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Invited review: Fresh pasta filata cheeses: Composition, role, and evolution of the microbiota in their quality and safety

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ABSTRACT

With a global market of around $55 billion (in US dollars; i.e. around the 57% of the global cheese market), pasta filata cheeses sales are rising approximately 2% per year worldwide and are expected to further increase to $65.01 billion by 2028. Among these groups of cheeses, fresh pasta filata cheeses, such as mozzarella and fior di latte, are the most consumed. Herein, we provide an overview of fresh pasta filata cheeses, their commodity-related and technological aspects, focusing on the composition, the role, and evolution of their microbiota along the dairy chain.
Key words: raw milk, fior di latte cheese, mozzarella, natural whey and milk cultures, microbiome

INTRODUCTION

The global pasta filata cheese market was valued at $55,492.8 billion (in US dollars) in 2018, and is expected to grow at a rate of 2.00% in the forecast period of 2021 to 2028, reaching $65.01 billion by 2028 (i.e., around the 57% of global cheese market; https://www.databridgemarketresearch.com/reports/global-pasta-filata-cheese-market).

Pasta filata cheeses, also known as spun paste or stretched-curd cheeses, include fresh products, among which the most important are mozzarella (made with water buffalo, goat or sheep milk) and fior di latte (made with cow milk) cheeses, and hard and semi-hard ripened products such as caciocavallo, Kashkaval, and provolone cheeses. However, a wide range of types, and even products of the same type but with different overall quality, arises depending on several parameters, including (1) the origin of the milk used (e.g., cow, water buffalo, and mixed); (2) the technology of production [Figure 1; raw, pasteurized, heat-treated, or biological milk, which, in turn, can produce different products depending on the composition of the autochthonous microbiota (natural fermentation), or the commercial starter, autochthonous starter, natural milk culture, or natural whey culture used; direct or mixed acidification; manual or mechanical stretching, and relevant time/temperature ratio used; manual or mechanical molding; and salting in paste or by brining]; (3) the type of intended use (direct consumption or as an ingredient in kitchens, pizzerias, and restaurants); (4) the nature of the packaging; and (5) the commercial duration envisaged by the manufacturer. Herein, we will provide an overview of the fresh pasta filata cheeses, their commodity-related and technological aspects, focusing on the composition, the role, and evolution of their microbiota along the dairy chain.

HISTORICAL AND COMMODITY-RELATED ASPECTS

Pasta filata cheeses originated in the Northern Mediterranean areas, encompassing Italy, Greece, Balkans, Turkey, and Eastern Europe. The production of soft, unripened pasta filata cheeses in Southern Italy has been documented since ancient times by the texts of Latin authors, such as Plinio and Columella (Addeo et al., 1996). The latter, in his *De re rustica*, wrote that the cheese, pressed by hand, congeals inside the tub while it is warm; then it is cut, and, throwing boiling water or throwing into it with hands, it is squeezed in the form of a lump. This description synthetizes the technology of production of pasta filata cheeses, whose typical common step of production is the submersion of the curd in hot water and its manually or mechanically stretching, followed by the molding.

Fior di latte has been a typical cheese of Southern Italy since ancient times; nevertheless, it is difficult to accurately date the nominal distinction between mozzarella produced with buffalo milk (or even goat or sheep milk), and fior di latte produced with cow milk (Addeo et al., 1996). In previous research on the historical origin of mozzarella, a document from the Episcopal Archives of Capua from the 12th century is cited, in which the "mozza or provatura" is mentioned, but the

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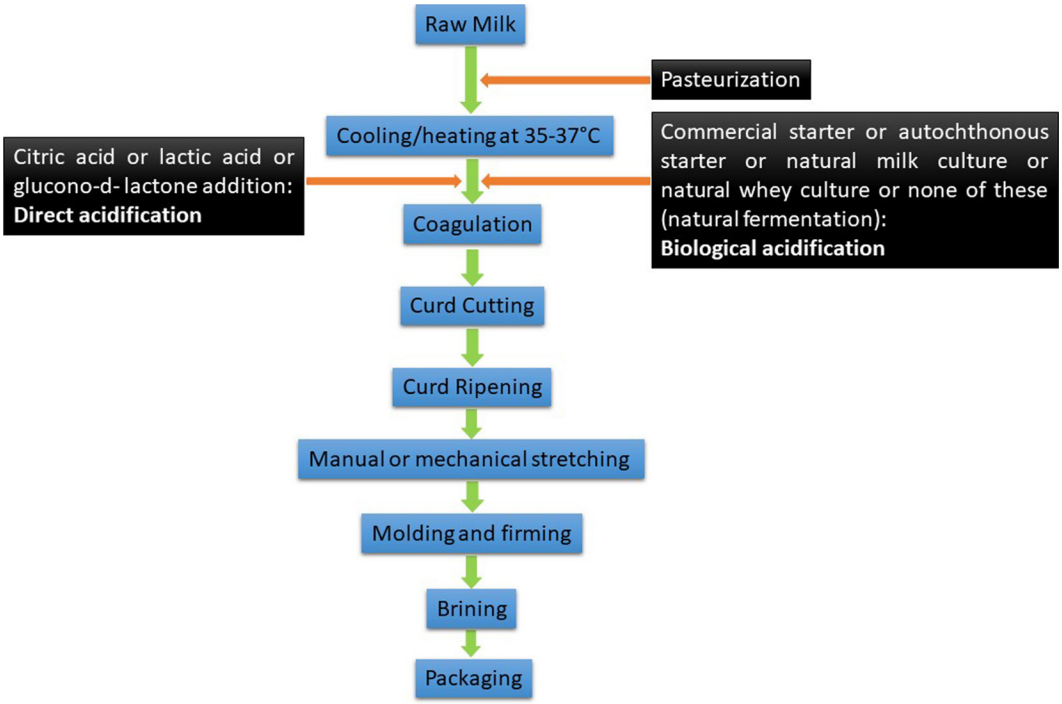

Figure 1. Flowchart of the cheesemaking process of fresh pasta filata cheeses.

type of milk with which the cheese was obtained is not specified (Addeo et al., 1996). The first certain information on the origin of the milk used for the production of stretched-curd cheeses dates back to the early 1900s. Romani (1911) made an accurate description of the manufacture of cow milk mozzarella produced in Basilicata; additionally, Besana (1916) states that mozzarella is mainly produced in Basilicata, Puglia, and Naples, and that it is more commonly produced with cow milk (Addeo et al., 1996). Thereafter, writings follow one another (Tosi, 1930; Savini, 1946) aimed at clarifying the subtle separation between products obtained from the stretching of cow curd and buffalo curd. Toward the beginning of the 1950s, to simplify the difference between dairy products, the mozzarella produced with cow milk began to be called fior di latte, allocating the name "mozzarella" to the fresh pasta filata cheese produced from buffalo milk (Addeo et al., 1996). However, currently such distinction is not maintained so that "traditional mozzarella" is a fresh pasta filata cheese made from cow milk.

According to the Code of Federal Regulations Title 21 (US-FDA, 2021a,b), mozzarella cheeses are distinguished in low- and high-moisture mozzarella cheeses. The low-moisture mozzarella cheese has a moisture content of 42 to 52% and can be made from cow milk, nonfat milk, cream, or the corresponding products of water buffalo origin, except that cow milk products are not combined with water buffalo products (US-FDA, 2021b). High-moisture mozzarella cheeses have a moisture content of 52 to 60% and can be made from cow milk, nonfat milk, or cream, as well as the corresponding products of water buffalo origin, except that cow milk products are not combined with water buffalo products (US-FDA, 2021a). The high-moisture mozzarellas are usually consumed fresh as table cheeses, whereas the low-moisture ones are usually used as ingredients or for pizza toppings.

As schematized in Table 1, the main fresh pasta filata cheeses existing on the market can be grouped into the following categories:

(1) Fior di latte di Agerola (made with whole cow raw milk; Coppola et al., 2006). For its production, no starter, neither natural whey nor milk cultures are added to the raw milk (milk from morning milking mixed with the refrigerated milk from the previous evening's milking). The clotting happens within 20 to 30 min from the addition of rennet to the raw milk heated at 35 to 37°C. Thereafter, the curd is cut into pieces of hazelnut size within 30 to 40 min, and the whey is drained while the curd is transferred on a draining table, where it ripens at room temperature for 10 to 12 h, according to the season, to reach the pH of about 5, which makes the

Table 1. Main categories of fresh pasta filata cheeses

Category	Type of milk	Type of acidification	Chemical or biological agent added
Fior di latte di Agerola	Raw cow milk	Biological	No addition of microbial cultures but natural fermentation of the autochthonous microbiota
Mozzarella di bufala campana	Raw, pasteurized, or heat-treated water buffalo milk	Biological	Natural whey culture
Mozzarella di Gioia del Colle	Raw, pasteurized, or heat-treated cow milk	Biological	Natural whey culture
Fior di latte Appennino Meridionale	Raw cow milk	Biological	Natural whey culture
Mozzarella tradizionale	Pasteurized cow milk	Biological	Natural milk culture
Mozzarella	Cow, water buffalo, ewe, or goat milk	Direct or mixed acidification	Lactic acid, citric acid, or glucono-delta–lactone

ripened curd stretchable (Coppola et al., 2006). Following is the milling, stretching in hot water (at 80–90°C), molding, hardening in cold drinkable water, brining, and packaging in plastic or paper bags (Coppola et al., 2006).

