high mortality rates, typically 20-30% [84]. *L. monocytogenes* is an opportunistic pathogen that has the ability to survive in wide variety of environments including low temperature (4 °C), high salt, and low pH [85]. Based on the resistance to stress conditions, the *L. monocytogenes* strain could survive in the cheese and is a significant safety concern. The DNA high-throughput sequencing techniques are extremely important to ensure safety of cheese, with the important results that can be achieved rapidly. An important outbreak by *L. monocytogenes* was registered in southern California in 1985, due to the ingestion of Mexican-style cheese, with pregnant women dying, some along with their unborn babies, and mothers losing their newborn infants [86,87]. An important listeriosis outbreak associated with Hispanic-style cheese occurred in 2013 [88]. Application of whole-genome sequencing revealed that cheese was the vehicle of infection and that a bacterial strain isolated from the surrounding environment, showed a high similarity with the outbreak isolate [45,88]. Between October 2002 and February 2003, two outbreaks caused by the strain *Escherichia coli* O157:H7 and associated with unpasteurized Gouda cheese from a dairy farm in Edmonton, Canada, infected children causing the hemolytic uremic syndrome. Further investigation revealed that the cheese was still contaminated with *E. coli* O157:H7 104 days after production [89]. In spring 2020, an outbreak of *Salmonella* Enteritidis occurred in the Marche region (Central Italy), due to the pecorino cheese "primo sale", produced with raw sheep milk. Microbiological investigation detected *Salmonella* Enteritidis in animal faeces and in environmental samples, as the raw-milk bulk tanks and in milk taken from single animals [81].

In 2014, an outbreak of *Staphylococcus aureus* occurred at a boarding school in Switzerland by consuming a soft cheese, Tomme, produced from raw cow milk. The outbreak caused symptoms of abdominal pain, violent vomiting, severe diarrhea, and fever [90]. *Campylobacter jejuni* is capable of contaminating raw milk and a member of this species has difficulties during isolation due to demanding growth requirements [91]. Outbreaks due to *Campylobacter* spp. can be detected in the consumption of raw milk (EFSA, 2018). In Germany, in 2018, a high percentage of all reported food-associated outbreaks originated from bacterial strains *Campylobacter* spp. and most of them resulted from consumption of raw milk. Strains *Campylobacter* spp. can survive unfavorable environmental conditions by shifting in the viable but non-cultivable state (VBNC), remaining viable and maintain their infectious potential [92]. Real-time PCR (qPCR) offered a highly sensitive culture-independent quantification method, allowing the determination of the highest possible residual risk present, while the minimal risk is indicated via plate count cultures, by colony-forming unit counts [93].

Defenses against Pathogenic Microorganisms

Aging for 60 days is believed to improve the antimicrobial properties of lactic acid bacteria. Lower levels of pathogenic bacteria, including *L. monocytogenes*, *E. coli* O157:H7, *Salmonella* spp. and *Campylobacter* spp. were found in cheese samples tested, supporting the hypothesis that 60-day maturation of raw milk cheese can improve microbiologically safe cheeses [94]. The Hazard Analysis Critical Control Point (HACCP) plan in the dairy sector have significantly contributed to the reduction of the incidence of foodborne illnesses linked to the consumption of cheese [95]. However, dairy products of unsatisfactory or borderline quality, according to European Community (EC) recommendations for the presence of pathogens *Salmonella* spp., *S. aureus*, *E. coli* and *L. monocytogenes*, can still be detected. Eukaryotic microorganisms such as yeasts and molds can be common contaminants of cheeses and dairy products. In particular, these microorganisms can pose a serious health hazard due to their ability to produce mycotoxins [6]. Control of microbiological safety of cheese can be achieved by the action of microorganisms with antimicrobial capabilities and protective effects in cheese [6]. Milk and dairy products colonization by natural microbial communities also helps to inhibit growth of pathogenic bacteria. Lactic acid bacteria are the most significant microorganisms of raw milk cheese and can inhibit the growth of pathogens [96]. Variation of the microbial composition of organic acid-producing bacteria in raw and pasteurized milk influence the survival of *L. monocytogenes*. In pasteurized milk, bacteria producing organic acid are more common and growth of *L. monocytogenes* is slower [97]. The contribution of lactic acid bacteria on inhibiting the growth of foodborne pathogens was highlighted in presence of *E. coli* that evidenced a decrease to less than 1% after the addition of commercial starter cultures [45,61]. Bacteriocins are peptides with antimicrobial activity produced by a wide diversity of bacteria. Those produced by Gram-positive bacteria are classified into class I, containing heavily modified (lanthionine-containing) peptides called lantibiotics, and class II, containing non-modified peptides or peptides with minor modifications, the latter consisting in disulfide bond formation or circularization [98]. Bacteriocins can be rapidly degraded by proteases in the gastrointestinal tract and thus they have no effects to human gut microbiota [99].

