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Potential growth of *Listeria monocytogenes* in Italian mozzarella cheese as affected by microbiological and chemical-physical environment

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ABSTRACT

In the present study, 33 brands of mozzarella cheese (pasteurized cow milk mozzarella obtained by direct acidification through the addition of food-grade citric acid or obtained by natural acidification through the addition of thermophilic starter cultures, mozzarella for pizza mainly obtained by addition of citric acid, and pasteurized buffalo milk mozzarella obtained by adding microbial rennet) were characterized for the factors potentially influencing the growth of *Listeria monocytogenes* (microbial populations, moisture, pH, and organic acids). Then, the growth potential of *L. monocytogenes* in mozzarella was investigated by challenge tests performed at different temperatures. The presence of heterogeneous microflora (lactobacilli, streptococci, *Pseudomonas* spp., and, for buffalo mozzarella, yeasts) was evidenced. Almost all the product typologies were classified as high-moisture mozzarella cheese because moisture was >52%. Moreover, pH varied from 5.32 to 6.43 depending on the manufacturing methodology applied. Organic acid concentrations too showed great variability depending on the mozzarella production method, with values ranging from less than limit of detection (LOD; 16 mg/kg) to 14,709 mg/kg, less than LOD (216 mg/kg) to 29,195 mg/kg, and less than LOD (47 mg/kg) to 1,725 mg/kg in the water phase of lactic, citric, and acetic acids, respectively. Despite this presence, the concentration of undissociated acids was lower compared with the minimum inhibitory concentrations estimated for *L. monocytogenes* by other authors. This was confirmed by the results of the challenge tests conducted inoculating the pathogen in mozzarella produced with the addition of citric acid, as the microorganism grew fast at each temperature considered (4, 9, 15, and 20°C). Good hygiene practices should be strictly applied, especially with the aim of avoiding postproduction contamination of mozzarella, as the presence of organic acids and microflora is insufficient to prevent *L. monocytogenes* growth.

Key words: *pasta filata* cheese, *Listeria monocytogenes*, growth potential, undissociated food-grade organic acids, pH

INTRODUCTION

Mozzarella is one of the most sold fresh cheeses in Italy, representing a 25% share of the cheese market. It is a soft, unripened *pasta filata* (spun paste) cheese manufactured from either pasteurized or unpasteurized milk. Bovine or buffalo milk are most often used. Two different acidification methods may be applied during its manufacture: direct acidification by addition of food-grade organic acids (typically, citric acid is added to pasteurized milk before the inclusion of rennet) or microbial acidification resulting from the growth of thermophilic starter cultures.

Unfortunately, listeriosis outbreak investigations are frequently associated with processed and ready-to-eat foods, such as meats, dairy products, cold smoked or gravad fish products, fruits, and vegetables (Jackson et al., 2011; McCollum et al., 2013; McIntyre et al., 2015; Gillesberg Lassen et al., 2016; Jensen et al., 2016). Listeriosis is a serious and sometimes lethal infection that may occur after eating food contaminated with *Listeria monocytogenes*. It is no surprise that some dairy products have been involved in listeriosis outbreaks because all fresh and soft cheeses, including mozzarella, could potentially represent a suitable growth environment for *L. monocytogenes* due to their physicochemical characteristics (high moisture and pH close to neutrality). Several alerts were also issued by Rapid Alert System for Food and Feed for the presence of *L. monocytogenes* in mozzarella during the period 2009 to 2017 (RASFF, 2018).

Contamination of milk by *L. monocytogenes* used for cheese production could occur during milking. However, if the pathogen is present in the milk, it is likely inactivated during the mozzarella production process due to the heat treatment applied during the spinning

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step, where temperatures may reach 90 to 95°C. Contamination could also occur during or after the remaining cheese-making process. The latter possibility was confirmed by Greco et al. (2014), who revealed a high prevalence of *L. monocytogenes* (24.4%) in mozzarella cheese due to cross-contamination.

Listeria monocytogenes growth in dairy products may be inhibited by the presence of short-chain food-grade organic acids in their undissociated form that may pass the cell membrane of the microorganism (Mitchell, 1961; Mejlholm et al., 2015; Wang et al., 2015; Wemmenhove et al., 2016). Organic acids may be present in dairy products as a product of hydrolysis of milk fat (free fatty acids such as acetic or butyric acids) or derive from ordinary bovine biochemical metabolism (citric, orotic, and uric acids). Direct addition of food-grade organic acids during the cheese-making process (mainly citric and lactic acids) is another option. Furthermore, organic acids can also derive from bacterial metabolism with lactic, acetic, pyruvic, propionic, and formic acids as the major products of carbohydrate catabolism of lactic acid bacteria (Tormo and Izco, 2004).