(2) Mozzarella di bufala campana [made with whole buffalo milk (raw, pasteurized, or heat-treated) and natural whey inoculum]. According to the protected designation of origin (**PDO**) specifications (EC, 2021a), mozzarella di bufala campana is produced from whole buffalo ("Italian Mediterranean buffalo" breed reared in the production area delimited in Article 2 of the specification) milk, which may be raw, pasteurized, or heat-treated, or both, with the maximum time between milking and the beginning of cheesemaking fixed at 60 h. The milk is added with a natural whey culture, consisting of the whey taken from earlier processing of buffalo milk in the same holding or in neighboring holdings, in the delimited production area, bringing the acidity of the mixture to 10°SH (Soxhlet-Henkel). Thereafter, the milk is heated at a temperature ranging from 33 to 39°C, and coagulation is obtained by adding natural calf's rennet. The curd is then cut into pieces of about 16 cm^3 and covered by the whey so that the "curd ripening" phase (Coppola et al., 1988), usually lasting 4 h, is finished. The whey is then removed from the vat, and part of it is stored at room temperature and used as a starter on the following day. The ripened curd is milled or cut into slices, stretched and molded into individual pieces of the appropriate shape and size, and then placed in drinkable water for varying lengths of time, depending on size, until they harden. Salting takes place in brine. This is immediately followed by packaging, which must be carried out in the same establishment as production. The packaged product must be kept in its protective liquid, which is acidic and may be salted, until final consumption. The characteristic acidity of the protective liquid may be achieved by adding lactic acid or citric acid (Coppola et al., 1988; EC, 2021a).

(3) Mozzarella di Gioia del Colle (made with whole cow milk and a natural whey culture). Mozzarella di Gioia del Colle (EC, 2021b) is a fresh stretched-curd cheese made with whole cow milk, which may be raw, pasteurized, or heat-treated, and is collected over 2 separate milking sessions and added with a whey starter culture. It is characterized by (1) the following chemical composition (values for fresh cheese): lactose $\leq$0.6%, lactic acid $\geq$0.20%, moisture 58 to 65%, and fat 15 to 21% on a wet basis. It is also characterized by (2) a taste reminiscent of slightly soured milk, with a pleasant aftertaste of fermentation or sour whey (stronger in freshly made cheese) and a sour milky aroma, sometimes accompanied by a slight hint of butter, and (3) the absence of preservatives, additives, and processing aids (EC, 2021b). All of the stages of the production process (rearing and milking the cows, collecting and processing the milk, and making the cheese itself) take place in the geographical area specified.

(4) Fior di latte Appenino Meridionale (made with cow milk and a natural whey culture). With the decree of March 1, 2002, of the Italian Ministry of Agricultural and Forestry Policies (2002a), transitional protection was granted at national level to the denomination of origin "Fior di Latte Appennino Meridionale," for whose recognition at European level the relative procedure is in progress. According with the production specification (Italian Ministry of Agricultural and Forestry Policies, 2002b), raw whole milk of the production area, coming from 1 or more consecutive milkings (which must be carried out within

16 h) is obtained from cattle fed with essences typical of the production area, and mainly consisting of fresh legumes and cereals. The use of concentrated feed and supplements is allowed. The milk must reach the dairy within 36 h of the first milking and cannot be thermized or pasteurized. The milk is heated to 33 to 38°C, a natural whey culture obtained by back slopping (resulting from the previous processing of raw cow milk coming from the production area), and a natural liquid calf rennet are added. Coagulation takes 20 to 40 min; afterward, the curd is broken down into hazelnut-size granules and put under whey to naturally ferment until its maturation, which takes place in 3 to 5 h from the addition of the rennet. The use of organic acids or chemical pH correctors is not allowed. At the end of the maturation, the pH is between 5 and 5.3; at this point, the curd is reduced into strips placed in a special vat, and, with the addition of almost boiling water, the curd is made to string. Once the mozzarella has been obtained, it is firmed by immersion in cold water and then the cheeses are immersed in brine (salting can also be carried out during the stretching of the curd). The use of preservatives is not allowed (Italian Ministry of Agricultural and Forestry Policies, 2002b).

(5) Mozzarella tradizionale (made with cow milk and a natural milk culture; UNI, 1995; EC, 1998; GU, 2019). In the case of "mozzarella tradizionale," cow milk, pasteurized at minimum 71.7°C for 15 sec (or any equivalent thermization treatment), is inoculated with natural milk culture, added with bovine liquid rennet (with pepsin activity between 20 and 30%), to achieve the presamic curdling at 35 to 39°C. Thereafter, the coagulum is cut into hazelnut-size pieces and drained. When curd has reached the pH of 5.0 to 5.4 (lactic fermentation of the curd), it is cut or shredded into slides, stretched by a thermomechanical operation using hot water, and eventually added with salt, with a final temperature of the curd between 58 and 65°C and molded. Then, cheese is firmed in cold water, eventually added with salt, and finally packaged (GU, 2019). According to the protocol described in the proposed modification of the product specification of the traditional specialty guaranteed "mozzarella tradizionale" (GU, 2019), the natural milk culture must be obtained as follows: (a) first natural milk culture: heat treatment of unrefrigerated raw milk, at a temperature not lower than 63°C for a minimum time of 15 min (or time/temperature combinations with equivalent minimum effect); cooling down at the incubation temperature of 42 to 50°C; incubation until the acidity is equal to 14 to 24°SH on 100 mL; cooling to a temperature below 8°C; refrigerated storage at a temperature not exceeding 4°C; and (b) subsequent natural milk cultures: inoculation of raw milk, which can also be refrigerated, with a minimum of 4% of the previous natural milk culture (back slopping); heat treatment as described for the first natural milk culture; cooling to the incubation temperature of 42 to 50°C; incubation up to acidity equal to 14 to 24°SH on 100 mL; cooling to a temperature below 8°C; refrigerated storage at a temperature not higher than 4°C. The ready-to-use natural milk culture must have (1) an acidity between 16 and 30°SH on 100 mL; (2) a minimal content of thermophilic streptococci of 10^8 cfu/mL; and (3) negative phosphatase activity. Moreover, it must be used in the mozzarella tradizionale cheesemaking within 3 d (GU, 2019).

(6) Mozzarella. While the second to the fifth product categories are governed by production regulations, the sixth brings together a great variety of cheeses, until now essentially united only by the unique name of "mozzarella." With this denomination are indicated mozzarella cheeses produced with citric acid (direct acidification) or with a mixed acidification technology, as well as some products for pizzerias (Addeo et al., 1996). The direct acidification for the production of fresh pasta filata cheeses foresees the addition of acid (usually citric acid) to pasteurized milk before the addition of rennet, so that when the pH of 5.6 to 5.8 is reached after the coagulation, the curd can be stretched. Apart from the shorter time for processing, the direct acidification provides a longer shelf life of the standardized products that moreover have a lower cost (Pisano et al., 2016). In addition, cheeses obtained by direct acidification have a lower degree of browning than those obtained by starter cultures (Oberg et al., 1991a). However, the direct acidification in absence of any fermentation activity confers to the final product a standardized bland taste, which is emphasized only by the addition of salt (Gulzar et al., 2020; Natrella et al., 2020a,b). Directly acidified mozzarella cheese has a sensory acceptability that deteriorates faster and obtains lower sensory scores than those obtained for mozzarella made with commercial starter cultures (*Streptococcus thermophilus*), or by complex defined starter cultures (De Angelis

et al., 2008). Natrella et al. (2020a) assessed the microbiological, sensory characteristics, and the volatile organic compound profiles by headspace solid-phase microextraction coupled with GC-MS of mozzarella made with raw milk and natural whey culture and mozzarella made with direct acidification with citric acid. The microbial loads of mesophilic and thermophilic lactic acid bacteria (**LAB**), yeasts, and molds in the cheese, obtained by direct acidification, were significantly lower than those found in the other type of mozzarella, whereas higher loads of *Enterobacteriaceae* were found in the mozzarella obtained with direct acidification. Concerning the sensory analysis, it allowed a clear differentiation between traditionally made and industrial mozzarella, with the traditional receiving higher intensity attributes. The volatile organic compound profile of traditionally made mozzarella was always more complex than the one of industrial mozzarella (Natrella et al., 2020a). Industrial mozzarella was moreover found to have a volatile organic compound profile more similar to the milk used for its production, which was less complex than that of mozzarella di Gioia del Colle made from the same raw milk, but with the addition of natural whey culture, following the PDO official protocol (Natrella et al., 2020b).