Nisin is a class I bacteriocin produced by lactococci and represents the best

characterized bacteriocin from lactic acid bacteria. Nisin, listed as E234, can be added as a food additive and is the only bacteriocin with a regulatory approval from the US Food and Drug Administration and European Union. Nisin presents a broad activity spectrum against different pathogenic bacteria, including *Listeria* sp., enterococci, staphylococci, streptococci, *Clostridium* sp., *C. jejuni*, *Helicobacter pylori* and antibiotic-resistant strains of *Neisseria gonorrhoeae* [100]. Stability of nisin depends on pH and can be subjected to degradation by proteolytic enzymes in cheese, as was observed in Cheddar cheese after 6 months of ripening, with a significant depletion of antimicrobial activity. Lactacin 3147 is a bacteriocin with two-component broad-spectrum antimicrobial peptide produced by *Lactococcus lactis* subsp. *lactis* DPC3147 evidencing a high stability over a wide range of pH. No decrease in lactacin antimicrobial activity was detected in Cheddar cheese over 6 months of ripening. Nevertheless, lactacin 3147 was not effective for controlling *L. monocytogenes* contamination on surfaces of smear-ripened cheese [101]. Pediocin AcH represents a broad-spectrum anti-listerial bacteriocin that belongs to class II, produced by *Pediococcus acidilactici* present in many cheeses, although with a low efficiency at pH higher than 5.0 [102]. Thermophilin, produced by some strains of *Streptococcus thermophilus*, is a class II bacteriocin with inhibitory activity against strains belonging to species of the genera *Streptococcus*, *Enterococcus*, *Lactococcus*, *Bacillus* and *Listeria* [103]. Macedocin is a bacteriocin produced by *Streptococcus macedonicus* ACA-DC 198 isolated from Kasserli, a Greek hard-cooked cheese, and belongs to the lantibiotic bacteriocins (Class I). Macedocin inhibits growth of bacterial strains of lactic acid bacteria and of the species *Clostridium tyrobutyricum*. *C. tyrobutyricum* strains can produce butyric and acetic acid, CO₂ and H₂ from fermentation of lactate, causing large defects in semi-hard and hard cheeses, such as Swiss-type or Gouda, damaging flavor and inducing late blowing of the cheese [104]. Enterocin 4 is produced by *Enterococcus faecalis* strain INIA 4 during the manufacture and ripening of the Spanish Manchego cheese and is able to inhibit growth of *L. monocytogenes* strains. This bacteriocin belongs to the heat-stable, non-lantibiotic class II [105]. The presence of bacteriocinogenic strains as "protective cultures" in cheese and dairy products can represent an interesting approach to improve safety of cheese and dairy products and to protect human health. The absence of acquired antibiotic resistance genes with transfer potential in these cultures must also be respected [6].

Probiotics

Probiotics are live microorganisms which confer a health benefit on the host when administered in adequate amounts. The most diffused probiotics are mainly microorganisms from species of the genera *Lactobacillus* and *Bifidobacterium*. Probiotics are defined by the FAO/WHO [106] 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 [76]. *Bifidobacteria* and *Lactobacillus acidophilus* are most used in functional dairy foods containing probiotics, especially milk, yoghurt, ice cream and desserts [107]. The viability of probiotics is a key parameter for their effectiveness, with the scientific acceptance that the minimum intake to provide a therapeutic effect requires at least 10⁸-10⁹ CFU g⁻¹ viable cells. Most cheeses seem to be suitable carriers for probiotic bacteria. It is worth mentioning that *Lactobacillus* sp. probiotics can inhibit the adhesion of *L. monocytogenes* to the human epithelium, thus avoiding subsequent infections [108].

Conclusion

In this mini-review the microorganisms involved in the development of cheeses and dairy products have been described, focusing on the key role that microorganisms play in the production of safe products and in maintaining their typicality. The most important microbial groups have been reported, from lactic acid bacteria involved in the fermentation and ripening of milk, to yeasts and molds active in imparting flavor during cheese ripening. A description of the pathogenic bacteria has also been reported, providing information on possible probiotics that produce antimicrobial compounds. Insights for future research in the contexts of dairy microbiology may include increased product diversification and in this context, the isolation of new microbial strains from the raw milk environment and the surrounding environment, followed by identification and characterization of isolates, could provide important bases for innovations and to improve the typicality of cheeses and dairy products. Other innovations have emerged, consisting of isolating lactic acid bacteria that produce new bacteriocins to be characterized and used against pathogens, possibly by bio-augmentation processes of lactic bacteria. Furthermore, metagenomic approaches represent recent methods which include DNA extraction from cheese and dairy products; amplification of microbial genes from extracted DNA and subsequent sequencing; followed by the comparison of the sequences and the elaboration of the whole picture of the microbiota. This powerful tool will allow to monitor microbial pathways during milk transformation processes



and to record possible contaminating microorganisms, including pathogens. Both the classic microbiological approach based on the culture of microorganisms and the latest metagenomic-based methodologies should be combined during the microbiological study of cheeses and dairy products, in order to acquire biotechnological opportunities and obtain a comprehensive view of microbiological characteristics. The combination of cultural and metagenomic approaches can offer important opportunities to improve the characteristics of cheeses and dairy products, while respecting traditions.

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