Previous studies that established MIC of undissociated acids for *L. monocytogenes* are available but limited and characterized by high variability between different strains. In a few studies, MIC were calculated under conditions relevant to dairy products (Coroller et al., 2005; van der Veen et al., 2008; Wemmenhove et al., 2016, 2018; Tirloni et al., 2019). All of these data are useful, especially in the determination of the ability of *L. monocytogenes* to grow in a specific substrate; this is a critical factor considered also by Commission Regulation (EC) No. 2073/2005 (European Commission, 2005), aiming to prevent the consumption of ready-to-eat foods with concentrations exceeding 100 cfu/g. Millet et al. (2006), for example, confirmed that low pH and the presence of short-chain organic acids help inhibit the growth of *L. monocytogenes* in Dutch-type cheeses such Gouda, Edam, and Maasdam, made from pasteurized milk with rennet-induced curd formation. Moreover, Tirloni et al. (2019) developed 2 cardinal parameters models that described the growth of *L. monocytogenes* in ricotta, including terms for temperature and pH, and evaluated another growth model including the effect of organic acids in the same substrate, showing a lack of antilisterial effect of organic acids in this product.

The objective of the present study was to characterize from a microbiological and physicochemical point of view several mozzarella brands on the Italian market representing a variety of product characteristics such as milk origin (cow or buffalo milk), acidification methods, and addition of organic acids. Then, the growth potential of *L. monocytogenes* in mozzarella was investigated at various temperatures to establish the influence of microbiological and chemical factors (in particular, the presence of organic acids).

MATERIALS AND METHODS

Microbiological Screening of Mozzarella Cheese

Mozzarella cheese samples from 33 producers (1–33) were obtained on the day of production. Part of these mozzarella samples were produced starting with pasteurized cow milk (1–27). The products intended for consumption as table cheese (1–20) were produced by natural acidification obtained through the addition of thermophilic starter cultures and cow rennet (1–12) or by direct acidification through the addition of food-grade citric acid (13–20). The samples classified as mozzarella for pizza (21–27) were mainly obtained by direct acidification through the addition of citric acid. Finally, some samples of pasteurized buffalo milk mozzarella obtained by adding microbial rennet (28–33) were considered. All mozzarella samples were packaged with a brine except for mozzarella for pizza; all were inserted in a package that was thermally sealed without modified-atmosphere packaging.

The weight of the samples varied from 100 to 250 g, depending on the producer, in forms of pearls (2–3 g), cherries (7–8 g), or whole pieces (from 100 up to 250 g). The stated commercial shelf life was between 25 and 35 d at 4°C depending on the brands considered. During transport to the laboratory, samples were maintained on ice and were analyzed within 4 h. Microbiological analyses were carried out at the beginning (**T0**) and at the end (**T final**) of the declared shelf life. All analyses were performed in duplicate. For microbial counts, 10 g of each sample was homogenized in 90 mL of pre-chilled sterile diluent solution (0.85% NaCl and 0.1% peptone) for 60 s in a Stomacher 400 (Seward Medical, London, UK), and appropriate serial 10-fold dilutions were performed. Total viable count was determined using spread plating on plate count agar (CM0325; Oxoid, Basingstoke, UK) according to ISO method 4833-1:2013 (ISO, 2013). *Pseudomonas* spp. were enumerated on *Pseudomonas* agar base supplemented with cetrimide, fucidin, cephaloridine supplement (Biogenetics, Ponte San Nicolò, Italy) incubated at 30°C for 48 h, *Enterobacteriaceae* were enumerated on violet red bile glucose agar (Biogenetics) incubated at 37°C for 24 h (ISO 21528-2:2004; ISO, 2004), and yeasts and molds were enumerated on Sabouraud dextrose agar (Biogenetics) incubated at 30°C for 96 h (ISO 21527-1:2008; ISO, 2008). Lactobacilli were enumerated on de Man,

Rogosa and Sharpe agar (Biogenetics) and incubated in anaerobiosis at 30°C for 48 h (ISO 15214:1998; ISO, 1998), lactococci were enumerated onto M17 agar (Biogenetics) supplemented with lactose (10 mL/L) and subsequently incubated at 20°C for 48 h, and streptococci were enumerated onto M17 and subsequently incubated at 37°C for 48 h. Qualitative detection of *L. monocytogenes* in 25 g of mozzarella was performed according to the AFNOR (1998) BRD 07/4-09/98 method at T0.