(7) Stretched and melted cheeses for pizza. This category refers to a product of imitation of mozzarella, used in the preparation of pizza or sandwiches. Actually, cheeses for pizza belong to the category of melted products; that is, those obtained from the fusion of ingredients of milk origin, curds, and possibly salts during melting (Addeo et al., 1996).

MICROBIOLOGICAL AND TECHNOLOGICAL ASPECTS

From a microbiological point of view, there are essentially 3 production technologies of fresh pasta filata cheeses involving the activity of microorganisms, which are as follows: one, which provides for the cheesemaking of pasteurized milk to which selected LAB are added; a second, which transforms the milk as it is, to which, before coagulation, natural whey or milk cultures are used to inoculate raw milk in the cheese vat; and a third, which uses raw milk, heated to about 35°C, added with rennet, and left to acidify without any addition of starter with a maturation that can last, depending on the season, for varying times.

The presence of microorganisms may be accidental if they enter milk and intermediates of production as contaminants (from milk; Fusco and Quero, 2014; Fusco et al., 2020) from the equipment and the environment of production (Scatassa et al., 2015; Stellato et al., 2015), or deliberately added due to the use of starters (natural or commercial). Moreover, the specific technology of production imposes selective processes that favor the colonization of the product by specific bacterial species or biotypes, which with their enzymatic patrimony, together with endogenous enzymes, contribute to the biochemical-physical transformation of the milk in cheese.

The current way of thinking about the microbiota occurring during cheesemaking is to not consider each individual microorganism and its own metabolic activities, but the whole community and its performances, so that the core microbiota of cheese is to be considered together with the totality of microbial interactions and metabolisms that happen among the microorganisms (Gobbetti et al., 2018).

Herein, we provide an overview of the main steps of cheesemaking that affect the composition and evolution of the microbiota of cheese.

Raw Milk Microbiota

Due to its high nutritious content, milk is a good substrate for the growth of numerous and diverse microorganisms. In addition to its endogenous microbiota (Fusco and Quero, 2014; Young et al., 2015; Addis et al., 2016), a copious and heterogeneous number of microbes originating from the udder skin, teat canal, tanks, milking machines, feed, air, water, soil grass, and other environments, as well as from (infected) workers, might contaminate the milk (Fusco et al., 2020; Parente et al., 2020; De Filippis et al., 2021). Apart from LAB, such as *Streptococcus*, *Lactococcus*, *Enterococcus*, *Weissella*, *Leuconostoc*, and *Lactobacillus* (for which a reclassification into 25 genera, including the emended genus *Lactobacillus*, has been proposed by Zheng et al., 2020; see the note for the reader), fresh raw milk may be inhabited by yeasts, molds, bacteriophages, and other gram-positive bacteria of the genera *Propionibacterium*, *Bacillus*, *Micrococcus*, *Mammaliicoccus*, *Microbacterium*, *Clostridium* and *Staphylococcus*, and gram-negative bacteria of the genera *Pseudomonas*, *Ralstonia*, *Stenotrophomonas*, *Sphingomonas*, *Psychrobacter*, *Acinetobacter*, *Chryseobacterium*, *Faecalibacterium*, *Porphyromonas*, *Bacteroides*, *Fusobacterium*, *Comamonas*, *Enterobacter*, *Hafnia*, *Aeromonas*, and *Klebsiella* (Coppola et al., 2006; Ercolini et al., 2012; Montel et al., 2014; Fusco et al., 2020; Parente et al.,

2020). The bulk tank milk transfer from the farm to the dairy, as well as the duration and conditions of storage, may further affect both the amount and structure of the microbiota of milk, affecting the loads of beneficial (starter and nonstarter cultures) and spoilage bacteria (psychrotrophic spore-formers, *Pseudomonas* spp., *Acinetobacter* spp.; Stellato et al., 2015; Faggiano et al., 2018; Parente et al., 2020).

Heat Treatment of Milk

Raw milk can be pasteurized if the addition of either citric acid, lactic acid, glucono-delta-lactone (directly acidified mozzarella; Kindstedt, 2004), or a commercial starter culture follows this step. In any case, the heat treatment, at 72°C and the subsequent chilling of milk at approximately 37°C, at which is foreseen the addition of the rennet and either acid or starter cultures, inactivate the microorganisms of raw milk, with the exception of thermoduric microorganisms (such as certain *Enterococcus*, *Brachybacterium*, *Streptococcus*, *Micrococcus*, *Kocuria*, and *Macrococcus* species), endospore-forming bacteria (such as *Bacillus* spp., *Clostridium* spp., and *Paenibacillus* spp.), whose proteolytic and lipolytic activities may cause undesired changes in the final cheeses, and thermophilic microorganisms, including LAB such as *Streptococcus thermophilus*, *Lactobacillus delbrueckii*, *Lactobacillus helveticus*, *Enterococcus faecium*, and *Enterococcus faecalis* (Delgado et al., 2013; Ribeiro Júnior et al., 2018).

Defined and Undefined Starter Cultures

Unlike the direct acidification process, the microbial-mediated acidification process, which foresees the addition to the pasteurized milk of natural whey/milk cultures, commercial undefined starters (mixed-strain starters derived from the best natural starters and reproduced under controlled conditions by specialized institutions and companies), or commercial defined starter cultures (whose species and strain ratios are defined; Parente, 2006; Parente et al., 2017), allows the slow acidification via fermentation of the curd that needs to reach a lower pH to be stretchable. This difference is mainly due to the intrinsic ability of the acid directly added to the milk to act immediately on micelles suspended in the water, and sequester part of the colloidal calcium phosphate present within them (Natrella et al., 2020a). Instead, when the defined or undefined cultures are added to the milk, the lactic acid is released slowly as it is produced by the fermentation, so that only after coagulation it reaches the micelles, which are concentrated into the paracaseinate network with a higher ration of colloidal calcium/casein that requires more acid for the sequestration reaction (Natrella et al., 2020a).

Pasteurized milk added with commercial or defined cultures of *S. thermophilus* alone, or in combination with *L. delbrueckii* ssp. *bulgaricus* or *L. helveticus*, or both, might be used for the production of industrial mozzarella or fior di latte cheeses (Coppola et al., 2001; De Candia et al., 2007). Defined starter cultures affect the rheological, melting, and physical properties of mozzarella cheese, as well as its microstructure, composition functional properties, and proteolysis (Oberg et al., 1991a,b; Yun et al., 1995; Merill et al., 1996; Dave et al., 2003). In particular, the use of *L. helveticus* together with *S. thermophilus* as adjunct starter allows mozzarella cheese to have functional properties, especially melting, superior to those achieved using *L. delbrueckii* ssp. *bulgaricus* (Oberg et al., 1991b). A symbiotic relationship occurs among coccus and rod thermophilic cultures, which is described as follows: the degradation of casein performed by lactobacilli releases peptides and AA, which are used by the weakly proteolytic streptococci that produce carbon dioxide and formic acid, which, in turn, stimulate the growth of lactobacilli (Radke-Mitchell and Sandine, 1984). Acid production and proteolysis with mixed thermophilic cultures is greater than the sum of the acid production and proteolysis with individual cultures (Barach et al., 1987; Marshall, 1987; Rajagopal and Sandine, 1990; Whitehead et al., 1991). A 3 to 4 times greater meltability of mozzarella cheese was achieved by using mixed culture of *S. thermophilus* and *L. helveticus*, in respect to the only 2 times greater meltability achieved by using *S. thermophilus* alone (Dave et al., 2003). Merill et al. (1996) produced reduced-fat mozzarella cheese using *S. thermophilus* and *L. helveticus*, and either total or partial replacement of *L. helveticus* with *Lactobacillus casei* (now *Lacticaseibacillus casei*; Zheng et al., 2020). Reduced-fat cheese made with *L. helveticus* and *S. thermophilus* showed the greatest stretch at both 1 and 7 d, compared with the cheese obtained by either total or partial replacement of *L. helveticus* with *L. casei* (Merrill et al., 1996). Regardless of the rod:coccus ratio, which was either *L. delbrueckii* ssp. *bulgaricus*:*S. thermophilus* 9:1, 1:1, or 1:9 and was used in the production of mozzarella, the cocci starter was dominant in the curd at milling and survived the curd stretching (at 57°C). A rapid proteolysis was achieved in stored mozzarella cheese, especially when obtained by using a rod:coccus ratio of 9:1. Lower springiness and apparent viscosity were achieved by using the rod:coccus ratio 9:1 as compared with the cheese obtained with the other 2 ratios of starter, whereas the meltability, hardness, and free oil properties of mozzarella were not affected by the different ratios (Yun et al.,

1995). However, using excess rods leads to an increased proteolysis and a softer cheese, but using cocci alone or in higher ratio than rods produces cheese with slower maturation rates (Oommen et al., 2002).