Physicochemical Screening of Mozzarella Cheese

The pH was measured at each sampling time in duplicate using a pH meter (Amel Instruments, Milan, Italy), water activity was determined at T0 (Rotronic Hygromer Aw-DIO, Basserdorf, Switzerland), and moisture was determined (Bradley and Vanderwarn, 2001) after homogenization by a rotary grater. Then, 3 ± 0.25 g of mozzarella was placed in aluminum moisture dish (5.5 cm) and dried in a forced-air oven at 100°C for 16.5 h.

Concentrations of organic acids were determined by HPLC (Tormo and Izco, 2004) according to the method previously described by Tirloni et al. (2019). Concentrations of undissociated organic acids in the water phase were estimated by the following equation:

$$[\text{undissociated acid}] = \frac{[\text{total acid}]}{1 + 10^{\text{pH}-\text{p}K_a}}. \quad [1]$$

Challenge Tests with Inoculated Mozzarella Cheese

Challenge tests were carried out to evaluate the growth potential of *L. monocytogenes* in a brand of mozzarella produced by direct acidification mainly due to the addition of citric acid. Based on the results obtained from Physicochemical Screening of Mozzarella Cheese, the brand most likely to favor growth of *L. monocytogenes*, considering its pH and content of organic acids, was chosen for subsequent challenge tests. Growth of *L. monocytogenes* was evaluated in this brand of mozzarella (packages containing a total of 150 g of mozzarella cherries of about 8 g each, immersed in 100 mL of brine) by conducting 7 challenge tests (from 7 to 15 sampling times for each challenge test) in triplicate at constant temperatures (2 challenge tests at 4°C, 2 challenge tests at 9°C, 2 challenge tests at 15°C, and 1 challenge test at 20°C), obtaining a total of 21 growth curves. A total of 177 samples of mozzarella were considered in this trial.

Bacterial Strains and Inoculation. Mozzarella samples used for challenge tests were inoculated with a mixture of 3 *L. monocytogenes* strains, including a human isolate (ATCC 7644), an isolate from a dairy product, and a strain responsible for a previous outbreak due to a contaminated dairy product. The subculturation of the 3 strains was performed using the method described by Tirloni et al. (2017a,b, 2018, 2019). The inoculation was performed in the original brine (100 mL), reaching a concentration of 3.7 to 4 log cfu/mL; the inoculum volume did not exceed 1% of the product weight. We considered an average surface of each mozzarella cherry of about 15 cm^2 (spheres about 2.2 cm in diameter) and hypothesized the adsorption of 0.1 mL of brine by each 1 cm^2 of mozzarella surface. Thus, we obtained a presumed concentration of 2 to 3 log cfu/g considering the whole mozzarella pieces (internal mass + surface). Afterward, inoculated samples were stored at a fixed temperature according to the experimental plan.

Microbiological Analyses. Inoculated samples were analyzed at settled intervals, and *L. monocytogenes* counts were determined in triplicate. For each series, between 8 and 17 sampling times were included depending on storage temperature. For microbiological analyses, 10 g of mozzarella was 10-fold diluted in prechilled sterile saline (0.85% NaCl and 0.1% peptone) and homogenized for 60 s in a Stomacher 400. Appropriate 10-fold dilutions were then made with prechilled sterile saline, and *L. monocytogenes* was enumerated by spread plating onto Palcam agar (Oxoid) with added Palcam selective supplement (Oxoid) and incubated at 37°C for 48 h. On the same samples, total viable count, *Pseudomonas* spp., *Enterobacteriaceae*, yeasts and molds, lactobacilli, lactococci, and streptococci were also enumerated according to the methods described in Microbiological Screening of Mozzarella Cheese.

For *L. monocytogenes*, the initial cell concentration (log cfu/g), lag time (h), maximum specific growth rate (μ_{max}, h^{-1}), and maximum population density (log cfu/g) were determined by fitting a primary model to the growth kinetics obtained. The maximum specific growth rate is an important parameter for the evaluation of the growth curve that allows one to estimate the concentration of the pathogen at any specific day of the shelf life knowing the starting concentration of *L. monocytogenes* in the product.

The Baranyi and Roberts (1994) model was fitted to the log-transformed growth data using the Excel (Microsoft Corp., Redmond, WA) add-in DMFit of the Institute of Food Research (Reading, UK). A conversion factor of ln 10 was multiplied with the DMfit-estimated growth rates, as the bacterial concentrations in DMfit were log transformed.

Physicochemical Analyses. During storage, temperatures were recorded by data loggers (Escort iLog, Escort Data Logging System Ltd., Aesch Bei Birmens-

Table 1. Physicochemical results obtained for 33 brands of mozzarella cheese[1]

Brand	pH	Moisture content (%)	Lactic acid (water phase)		Citric acid (water phase)		Acetic acid (water phase)	
			Mean (mg/kg)	SD	Mean (mg/kg)	SD	Mean (mg/kg)	SD
Cow milk with starter cultures								
1	5.86	67.13	2,289	324	6,835	1,234	169	12
2	6.12	70.65	1,183	29	4,236	223	161	117
3	5.74	68.78	2,527	72	6,992	65	213	79
4	5.78	61.90	4,073	204	9,843	41	343	154
5	6.04	64.81	2,059	0	<LOD	—	238	337
6	5.83	66.52	2,553	0	<LOD	—	559	0
7	6.00	60.21	1,821	3	6,283	42	177	11
8	5.95	60.02	2,499	7	5,561	253	206	6
9	5.68	65.68	2,954	0	3,347	4,733	266	375
10	5.69	59.46	4,087	144	10,060	429	297	14
11	5.77	59.86	4,349	50	14,261	715	365	16
12	6.23	61.65	1,875	11	20,815	141	<LOQ[2]	31
Cow milk with citric acid								
13	6.13	65.88	311	16	13,318	451	421	85
14	5.49	63.81	<LOD	—	1,409	—	<LOD	—
15	6.38	63.18	<LOD	—	1,287	—	914	—
16	6.13	67.41	1,230	56	9,734	525	267	7
17	5.77	62.15	4,255	368	10,976	265	317	92
18	6.41	63.83	<LOD	—	9,024	275	0	—
19	5.43	67.17	122	68	11,333	67	459	5
20	6.43	62.70	175	11	7,803	797	364	30
Cow milk for pizza								
21	5.77	53.55	10,955	484	20,502	627	782	33
22	5.90	49.34	14,709	304	29,195	351	1,725	37
23	5.69	49.96	14,677	1,236	28,435	1,254	1,105	98
24	6.23	55.31	650	79	26,120	1,003	717	25
25	6.05	61.07	814	12	21,752	381	543	11
26	5.72	48.47	14,312	749	25,223	1,207	1,583	114
27	6.48	63.84	2,970	18	32,606	221	239	49
Buffalo milk with microbial rennet								
28	5.32	65.30	4,755	512	12,321	1,412	<LOD	—
29	5.47	65.00	4,774	80	10,800	422	<LOD	—
30	5.33	62.37	4,301	0	5,226	7,390	<LOD	—
31	5.56	65.77	1,756	3	21,173	524	<LOD	—
32	5.42	62.15	5,782	40	1,930	237	647	13
33	5.37	61.65	4,647	39	8,339	306	<LOD	—

[1]LOQ = limit of quantification (722 mg/kg for citric acid, 52 mg/kg for lactic acid, and 157 mg/kg for acetic acid). LOD = limit of detection (216 mg/kg for citric acid, 16 mg/kg for lactic acid, and 47 mg/kg for acetic acid).
[2]LOQ = 152.

dorf, Switzerland), and pH was measured at T0 and T final in triplicate using a pH meter (Amel Instruments). Organic acid concentrations were determined at T0 and on the last sampling day in triplicate as described previously.

RESULTS AND DISCUSSION

Physicochemical Screening of Mozzarella Cheese

Mozzarella cheese comprises a wide range of product typologies obtained with different technologies. These differences are completely reflected in the intrinsic characteristics of each brand considered, as shown by the results obtained from the physicochemical and microbiological analyses (Tables 1 and 2). To our knowledge, no previous studies focused on a broad variety of mozzarella brands as in the present study, and the physicochemical characterization of a high number of mozzarella cheese brands aimed to supply more complete information about the products available on the market.

All the samples produced with cow milk by adding citric acid or starter cultures and those produced with buffalo milk had a moisture content >52% (all >60%); thus, according to the Italian Dairy Association (Assolatte, 2009), they could be classified as high-moisture mozzarella cheese, mainly used fresh as a table cheese.

The samples produced from cow milk and defined as mozzarella for pizza (samples 21–27) showed significant variability in moisture content: 3 out of 7 brands had values between 42 and 52%. Thus, they should be classified as low-moisture mozzarella, mainly used as a pizza topping or as an ingredient in other recipes. The other 4 samples had a higher moisture (>52%); these data confirmed the high variability among cheeses in this category.