Exopolysaccharides produced by LAB can enhance the texture, viscosity, and mouthfeel of cheeses, and prevent syneresis (Perry et al., 1997, 1998; Bhaskaracharya and Shah, 2000; Petersen et al., 2000; Amatayakul et al., 2006; Purwandari et al., 2007), in addition to their beneficial effects on the host (Prete et al., 2021). However, although a qualitative and technological standardization of the products is achieved by the use of commercial starter cultures, the resulting flavors of these products may be flattened (Broome, 2007; Braghieri et al., 2018). Moreover, the proteolytic activity of the thermophilic starters produces small peptides and AA able to react with residual sugars in the system when subjected to heating, causing browning via the Maillard reaction (Lee et al., 2014; Igoshi et al., 2017). In particular, most LAB used in the production of mozzarella cheese, such as *L. delbrueckii* ssp. *bulgaricus*, do not have galactosidase; thus, they only ferment the glucose of the lactose molecules, releasing the galactose in the curd, which enters the Maillard reaction during high-temperature cooking of mozzarella cheese (Igoshi et al., 2017). Some other LAB, such as *L. helveticus*, being endowed with the β-galactosidase, release less galactose in the curd, resulting in less browning and blistering during cooking (Johnson and Olson, 1985; Hutkins et al., 1986; Oberg et al., 1991b; Ma et al., 2013).

For all the aforementioned reasons, galactose-fermenting, exopolysaccharide-producing, or proteolytic LAB strains, or all of these, mainly belonging to the *Lactobacillus*, *Streptococcus*, and *Enterococcus* genera, have been selected and evaluated for their use as starter culture in the production of mozzarella and fior di latte cheese (Mukherjee and Hutkins, 1994; De Angelis et al., 2008; Speranza et al., 2015; Silva et al., 2020). Moreover, given the occurrence of probiotics in fermented foods and the well demonstrated capability of dairy products to vehicle such beneficial microorganisms (Fusco et al., 2021a,b, 2022a,b), many authors are focusing on the use of autochthonous or commercial probiotic starter cultures for the production of probiotic mozzarella and fior di latte cheeses (Minervini et al., 2012; Ortakci et al., 2012; Jeronymo-Ceneviva et al., 2014; de Paula et al., 2015; Casarotti et al., 2017; de Souza et al., 2019; Mukhtar et al., 2020).

Defined starter cultures have highly reproducible performances and phage resistance, which allow the obtainment of products with standardized quality, thus avoiding the fluctuations associated with the use of undefined starter cultures. For these reasons, defined starter cultures are used for the production of numerous cheese varieties.

Although defined starter cultures are used to make most low-moisture mozzarella and fior di latte cheeses, natural whey or milk cultures are employed for the production of various types of high-moisture traditional fresh pasta filata cheeses. The microbial composition of natural whey cultures (**NWC**) used for the production of traditional water buffalo mozzarella cheese has been investigated by both culture-dependent and culture-independent methods. In particular, Coppola et al. (1988) used plating and biochemical identification to assess the composition of 16 NWC from 3 different mozzarella cheese plants, whereas Ercolini et al. (2004) used PCR denaturing gradient gel electrophoresis (**DGGE**) of NWC and of bulk colonies, collected from the agar plates used to enumerate mesophilic and thermophilic LAB. Moreover, Ercolini et al. (2012) and De Filippis et al. (2014) used next generation sequencing to investigate the composition of the microbiome of NWC collected from 2 dairies producing traditional water buffalo mozzarella cheese and 12 dairies producing traditional water buffalo mozzarella PDO cheese, respectively. The enumeration on selective media revealed the prevalence of thermophilic lactobacilli and streptococci, followed by enterococci, mesophilic streptococci, and lactobacilli, on micrococci, coliform bacteria, yeasts, and leuconostocs (Coppola et al., 1988). Subsequent studies have typed microorganisms isolated from NWC and demonstrated their technological attributes (Coppola et al., 1990; Moschetti et al., 1996, 1998; Cocconcelli et al., 1997; Villani et al., 1997; Mauriello et al., 1998; Blaiotta et al., 2002; De Filippis et al., 2014). Polymerase chain reaction-DGGE of the bulks (Ercolini et al., 2001) revealed the presence of *S. thermophilus*, *Enterococcus faecalis*, *Escherichia coli*, *L. delbrueckii*, *Lactobacillus crispatus*, *Lactobacillus fermentum* (now *Limosilactobacillus fermentum*; Zheng et al., 2020), and *Lactococcus lactis*, whereas the PCR-DGGE of DNA directly isolated from NWC revealed the presence of *Lact. lactis*, *S. thermophilus*, *L. delbrueckii*, *L. crispatus*, *Alishewanella fetalis*, and microorganisms of the Gamma proteobacterium group (Ercolini et al., 2001). Culture-independent PCR-DGGE for microbial community fingerprinting and neutral volatile compounds identification by high-resolution GC-MS coupled with multivariate statistical analysis were used to characterize 29 NWC used in the production of mozzarella di bufala campana, from 29 cheese factories located in Campania region (17 from the province of Salerno and 12 from the province of Caserta) by Mauriello et al. (2003). A relationship between the geographical origin, microbial composition, and flavoring capabilities of the NWC analyzed was highlighted, leading to suggest the

use of molecular characterization of typical mozzarella cheeses to support the traceability criteria of these products (Mauriello et al., 2003). By high throughput sequencing, *S. thermophilus*, *L. delbrueckii*, and *L. helveticus* were the main operational taxonomic unit (**OTU**) in 1 of the 2 NWC analyzed, whereas *S. thermophilus* and *L. delbrueckii* were the most abundant OTU in the other one NWC, as found by Ercolini et al. (2012). De Filippis et al. (2014) additionally found *L. fermentum* and *Lact. lactis* at a lower concentration and not in all NWC and curd samples analyzed by high throughput sequencing. Moreover, they found many environmental contaminants such as *Escherichia* sp., *Enterobacter cowanii*, and other OTU belonging to *Enterobacteriaceae* family as subdominant OTU (De Filippis et al., 2014). A different composition in LAB of the natural whey starter used for the production of mozzarella di bufala campana in 3 factories of the provinces of Salerno, Latina, and Caserta was found by Devirgiliis et al. (2008), who used 16S rRNA gene sequencing and amplified ribosomal DNA restriction analysis to identify the following isolates: *L. fermentum* and *L. delbrueckii* were found in natural whey culture of Salerno and Caserta, whereas *L. fermentum*, *L. delbrueckii*, *Lactobacillus paracasei* (now *Lacticaseibacillus paracasei*; Zheng et al., 2020), and *Lactobacillus plantarum* (now *Lactiplantibacillus plantarum*; Zheng et al., 2020) were found in the NWC of Latina.

No deep detection and characterization of yeasts, which are present in NWC, in the intermediate products, and in the final water buffalo mozzarella cheese, has been conducted. However, Suzzi et al. (1998) and Romano et al. (2001) revealed the dominance of *Saccharomyces cerevisiae* followed by *Kluyveromyces marxianus*, *Candida sphaerica*, *Candida kefir*, and *Kluyveromyces lactis* in water buffalo mozzarella produced in the Basilicata region, which have specific fermentative and biochemical patterns, and could contribute mainly to the organoleptic properties of this pasta filata cheese (Coppola et al., 1990; Moio et al., 1993; Tofalo et al., 2020). Fermenting yeasts such as *K. marxianus*, followed by *Sacc. cerevisiae*, have been found as the dominant species also in 16 samples of water buffalo mozzarella produced in the provinces of Salerno, Caserta, and Frosinone (Campania and Lazio regions in Italy; Aponte et al., 2010). Other species seldom or never reported in dairy products, such as *Candida butiry/aaseri*, *Candida pararugosa*, *Candida sorbophila*, *Pichia pastoris*, *Pichia cactophila*, *Pichia barkeri*, *Pichia norvegensis*, and *Clavispora lusitaniae* were found by Aponte et al. (2010). However, *Debaryomyces hansenii*, *Clavispora lusitaniae*, and *Candida parapsilosis* were the prevalent yeast species isolated from water buffalo mozzarella cheese produced in Minas Gerais (Brazil), whereas opportunistic yeast species such as *Candida guilliermondii*, *Candida tropicalis*, *Candida parapsilosis*, *Clavispora lusitaniae*, *Candida catenulata*, *Candida rugosa*, and *Candida krusei* were less frequently isolated from the final product (Facchin et al., 2013). Among the intermediate of production, the acidified curd presented the highest numbers of yeasts, with *Candida catenulata* as the dominant species (Facchin et al., 2013).