Mozzarella samples produced from cow milk by adding citric acid were characterized by an average pH of 6.02 ± 0.41. As a consequence of its addition during production, this food-grade acid was present in large amounts in the water phase, with an average concentration of 8,110 mg/kg (values from 1,287 to 13,318 mg/kg). However, citric acid is considered a mild antimicrobial agent, and to obtain an inhibiting effect it should be used in combination with other organic acids. In that case, an extension of the lag phase of the spoilage microorganisms could be obtained, leading to a reduction of total count (Stella et al., 2014).

Lactic acid is recognized as a more efficient antimicrobial agent than citric acid, and it possesses an inhibitory action against pathogenic microorganisms

Table 2. Main microflora (expressed in parentheses as log cfu/g) present at the beginning (T0) and end (T final; assigned by producer) of the declared shelf life in 33 brands of mozzarella cheese

Brand	T0	T final
Cow milk with starter cultures		
1	Streptococci (>5), *Pseudomonas* spp. (>5)	*Pseudomonas* spp. (>8)
2	Streptococci (>5)	*Pseudomonas* spp. (>8)
3	Streptococci (>8)	Streptococci (>8)
4	Streptococci (>7)	Streptococci (>7)
5	Lactobacilli (>7)	Streptococci (>8), lactobacilli (>8)
6	Streptococci (>7), lactobacilli (>7)	Streptococci (>8), lactobacilli (>8)
7	Streptococci (>5)	Streptococci (>8), lactobacilli (>8), *Pseudomonas* spp. (>8)
8	Streptococci (>8), lactobacilli (>7), *Pseudomonas* spp. (>5)	Streptococci (>8), lactobacilli (>8), *Pseudomonas* spp. (>8)
9	Streptococci (>8), lactobacilli (>7), *Pseudomonas* spp. (>5)	Streptococci (>8), lactobacilli (>8), *Pseudomonas* spp. (>7)
10	Streptococci (>6), *Pseudomonas* spp. (>5)	Streptococci (>7), lactobacilli (>7)
11	Streptococci (>7)	Streptococci (>7)
12	Streptococci (>7), lactobacilli (>7), *Pseudomonas* spp. (>7)	Streptococci (>8), lactobacilli (>7), *Pseudomonas* spp. (>7)
Cow milk with citric acid		
13	Lactobacilli (>5)	Streptococci (>7), lactobacilli (>6)
14	Lactobacilli (>5)	Lactobacilli (>6)
15	Streptococci (>7), *Pseudomonas* spp. (>7)	Streptococci (>7), *Pseudomonas* spp. (>7)
16	Streptococci (>5)	Streptococci (>5), lactobacilli (>5), *Pseudomonas* spp. (>6)
17	Streptococci (>5), *Pseudomonas* spp. (>5)	Streptococci (>5), lactobacilli (>5)
18	—	Streptococci (>6)
19	Lactobacilli (>5)	Lactobacilli (>6)
20	—	Streptococci (>5), lactobacilli (>6)
Cow milk for pizza		
21	Streptococci (>5)	Streptococci (>8)
22	Streptococci (>7), lactobacilli (>5)	Streptococci (>8), lactobacilli (>6)
23	Streptococci (>7)	Streptococci (>8)
24	Lactobacilli (>5)	Lactobacilli (>6)
25	Lactococci (>5)	Streptococci (>6), lactobacilli (>7), *Pseudomonas* spp. (>7), lactococci (>6)
26	Streptococci (>5), lactobacilli (>5)	Streptococci (>6), lactobacilli (>7)
27	Streptococci (>5), lactobacilli (>5), *Pseudomonas* spp. (>5), lactococci (>5)	Streptococci (>6), lactobacilli (>6), *Pseudomonas* spp. (>6), lactococci (>6)
Buffalo milk with microbial rennet		
28	Yeasts (>5)	Yeasts (>7), lactobacilli (>6)
29	Yeasts (>5), lactobacilli (>5), lactococci (>5)	Yeasts (>7), lactobacilli (>6), lactococci (>6), streptococci (>6)
30	Yeasts (>5), lactobacilli (>5), lactococci (>5), streptococci (>5)	Yeasts (>7), lactobacilli (>6), lactococci (>6), streptococci (>6)
31	—	Yeasts (>5), lactobacilli (>7), streptococci (>5)
32	Yeasts (>5), lactobacilli (>7), lactococci (>6), streptococci (>6)	Yeasts (>6), lactobacilli (>8), lactococci (>7), streptococci (>7)
33	Yeasts (>5), lactobacilli (>5), lactococci (>5)	Yeasts (>6), lactobacilli (>6), lactococci (>6)

(Seyfert et al., 2007). The analyses showed the presence of variable amounts of this acid in the samples [mean value = 1,016 ± 1,647 mg/kg, with values ranging from less than limit of detection (**LOD**; 16 mg/kg) to 4,255 mg/kg in the water phase]. Low concentrations of acetic acid were also detected (maximum value determined = 459 mg/kg); as already stated, the efficient antimicrobial action of this acid is due to its ability to reduce the pH and consequently to compromise the bacterial cell walls (Lück and Jager, 1997). In any case, the amount of lactic and acetic acids detected in the samples did not seem to represent a significant hurdle for the prevention of *L. monocytogenes* growth if postprocessing contamination eventually occurs.

Mozzarella samples produced from cow milk by adding starter cultures were characterized by an almost constant pH (mean value = 5.89 ± 0.18); citric acid was present with an average concentration of 8,823 mg/kg in the water phase, with values ranging from less than LOD (16 mg/kg) to 20,815 mg/kg. The concentration of lactic acid was significantly higher (P = 0.04) compared with cow milk mozzarella produced by adding citric acid (average = 2,689 mg/kg in the water phase, with values ranging from 1,183 to 4,349 mg/kg). Low concentrations of acetic acid were also measured (maximum value determined = 559 mg/kg), confirming its small role in this product typology.

In cow milk mozzarella for pizza, mean pH determined was 5.98 ± 0.29; moreover, high mean concentrations of citric and lactic acids were detected (26,262 ± 4,246 and 8,441 ± 6,680 mg/kg, respectively, in the water phase). A higher concentration of acetic acid was also revealed in these samples, with values ranging from 239 to 1,725 mg/kg. A very high heterogeneity was revealed within the brands present in this category.

Finally, buffalo milk mozzarella showed very different characteristics compared with the samples belonging to the other 3 categories. First, the average pH was significantly lower (5.41); although lactic and citric acid concentrations were generally in line with the mozzarella samples of the other typologies, acetic acid concentration was below the LOD (47 mg/kg) for almost all the brands.

Few studies established accurate MIC of undissociated lactic, acetic, and citric acids for *L. monocytogenes*. Wemmenhove et al. (2016) calculated the MIC for *L. monocytogenes* in broth in a range of pH that included relevant conditions for Dutch-type cheeses (5.2–5.6) at 30°C: the MIC of undissociated lactic, acetic, and citric acids were estimated on average to be 5.0 ± 1.5, 19.0 ± 6.5, and 3.8 ± 0.9 mM, respectively. The concentrations of undissociated organic acids calculated applying the equation described in Physicochemical Screening of Mozzarella Cheese (Table 1) were lower in the mozzarella samples from all the different brands compared with the MIC values reported by Wemmenhove et al. (2016; Table 3). Tirloni et al. (2019) showed that a concentration of undissociated acetic acid >8.8 mM inhibited the growth of *L. monocytogenes* in ricotta; the same effect was obtained with a concentration of undissociated citric acid >24.0 mM and of lactic acid from 5.1 to 14.7 mM. Thus, *L. monocytogenes* may likely be able to grow on any mozzarella cheese substrate independently from the milk origin or the technology used to produce it.

The MIC proposed by Wemmenhove et al. (2016) and Tirloni et al. (2019) were generally in agreement with those reported by Oh et al. (2016) for this pathogen and measured in broth at 37°C. To our knowledge, MIC values for organic acids in real dairy substrates have not been investigated previously; apart from the strain specificity (as described by Wemmenhove et al., 2016), an effect of food structure on the chemical redistribution of organic acids is also likely to occur in these complex matrices (Brocklehurst et al., 1993; Brocklehurst and Wilson, 2000). Structural features of the water phase of a food could be important for microbial growth, and the effect of food structure often results in a fail-safe behavior, with slower bacterial growth compared with that observed in broths (Wilson et al., 2002).

Finally, results obtained from the water activity determination, an important physicochemical parameter that has an effect on bacterial growth, showed constant values among the mozzarella samples belonging to the same category, with average values equal to 0.988 ± 0.02, 0.982 ± 0.01, 0.974 ± 0.07, and 0.984 ± 0.06 for mozzarella cheese obtained by direct acidification, natural acidification through the addition of thermophilic starter cultures, mozzarella for pizza, and buffalo mozzarella, respectively. All these results confirmed the potential ability of *L. monocytogenes* to grow on these substrates, as the reported limit of growth was 0.92, far lower compared with our results (Cole et al., 1990).