De Candia et al. (2007) enumerated, identified and typed the LAB population of 21 samples (different batches) of NWC from 7 different factories used for the production of mozzarella cheese in the Apulia region (Italy). The loads of presumptive LAB were different among the 7 NWC analyzed. In particular, thermophilic lactobacilli ranged from 2.7 to 7.7 log cfu/g, whereas mesophilic lactobacilli were few in 4 out of the 7 NWC (ranging from 1.2 to 3.3 log cfu/g) and from 6.0 to 7.7 log cfu/g in the remaining 3 NWC. Streptococci and lactococci ranged from absent in 3 NWC, to 3.6 to 4.3 log cfu/g in 2 NWC and to 6.0 to 7.2 log cfu/g in the remaining 2 NWC. Enterococci ranged from 3.8 to 5.5 log cfu/g in 6 NWC, whereas they were absent in 1 NWC. A total of 145 isolates were identified by partial sequencing of the 16S rRNA gene and were found to belong to the following species: *L. fermentum*, *L. plantarum*, *L. casei*, *L. delbrueckii* ssp. *lactis*, *L. helveticus*, *L. delbrueckii* ssp. *bulgaricus*, *Lact. lactis*, *Lactococcus garvieae*, *S. thermophilus*, *Enterococcus durans*, *Enterococcus faecium*, *Enterococcus faecalis*, and *Enterococcus* spp. (De Candia et al., 2007). Lesser loads and biodiversity of the lactobacilli population at both species and strain level was found in the NWC used for the production of mozzarella cheese in the Apulia region by Morea et al. (1998). In particular, the natural whey culture at pH 4, obtained by spontaneous 24 h fermentation of the whey derived from the cheesemaking of the previous day, contained 8.6×10^5 and 4.6×10^4 cfu/mL of mesophilic and thermophilic presumptive lactobacilli, respectively. The isolates were identified as *L. fermentum*, *L. casei*, and *L. plantarum*. However, it should be mentioned that Rogosa agar (Oxoid) was used for the enumeration and isolation of lactobacilli, and it is well known that due to its high salt content, this medium does not allow the growth of *L. delbrueckii* ssp. *bulgaricus* and ssp. *lactis* (Blaiotta et al., 2017). The M17 agar incubated at 30 and 42°C was instead used to investigate the dominant bacterial populations in traditional mozzarella cheese processing in the Apulia region by Morea et al. (1999); however, it is well known that this medium allows the selective enumeration and isolation of lactococci and streptococci (Blaiotta et al., 2017). Indeed, in the natural whey culture analyzed, Morea et al. (1999) found strains of *S. thermophilus*, *Enterococcus faecalis*, *Leuconostoc mesen-*

teroides ssp. *mesenteroides*, *Staphylococcus epidermidis*, and *Aerococcus viridans* as typed by random amplified polymorphic DNA-PCR and identified by partial sequencing of the 16S rRNA gene.

Parente et al. (1997) and Guidone et al. (2016a) investigated the composition of natural whey and milk cultures used for the production of traditionally made mozzarella cheese of the Basilicata region. Different procedures were used for the production of NWC, analyzed by Parente et al. (1997), and are as follows: (1) 4 NWC obtained from the same dairy over a period of 5 mo were prepared by heat treating the cheese whey at 70°C for 15 s, cooling it thereafter at 45°C, inoculating it with 1 to 2% of the previous day's whey culture, and then incubating the resulting mixture overnight in an insulated tank; (2) 4 other whey cultures obtained from 4 different plants were prepared each day by incubating the whey at the end of the cheese manufacturing at 37 to 42°C overnight in insulated containers without temperature control; (3) a natural milk culture coming from a different plant in Basilicata was prepared each day by heating the raw milk at 63°C for 20 min, cooling it to 45°C, and incubating overnight without temperature control (Parente et al., 1997). The incubation conditions used to prepare the natural cultures affected both their composition and technological properties. Indeed, results for the NWC incubated at a temperature higher than 45°C was dominated by *L. helveticus*, that is, an acid-tolerant, thermophilic, and homofermentative species. The natural milk culture, as well as the NWC obtained by incubation at 37 to 52°C overnight, were dominated by cocci, some of which were identified as *S. thermophilus*, *Enterococcus faecalis*, *Enterococcus* spp., *Lact. Lactis*, and *Lact. garvieae*. These natural cultures were also contaminated by a high load of coliforms, probably due to the higher pH. However, as demonstrated by Parente and Coppola (1987), Parente et al. (2016), Serraino et al. (2012), and Villani et al. (1996), changes in the composition of the culture and eradication of coliforms and pathogenic bacteria such as *Listeria monocytogenes* and *Salmonella typhimurium* can be achieved by controlling the temperature and the pH during the production of the natural cultures. Natural milk culture, created in the laboratory by heating 500 mL of raw milk at 63°C for 15 min, followed by incubation at 42°C to reach a pH of 4.0 to 4.2 and 14 to 24 °SH, was propagated by back slopping; that is, by inoculating (0.5% vol/vol) the culture from the previous cycle in raw milk obtained daily from a dairy plant located in the Basilicata region for the production of mozzarella cheese, followed by heating at 63°C for 15 min and incubation at 42°C for 16 h (Guidone et al., 2016a). The resulting culture was refrigerated at 4°C, for up to 3 d, before use for inoculation of the following cycle (Guidone et al., 2016a). Enterococci and coliforms were found at loads between 10^2 and 10^5 cfu/mL only in natural milk cultures provided by the dairy plant, whereas the presumptive lactobacilli were always below 10^3 cfu/mL, and presumptive streptococci were 8.58 log cfu/mL (median value; Guidone et al., 2016a). The natural milk cultures obtained by this repeated propagation cycle were dominated by *S. thermophilus*, *L. delbrueckii*, and, seldom, *L. helveticus*. *Enterococcus casseliflavus*, *Enterococcus gallinarum*, *Enterococcus faecalis*, and *Lact. garvieae* were also found (Guidone et al., 2016a). Variability of all cultures and of their performances over time were found with those reproduced at the dairy plant as the most variable, thus demonstrating that improved control of the conditions used to prepare and reproduce the cultures may reduce such variability (Guidone et al., 2016a). Similar results were also achieved by Parente et al. (2016), who analyzed undefined milk starter cultures prepared in laboratory by using culture-dependent (random isolation and molecular characterization of isolates) and -independent [amplicon targeted next generation sequencing of the 16S rRNA gene and partial sequencing of the phosphoserine phosphatase gene (*ser*B) of *S. thermophilus*, as well as quantitative real time PCR and multiplex PCR both used for the detection of bacteriophages] methods, as done by Guidone et al. (2016a), starting from raw milk obtained from 8 different pasta filata dairy plants in Campania and from 1 dairy plant in Basilicata. The natural milk cultures were composed mainly by thermophilic LAB, but LAB population and acid production activity varied over reproduction cycles. The cultures were dominated by *S. thermophilus* or *L. delbrueckii* ssp. *lactis*, or both, but subdominant mesophilic species including *Lact. lactis*, *Lactococcus raffinolactis*, *and Lactococcus* spp., as well as spoilage bacteria such as *Enterobacteriaceae*, *Raoultella* spp., and *Streptococcus parauberis*, persisted at low levels (Parente et al., 2016). The *S. thermophilus* populations were composed by a moderate number of *ser*B sequence types, and a single sequence type dominated all the cultures but 2 (Parente et al., 2016). Only in 2 cases, high titers of *cos*-type *S. thermophilus* bacteriophages were detected, whereas *Lact. lactis* and *L. delbrueckii* bacteriophages were below the limit of detection (Parente et al., 2016).

All these findings highlight the importance of adopting controlled conditions for the preparation and reproduction of natural whey and milk cultures, in order to reduce its intrinsic variability in terms of composition and performances that in turn cause fluctuations in the quality and safety of the final products. However, the use of natural whey/milk cultures even from specific areas of productions confers typical microbiological, sensory, aromatic, and metabolomics profiles to PDO

and non-PDO high-moisture mozzarella and fior di latte cheeses that can be used to assess their authenticity and traceability (Mauriello et al., 2003; Guidone et al., 2016a,b; Pisano et al., 2016; Natrella et al., 2020a,b; Salzano et al., 2020).

Curdling and Curd Ripening

The technology of pasta filata cheese is characterized by a start that is presamic (rennet-based), but with acid support (natural or acquired), which enhances the creation of a good, soft, elastic clot, which lends itself well to breaking, so to obtain glomerular uniformity, with reduced caseous losses and with an optimal predisposition to homogeneous draining (Ghitti et al., 1996). Such a goal is realized in the vat, from the coagulation of milk to the curd cutting and ceases with the drying of the curd. Thereafter, the fermentation activity occurring during the curd ripening is essential to achieving the lactic address, which confers the curd rheological characteristics necessary for a good stretching and modeling of the dough. In other words, a rennet-lactic start is followed by a phase diversification, which leads to a completely lactic approach (Ghitti et al., 1996). In physico-chemical terms, the initial presamic phase realizes a polymerization reaction with enrichment and neutralization of the caseous micelle, which provokes the loss of the hydrophilic glycomacropeptide (which acted as a protective colloid of the whole casein) that passes into solution, allowing its dispersion in water. The rennin or chymosin of rennet, by splitting the peptide bond between the AA phenylalanine and methionine in position 105 and 106 of phosphoprotide, provokes the conversion of the κ-CN in the hydrophobic para-κ-CN. The para-κ-CN is transformed into dicalcium phospho-paracaseinate and then into curd (Ghitti et al., 1996). The subsequent stages of processing consist of cutting and extracting the curd from the whey. The stopover of the curd is the time between the appearance of the gel and the breaking of the curd. During this pause, and in proportion to it, the consistency and viscosity of the curd increases, so the most appropriate moment to start cutting the gel into pieces depends on the type of product to be obtained: for instance, to produce fresh stretched-curd cheeses, the gel is cut after about 15 to 20 min, when it has a certain consistency and is reduced to pieces of curd that are the size of a walnut (Bossini et al., 1993).