Microbiological Characterization

From a microbiological point of view, a high heterogeneity was found within the brands of the same category (Table 2) considering the main bacterial populations (present with loads >5 log cfu/g in the product). At the end of the shelf life, the microflora mainly comprised lactobacilli and streptococci in 3 out of 8 cow milk mozzarella brands obtained by addition of citric acid. The presence of *Lactobacillus* alone was revealed in 3 brands, and in the remaining 2 brands a combination of streptococci and *Pseudomonas* spp. and a combination of streptococci, lactobacilli, and *Pseudomonas* spp. were detected.

Table 3. Undissociated (UN) organic acid concentrations calculated in mozzarella cheese samples

Item	UN lactic acid (m*M*)	UN citric acid (m*M*)	UN acetic acid (m*M*)
MIC			
Wemmenhove et al. (2016)	5.0 ± 0.5	19.0 ± 6.5	3.8 ± 0.9
Tirloni et al. (2019)	5.1–14.7	>24	>8.8
Cheese sample			
1	0.25	0.06	0.21
2	0.07	0.02	0.11
3	0.36	0.08	0.34
4	0.54	0.11	0.50
5	0.15	0.00	0.20
6	0.30	0.00	0.73
7	0.15	0.04	0.16
8	0.22	0.04	0.21
9	0.49	0.05	0.47
10	0.66	0.13	0.52
11	0.59	0.16	0.54
12	0.09	0.08	0.08
13	0.02	0.06	0.29
14	0.00	0.03	0.00
15	0.00	0.00	0.36
16	0.07	0.05	0.18
17	0.57	0.12	0.47
18	0.00	0.02	0.00
19	0.04	0.27	1.35
20	0.01	0.02	0.13
21	1.48	0.23	1.16
22	1.48	0.24	1.94
23	2.37	0.38	1.94
24	0.03	0.10	0.39
25	0.06	0.13	0.44
26	2.16	0.31	2.60
27	0.08	0.07	0.07
28	1.77	0.38	0.00
29	1.27	0.24	0.00
30	1.56	0.16	0.00
31	0.38	0.38	0.00
32	1.72	0.05	1.93
33	1.55	0.23	0.00

In cow milk mozzarella brands obtained by the addition of starter cultures, the main microflora comprised a combination of lactobacilli, streptococci, and *Pseudomonas* spp. in 4 out of 12 brands analyzed. Five brands showed the presence of high loads of a single microbial group (streptococci in 3 brands and *Pseudomonas* spp. in 2 brands), and the remaining 3 brands showed a microflora dominated by a combination of streptococci and lactobacilli.

In cow milk mozzarella for pizza, the main microflora was dominated by a single group in 3 out of 7 brands (streptococci in 2 brands and lactobacilli in 1 brand). A microflora mainly comprising lactobacilli, streptococci, and *Pseudomonas* spp. was observed in 2 brands, and a combination of lactobacilli, streptococci, lactococci, and *Pseudomonas* spp. was detected in the remaining 2 brands.

Finally, buffalo mozzarella samples were characterized by a constant presence of high counts of yeasts (up to 7 log cfu/g) in combination with lactococci (1 out of 6 brands analyzed); lactobacilli (1 brand); lactobacilli and streptococci (1 brand); or lactobacilli, streptococci, and lactococci (3 brands).

Generally, in the mozzarella samples analyzed, at T0 the natural microflora (lactic acid bacteria or spoilage) was already present, although in lower loads, thus probably reflecting the hygienic conditions of the cheese-making process. In few cases, at T0 the microflora was below the detection limit (samples 18 and 31); *Enterobacteriaceae* loads were always below the detection limit (2 log cfu/g). *Listeria monocytogenes* was never detected in any of the samples analyzed at T0 or T final.

Generally, results obtained from the present study were in agreement with those stated by previous studies where micrococci and butyric and propionic acid bacteria occurred only occasionally, whereas lactobacilli, streptococci, lactococci, *Carnobacterium* spp., *Leuconostoc* spp., *Aerococcus* spp., enterococci, and staphylococci represented the dominant bacterial com-

munities in cow milk mozzarella (Coppola et al., 1988, 2001; Ercolini et al., 2001). For buffalo milk mozzarella, yeasts should also be included in the main resident microflora. Moreover, previous studies highlighted microbial diversity among different typologies of mozzarella, discerning those produced in industrial, semiartisanal, or traditional scale by progressively complex DNA profiles (Coppola et al., 1988, 2001; Morea et al., 1999; Ercolini et al., 2001).