The first cut of the curd, operated manually or mechanically, is rather coarse (generally cross-shaped): in fact, it determines an important separation of the whey from the mass that is localized between the curd granules acting as a lubricant of the structure. Another part of the whey, in contrast, still remains incorporated in the granules, along with fat, bacteria, and air: the greater the quantity of whey included in the curd, the lower the consistency of the finished product (in fact, to obtain hard cheeses, the cutting of the gel is pushed to obtain pieces the size of rice grains; Addeo et al., 1996).

The maturation of the curd, under or outside the whey, takes place by the lactic acid bacteria added to the raw or pasteurized milk, before coagulation. The lactic acid (or other acids, or both) produced, thanks to the H^+ ions that release Ca^{++} from the dicalcium phospho-paracaseinate, determines a progressive depolymerization and demineralization action of the rennet curd, forming mono-calcium phospho-paracaseinate, which is ready to be stretched, and lactates or calcium citrates (Kosikowski, 1958).

Overall, the microbial composition of the curds reflects that of the natural whey/milk cultures used for the mozzarella and fior di latte cheese production, but it seems that during the curdling, the draining (in cases where the ripening of the curd is not achieved under whey) and the ripening of the curd, which is usually realized at room temperature, an increase in the mesophilic LAB population of the milk and NWC/NMC is favored. The curd obtained during the production of water buffalo mozzarella (mozzarella di bufala campana) contained loads of presumptive thermophilic lactobacilli and streptococci similar to those found in the relevant natural whey culture, whereas the loads of presumptive mesophilic rod- and coccus-shaped LAB were higher than those in the natural whey culture (Ercolini et al., 2004). The community fingerprint obtained by culture-independent PCR-DGGE of the curd reflected that of the natural whey culture (Ercolini et al., 2004). A microbial composition of the curd similar to that of the relevant natural whey culture was found also by next generation sequencing approach (Ercolini et al., 2012). However, Ercolini et al. (2004) demonstrated that mesophilic LAB were the microbial group more numerous and biodiverse in the curd. In fact, apart from being present in highest number, they resulted in the highest biodiversity because they had the highest number of DGGE bands obtained by the analysis of the bulk. In the fermented curd obtained during the production of traditionally made mozzarella cheese in the Apulia region a prevalence of mesophilic milk fermenting bacteria over the thermophilic population was found, with several mesophilic and thermophilic lactobacilli slightly lower than that in the natural whey culture (Morea et al., 1998). *Lactobacillus fermentum*, *L. plantarum*, *L. helveticus*, and *L. casei* were isolated and identified by partial 16S rRNA gene sequencing from the curd (Morea et al., 1998). *Lactobacillus casei*, *Enterococcus* spp., *Leuconostoc citreum*, and *Lact. lactis* were the dominant species found in the curd obtained

during the production of Brazilian buffalo mozzarella cheese (Silva et al., 2021).

In the fior di latte di Agerola cheesemaking, where the sole microbiota of raw milk is involved in the transformation of raw milk in cheese, microbial loads of the various group of LAB in the curd were similar to those of the milk before cheesemaking, with the exception of presumptive streptococci and enterococci, whose loads increased approximately 1 log cfu/g (Coppola et al., 2006). After 10 to 12 h of ripening at room temperature, both mesophilic and thermophilic LAB loads increased significantly with the exception of enterococci. Presumptive *Enterobacteriaceae* increased significantly during the curdling and the curd ripening, whereas presumptive *Staphylococcus aureus* increased approximately 1 log cfu/g in the curd but remained stable during the curd ripening (Coppola et al., 2006). The high diversity at species and strain level found in the curd reflects that of the raw milk, but decreases in the ripened curd where the most abundant species were *L. helveticus*, *Lact. lactis* ssp. *lactis*, *S. thermophilus*, *Enterococcus faecalis*, and *Leuconostoc mesenteroides* ssp. *mesenteroides* (Coppola et al., 2006).

Stretching, Molding, Brining, and Packaging

Stretching is a technique based on the property of casein to assume a filamentous plastic structure under appropriate conditions of temperature and acidity. For the production of buffalo (mozzarella) and cow (fior di latte) pasta filata cheeses, it is necessary to achieve an acidification of the curd up to the pH of stretching, variable between 4.9 and 5.4, in a time between 3 and 5 h. At the optimum pH, the calcium content of the curd is reduced by about 75% compared with the original content (Addeo et al., 1996).

The stretching of the curd accomplishes the task of giving the typical structure to the different pasta filata products; in particular, it can be manual or mechanical. In the latter case, the curd mass is finely cut by a special grinder and the individual pieces are sent to the so-called "macero (pulping mill)," where hot water (80–90°C) is introduced (Addeo et al., 1996).

The stretching phase has been frequently proposed as a microorganism inactivation process but, as mentioned above, it is merely a technological step that confers the desired structure to the final products. Moreover, variables such as temperature and time of the heat treatment, which in turn may vary with the rheological attributes of the curd (Trevisani et al., 2017), as well as the initial level of milk contamination, makes it unsuitable to ensure the safety of the final product (Addeo and Coppola, 1983; Addeo et al., 1996; Addeo and Schiavi, 1997). Up to 5 log reduction can be achieved for O157 and O26 Shiga toxin-producing *E. coli* only when the temperature of the curd during stretching is of 78 to 80°C (Trevisani et al., 2017), whereas a stretching at ≥80°C for 5 min of the ripened curd during the laboratory-scale production of mozzarella cheese inactivate *E. coli* O157:H7 (Spano et al., 2003). Stretching might be able to significantly reduce the loads of *Salmonella typhimurium* (Serraino et al., 2012), but might be unable to definitely inactivate this microorganism (Serraino et al., 2012) and *Salmonella senftenberg* (Cortesi et al., 1998) in water buffalo mozzarella cheese, as well as *Salmonella javiana* in mozzarella cheese (Eckner et al., 1990). Villani et al. (1996) produced traditional water buffalo mozzarella cheese in a pilot plant starting from raw buffalo milk, artificially contaminated with a mixture of 4 *Listeria monocytogenes* strains at a final concentration of approximately 10^5 and 10^3 cfu/mL of vat milk, and stored the resulting final products in a conditioning liquid composed of skim water resulting from the stretching, diluted with skim whey from previous manufacture (5–6°SH, pH 3.8–4.2), and added with 1% NaCl. Although the curd ripening did not significantly affect the load of *Listeria monocytogenes*, the stretching of the curd in hot water (95°C) did not completely kill the pathogenic population (Villani et al., 1996). Increasing stretching time and temperature is of outmost importance in controlling *Listeria monocytogenes* (Buazzi et al., 1992; Kim et al., 1998) during the production of mozzarella cheese.

As demonstrated by Ricci et al. (2021), heat resistance of *Listeria monocytogenes* may be strain dependent, and using the same heat resistant strain a different response to the different time-temperature conditions was obtained depending on the different matrices considered, with drained cheese curd causing a noticeable increase in the heat resistance. Murru et al. (2018) produced traditional water buffalo mozzarella cheese at laboratory-scale using water buffalo raw milk, contaminated either with a wild-type or a reference strain of *Listeria monocytogenes*, to reach loads of around 10^3, 10^4, and 10^5 cfu of wild strain, and approximately 10^6 and 10^7 cfu of reference strain per mL of milk, in 5 different trials respectively, finding that the load of the reference strain decreased below 100 cfu/g with the stretching, whereas that of the wild-type strain remained almost the same. Thus, the curd stretching may result in incomplete inactivation of pathogens, whose thermal resistance varies with the strain and the characteristics of the matrix they inhabit. However, all these authors, to enumerate the target pathogens, used selective media, which do not allow the quantitative detection of viable, but not culturable, microbes (Fusco et al., 2012; Fusco and Quero, 2014; Quero et al., 2014). Fusco et al. (2012) used the thin agar layer method (Kang and

Fung, 1999) and the most probable number technique combined with PCR to enumerate viable and stressed *E. coli* O157:H7 cells, subjected to thermal stress in tryptone soya broth, in pasteurized milk and during a laboratory-scale production of fior di latte cheese, made either with raw or pasteurized milk added with a starter culture. These authors demonstrated that the raw milk matrices, most likely due to their specific structural and chemico-physical compositions as well as the microbial adjunct and thermal, acid, and osmotic stresses to which it was subjected during the cheesemaking, protected the *E. coli* O157:H7 strain better than the pasteurized ones (Fusco et al., 2012).

The curd is then conveyed from the stretching section to the molding section by means of screw systems. The firming of the product obtained, by immersion in cold water, is necessary to guarantee the maintenance of the shape imparted to it during the forming phase.

The last stages of processing are salting and packaging (the latter can be done by hand or using appropriate equipment). Salting, in the traditional system, takes place by immersion in brine with 12 to 15% salt for variable times according to the size of the cheese. Salting, on an industrial level, takes place during the stretching by adding 1 to 2% NaCl salt water, or by adding small quantities of saturated saline solution to the melted curd before forming, or by sprinkling with salt. The salting process is sometimes completed by adding salt (1–1.5%) to the preserving liquid (Addeo et al., 1996).

To the best of our knowledge, no studies have assessed the evolution of microbial dynamics during these last steps of the production of fresh pasta filata cheeses. Most studies assessed the microbial composition of the final mozzarella cheese.

Culture-independent approaches, such as 16S-23S spacer region analysis, PCR-DGGE, and pyrosequencing of the DNA directly extracted from industrial mozzarella cheese (made with pasteurized cow milk and commercial starter cultures), fior di latte cheese (made with raw milk and natural thermophilic milk cultures), traditional fior di latte cheese (obtained from ripened raw milk without any addition of starter cultures), and mozzarella di bufala campana (obtained from raw milk and natural why cultures; Coppola et al., 2001; Ercolini et al., 2004, 2012) revealed a microbial diversity reflecting that of the (natural) starter cultures used, or the raw milk in the case of the traditional fior di latte cheese production, with DNA profiles complexity increasing from industrial, to semi-artisanal to traditional cheeses, respectively. The next generation sequencing approach applied by Ercolini et al. (2012) revealed the presence in the buffalo mozzarella cheese of *L. delbrueckii*, *L. helveticus*, *S. thermophilus*, *Streptococcus macedonicus*, *Lact. lactis*, and *Pseudomonas* spp., (which were also present in the raw milk, NWC, and curd, or curd at the end of ripening, or both), and *Lactobacillus kefiranofaciens*, which was present only in the final product, most likely originating from the governing liquid.

Lactobacillus casei, *Enterococcus* spp., and *L. delbrueckii* ssp. *bulgaricus* were found as the dominant LAB in Brazilian mozzarella cheese obtained from whole raw milk and natural whey culture (Silva et al., 2021).

In the cow mozzarella cheese samples analyzed by de Candia et al. (2007), LAB, especially the thermophilic ones, had loads similar or higher than the natural whey starter cultures used for their production, whereas the molecular typing by RAPD-PCR confirmed the presence of the same strains inoculated with the relevant natural whey starter cultures in the mozzarella samples, leading to the hypothesis that stretching at 90°C (curd temperature 58–65°C) only slightly, or not at all, inactivated the LAB population. Similar results were achieved also by other authors (Bianchi Salvatori et al., 1999, 2000). An overall reduction of the mesophilic and thermophilic LAB populations was found following stretching (at 80°C), molding, firming, brining, and packaging within the production of traditionally made mozzarella cheese in the Apulia region (Morea et al., 1998, 1999). Packaged fior di latte di Agerola samples collected after stretching, molding, firming, salting, and packaging resulted in loads of presumptive enterococci and mesophilic and thermophilic lactobacilli, lactococci, and streptococci similar to that of the curd after ripening. Presumptive *Leuconostoc*, yeasts, and coagulase positive staphylococci were undetectable in the final products, whereas the load of presumptive *Enterobacteriaceae* decreased approximately 5 log cfu/g in the packaged fior di latte cheese, but the isolates from the final cheese samples were identified as belonging to the *S. thermophilus*, *Lact. lactis* ssp. *lactis*, and *Enterococcus faecalis* species, with certain strains occurring in all the cheesemaking steps and some others occurring only in the final products (Coppola et al., 2006), leading to hypothesize that certain strains were dominant throughout the whole cheese production, whereas the others were acquired from either the brine, the environment, or the cheesemakers. Significantly different microbiomes were found in industrial high-moisture cow mozzarella cheese (made by direct acidification with citric acid), and high-moisture mozzarella cheese made either with thermophilic defined starter or undefined starters (natural milk cultures), with most differences in dairies mainly due to the starter system used, whereas the lot-to-lot variability was responsible for the difference due to psychrotrophic contaminants (Guidone et al., 2016b). In particular,

high-moisture industrial cow milk mozzarella cheese (direct acidification) had a microbiome including LAB and psychrotrophic γ-proteobacteria, whereas the microbiome of artisanal cheese produced with undefined cultures included dominant thermophilic LAB such as *S. thermophilus*, *L. delbrueckii*, and *L. helveticus*, as well as subdominant LAB in addition to psychrotrophic bacteria and *Enterobacteriaceae*, whereas the microbiome of those produced with defined starter cultures were dominated by *S. thermophilus* (Guidone et al., 2016b). Similar results were achieved by Marino et al. (2019), who found a dominance of *S. thermophilus* in cow and buffalo high-moisture mozzarella cheese obtained with commercial cultures, whereas a prevalence of *L. delbrueckii* ssp. *bulgaricus*, *L. helveticus*, and *S. thermophilus*, and a subdominance of nonstarter LAB, was found in buffalo samples made with NWC but with a higher diversity of the microbiome in cow than in buffalo mozzarella. Cow mozzarella cheeses in which a higher prevalence of psychrophilic genera such as *Anoxybacillus*, *Flavobacterium*, *Brochothrix*, *Shewanella*, *Pseudomonas*, and *Thermus* was found, clustered apart from buffalo mozzarella cheeses, which showed a prevalence of *Lactobacillus* and *Streptococcus* taxa (Marino et al., 2019). The prevalence of the psychrotrophic microorganisms in cow mozzarella led to a hypothesis that there is more use of refrigeration during processing or storage, or both, of this type of product, compared with buffalo mozzarella cheese. Most likely due to longer exposure to suboptimal temperatures or longer production-to-consumption times, samples collected at retail level from the mass distribution circuit had a higher prevalence of psychrophilic taxa and potential spoilage microorganisms than those taken from the local market (Marino et al., 2019).

Concerning the mycobiota of fresh pasta filata cheeses, microbial loads of yeasts ranging from approximately 4 to 5 log cfu/mL or g were detected along the fior di latte di Agerola cheesemaking, but were absent in the final products (Coppola et al., 2006), whereas loads ranging from 10^4 to 10^6 were detected along the production of water buffalo mozzarella cheese by Coppola et al. (1988) and Romano et al. (2001), whereas Aponte et al. (2010), by plating on WL Agar (Oxoid), found from 1 to 4 log cfu/g of yeasts in the 16 water buffalo mozzarella samples analyzed. Stellato et al. (2015), in an attempt to characterize the microbiota occurring in a dairy processing environment found that the most abundant species, which were found to occur also in the swabs taken from the environment and equipment of production, were *K. marxianus*, *Yamadazyma triangularis*, *Trichosporon fecale*, and *Debaryomices hansenii*. However, the metagenomic approach used by these authors did not provide information on the actual prevalence of metabolically active mycobiota because it targeted DNA of both viable and dead yeasts. Aponte et al. (2010) found a prevalence of lactose-fermenting *K. marxianus* (23 strains) and galactose-fermenting *Sacc. cerevisiae* (13 strains) in 16 samples of water buffalo mozzarella cheeses produced in 16 farms located in the Campania and Lazio regions (Southern Italy), followed by 7 strains of *Pichia barkeri*, 4 of *Clavispora lusitaniae*, 4 of *Pichia norvegensis*, 2 of *Candida pararugosa*, 2 of *Pichia cactophila*, 1 of *Candida butiry/aaseri*, and 1 of *Pichia pastoris*.

Saccharomyces cerevisiae (37 isolates) and *K. marxianus* (22 isolates) were found as dominant yeasts in 10 water buffalo mozzarella samples from 3 farms located in the Basilicata region, followed by *C. sphaerica* (21 isolates), *C. kefir* (16 isolates), and *K. lactis* (9 isolates; Romano et al., 2001). A lower diversity at strain level was found within these latest isolates in respect to that found by Aponte et al. Blaiotta (2010) most likely due to the lower number of farms from which the samples were collected, and the smaller geographic area targeted by Romano et al. (2001).

Saccharomyces cerevisiae, *K. marxianus*, and *K. lactis*, which produce primarily esters, aldehydes, and alcohols, occur frequently in the yeast biota of various kinds of cheese and may contribute to their sensory characteristics mainly through the liberation of acetic, fruity, ester, and alcoholic notes (Tofalo et al., 2020). However, no studies focusing on the aroma compound production by yeasts in fresh pasta filata cheeses have been published so far.

Nonetheless, it should be highlighted that *K. marxianus/C. kefir* has been recognized as significant opportunistic fungus, although for *K. marxianus*, the qualified presumption of safety status has been recently confirmed (Ricci et al., 2018), and that certain strains of *Sacc. cerevisiae*, even isolated from foods, are emerging as opportunistic pathogens (Tofalo et al., 2020).

Storage

Usually, high-moisture mozzarella and fior di latte cheeses are packaged in a conditioning liquid, containing whey, water, brine, and stretching water, and they are subjected to refrigerated storage at 4°C for a shelf life of ≤5 d. This short shelf life is due to their physicochemical attributes, in particular the high humidity (50–60%), relatively low presence of NaCl (expressed in the aqueous phase), and, therefore, the high water activity, even in the presence of a sufficiently acidic pH (5.2–5.5), which are ineffective to limit the growth of spoilage (such as psychrophilic and proteolytic *Pseudomonas*, *Acinetobacter*, *Rahnella*, and *Psychrobacter*; Cantoni et al., 2000, 2001, 2006; Baruzzi et al., 2012;

Ricciardi et al., 2015; Stellato et al., 2015; Meier et al., 2018; Carminati et al., 2019; Marino et al., 2019) and pathogenic bacteria (such as *Listeria monocytogenes*, pathogenic *E. coli*, *Staphylococcus aureus*, *Salmonella* Typhimurium, *Cronobacter* spp., and *Bacillus cereus*; Villani et al., 1991; Spolaor and Disegna, 1995; Finazzi et al., 2011; Serraino et al., 2012, 2013; Casalinuovo et al., 2014; Greco et al., 2014; Nobili et al., 2016; Tirloni et al., 2019; Montone et al., 2020). Apart from the inability of stretching to ensure the complete inactivation of certain pathogens, as discussed above, sublethally injured cells of pathogenic bacteria may resuscitate and restart to multiply if the further processing steps, as well as the conditions of the final products, are favorable. Moreover, postprocess contaminations may occur so that pathogenic bacteria such as *Listeria monocytogenes*, pathogenic *E. coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Cronobacter* spp., and *Bacillus cereus* have been detected in mozzarella cheeses (Villani et al., 1991; Spolaor and Disegna, 1995; Finazzi et al., 2011; Serraino et al., 2012, 2013; Casalinuovo et al., 2014; Greco et al., 2014; Nobili et al., 2016; Tirloni et al., 2019; Montone et al., 2020). Concerning the spoilage of high-moisture fresh pasta filata cheeses, Baruzzi et al. (2012) investigated the prevalence of non-LAB (**NLABP**) occurring in 3 high-moisture mozzarella cheeses, packaged in governing liquid at d 0 and after 7 d of storage at 4°C and their proteolytic activity. In 2 samples, psychrotrophic NLABP were a subpopulation of mesophilic NLABP, whereas in the remaining sample, similar microbial loads of pyschrotrophic and mesophilic NLABP were already detected at d 0 (Baruzzi et al., 2012). By means of RAPD-PCR of the isolates, 66 strains were distinguished and identified by 16S rRNA and *rpo*B genes, sequencing into 25 species, conducible to 15 genera, with *Pseudomonas*, *Acinetobacter*, and *Rahnella* as main genera (Baruzzi et al., 2012). A moderate proteolytic activity in skim milk was demonstrated in 15 strains, of which 11 caused total or partial vanishing of 1 or more caseins on the outer surface of mozzarella, where a concomitant wrinkling and successive exfoliation became visible (Baruzzi et al., 2012). However, no linkage between the type of high-moisture mozzarella cheesemaking or the type of cheese and the metabiotic spoilage association could be outlined, as the authors only mentioned that all 3 mozzarella cheeses analyzed were made with natural whey or commercial starter cultures, and did not specify whether the 3 mozzarella cheeses analyzed were from raw or pasteurized cow or water buffalo milk. Moreover, no information about the composition of the governing liquid was provided by the authors and, as mentioned by the authors, the 2 samples per each mozzarella type were purchased at the market at least 10 d before the expiration date, leading to suppose all samples were taken at diverse time from the delivery to the market, that is, at different times of their own shelf lives. All the samples were instead purchased the same day they were delivered from cheesemaking plants and analyzed for their microbiological and physico-chemical composition, immediately or after 5 d of refrigerated storage at 10°C, by Ricciardi et al. (2015). The 20 samples of high-moisture cow mozzarella cheese were of 14 different brands, of which 5 used direct acidification with citric acid, 4 used the starter cultures, and the remaining 4, that provided no indication about the cheesemaking process used, were supposed to have been produced artisanally with NWC, based on results of Ricciardi et al. (2015) and pyrosequencing results by Guidone et al. (2016b). Anyway, among the parameters assessed (pH, microbiological quality, proteolysis, color, and head space composition), a high variability was found in all types of mozzarella analyzed. No significant differences were found in the microbiological quality of the samples at the beginning of storage, regardless the industrial or artisanal production and the different acidification methods used (direct acidification/defined starters/undefined starters use), whereas at the end of the storage, all parameters were affected by the diverse mode of acidification, with mozzarella cheese produced by direct acidification having a significantly lower microbiological quality (Ricciardi et al., 2015). However, after 5 d of refrigerated storage, most samples had microbial loads of psychrotrophs higher than 10^7 cfu/g, and a significant correlation between the microbial counts and the remaining shelf life was demonstrated (Ricciardi et al., 2015). Various groups of psychrotrophic bacteria of the genera *Flavobacteriaceae*, *Pseudomonadaceae*, *Shewanellaceae*, *Listeriaceae*, *Enterobacteriaceae*, *Aeromonadaceae*, *Moraxellaceae*, *Aeromonas*, *Pantoea*, *Raoultella*, *Serratia*, *Acinetobacter*, and *Brochothrix* were found in all samples analyzed, with a more abundance in direct-acidified mozzarella (Guidone et al., 2016b). These microorganisms, whose sources in milk and cheeses may be contaminated water and soil, milking surfaces, processing, storage, and transporting equipment, have been associated with spoilage, mainly consisting in discoloration (as the blue discoloration of mozzarella cheese due to *Pseudomonas fluorescence*; Gennari and Dragotto, 1992; Sechi et al., 2011; Carrascosa et al., 2015), loss of structure, and off-flavors (Faggiano et al., 2018; Odeyemi et al., 2020; Carrascosa et al., 2021). A high variability in the microbial counts of psychrotrophic microorganisms in the different samples at the beginning of storage has been detected by other researchers (Conte et al., 2007, 2013; Gammariello et al., 2008; Sinigaglia et al., 2008; Del Nobile et al., 2009; Faccia et al., 2013; Lucera et al., 2014), although a

limited variety of samples was used in their studies. A higher prevalence of psychrophilic taxa and potential spoilage microorganisms was found in samples collected at retail level from the mass distribution circuit than in those taken from the local market, most likely due to longer exposure to suboptimal temperatures or longer production-to-consumption times (Marino et al., 2019).

To control the spoilage and prolong the shelf life of mozzarella and fior di latte cheeses, several technologies are being explored including the addition of preservatives (either in the brine or in the governing liquid or in the material used for packaging) or coating, as well as the employment of modified atmosphere for the packaging by several researchers (Conte et al., 2007; Gammariello et al., 2008; Sinigaglia et al., 2008; Del Nobile et al., 2009; Lucera et al., 2014; Mastromatteo et al., 2014; Braghieri et al., 2018; Faccia et al., 2019; Zappia et al., 2020a,b; Chang et al., 2021; Deshwal et al., 2021; Quintieri et al., 2021).

CONCLUSIONS

Fresh pasta filata cheeses are produced worldwide. However, high-moisture fior di latte and mozzarella cheeses, mainly produced in Italy and protected by quality brands such as the European PDO, protected geographical indication (PGI), and traditional specialty guaranteed (TSG), are characterized by a complex microbiota consisting of a consortium of prokaryotic and eukaryotic populations whose succession, interactions, and metabolic activities are mediated by a wide range of biotic and abiotic factors that occur during production and storage of these cheeses and affect the overall safety and typical quality of the final products. Most information on the complex composition and activity of such consortia has been obtained in the latest years thanks to the omics technologies. However, all the metagenomics approach used in these studies have targeted DNA, thus, not only viable, but also dead microbes present in the cheese matrices. Therefore, culturomics and metatranscriptomic approaches are needed, being more appropriate to describe the actual metabolically active microbiota involved.

NOTE FOR THE READER

A reclassification of the genus *Lactobacillus* into 25 genera, including the emended genus *Lactobacillus*, *Paralactobacillus*, and 23 novel genera (*Holzapfelia*, *Amylolactobacillus*, *Bombilactobacillus*, *Companilactobacillus*, *Lapidilactobacillus*, *Agrilactobacillus*, *Schleiferilactobacillus*, *Loigolactobacillus*, *Lacticaseibacillus*, *Latilactobacillus*, *Dellaglioa*, *Liquorilactobacillus*, *Ligilactobacillus*, *Lactiplantibacillus*, *Furfurilactobacillus*, *Paucilactobacillus*, *Limosilactobacillus*, *Fructilactobacillus*, *Acetilactobacillus*, *Apilactobacillus*, *Levilactobacillus*, *Secundilactobacillus*, and *Lentilactobacillus*), has been proposed by Zheng et al. (2020). Herein, we provide the new nomenclature for the taxa that have been proposed to be reclassified when they are encountered for the first time in the text. Moreover, in the present manuscript, the reference to the genus *Lactobacillus* is intended to be *sensu lato*, following the traditional nomenclature used before the proposed reclassification.

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