Challenge Tests with Inoculated Mozzarella Cheeses

In the present study, postprocess contamination by *L. monocytogenes* was simulated in a brand of cow milk mozzarella produced by adding citric acid, and its behavior was evaluated during the shelf life at different storage temperatures. Temperatures included optimal refrigeration (4°C) as well as mild (9°C) and strong (15 and 20°C) thermal abuse conditions. Generally, mozzarella producers affirm that the suggested temperature of product storage, useful to promote its flavor, should be around 12 to 15°C.

It also should be noted that if the heat treatment applied during mozzarella production is sufficient to inactivate low concentrations of *L. monocytogenes* that could be present in the curd, postprocess contamination may also happen (Stecchini et al., 1995; Villani et al., 1996). Previous studies have attested a prevalence of this pathogen in around 6% of mozzarella samples (Castellucci et al., 1996).

Listeria monocytogenes grew fast in the cow milk mozzarella selected for the challenge tests independently from the temperature considered (Figure 1; Table 4). At 4°C, an increase in loads between 3.36 and 3.99 log cfu/g was obtained after 13 and 17 d, respectively (μ_{max} from 0.026 to 0.053 log cfu/h), whereas at 9°C an increase of 4.43 and 4.75 log cfu/g was obtained after less than 6 d of storage (μ_{max} from 0.084 to 0.096 log cfu/h). No lag was estimated for kinetic growth at 4°C, whereas a lag of 1 and 2.4 h was estimated at 9°C.

Considering a strong thermal abuse (15°C), an increase in loads of 4.26 and 4.99 log cfu/g was obtained in less than 4 d of storage (μ_{max} = 0.21 log cfu/h for both series), whereas at 20°C an increase in loads of 4.35 log cfu/g was obtained in just over 2 d of storage (μ_{max} = 0.39 log cfu/h). Lag time was estimated to be 3.4 and 6.5 h at 15°C and 4.4 h at 20°C. If we consider possible household contamination of the product occurring after the opening of a package and subsequent storage, it is clear that at refrigeration conditions as well as in thermal abuse, the pathogen would be able to reach loads of concern in a brief time without any strong hurdle.

In the evaluation of the product characteristics influencing the growth of *L. monocytogenes*, the natural microflora was enumerated during all the sampling times. The main bacterial population comprised streptococci and lactobacilli at T0 (loads from 3.3 to 3.45 log cfu/g) according to the results obtained in the characterization section. This microflora showed an evident growth during all the challenge tests performed at the different temperatures, reaching values above 7 log cfu/g. The antagonistic activity of natural microflora, and in particular of lactic acid bacteria, toward *L. monocytogenes* has been described in several dairy products, showing a potential biopreservative role (Favaro et al., 2015; Tirloni et al., 2017b) due to nutritional competition and the production of active compounds such as bacteriocins, H_2O_2, organic acids, bacteriocin-like substances, dyacetil, carbon dioxide, reuterin, and ethanol. Such antagonistic activity was not shown in the substrate and temperature conditions considered in this study, as *L. monocytogenes* grew faster compared with streptococci and lactobacilli. These results can be explained by the fact that, to exert an efficient antagonistic activity (thus stopping listerial growth, the phenomenon described as the Jameson effect), the natural microflora should quickly reach high counts, thus being favored in the microbial competition (Jameson, 1962). Such a situation did not occur in our case because the starting bacterial loads were too low to overgrow the *L. monocytogenes* inocula in a very favorable substrate.

Moreover, pH confirmed permissive values for *L. monocytogenes* growth; in particular, the trend of this parameter showed values from 6.42 to 6.60 at T0. These data slightly decrease up to values from 6.25 to 6.48 after the storage period that varied depending on the temperature. These findings should be carefully analyzed considering a possible opening of the package with potential contamination by the handler and subsequent storage, especially if contamination occurs in the first part of the shelf life, when natural microbial counts are lower and pH values are more permissive. This possibility is especially likely to occur for mozzarella cherries, such as the product considered for the evaluation of *L. monocytogenes* growth potential.

A significant part of the water-soluble portion of cheese, such as mozzarella, includes short-chain organic acids resulting from glycolysis or added during the production process. These organic acids play an important role in the acquisition of flavor of a cheese, especially if submitted to ripening, as demonstrated by Hough et al. (1996), who showed that total aroma intensity of Reggianito cheese was correlated with organic acid concentrations. Although in mozzarella no ripening is expected, lactic acid plays an essential role for good

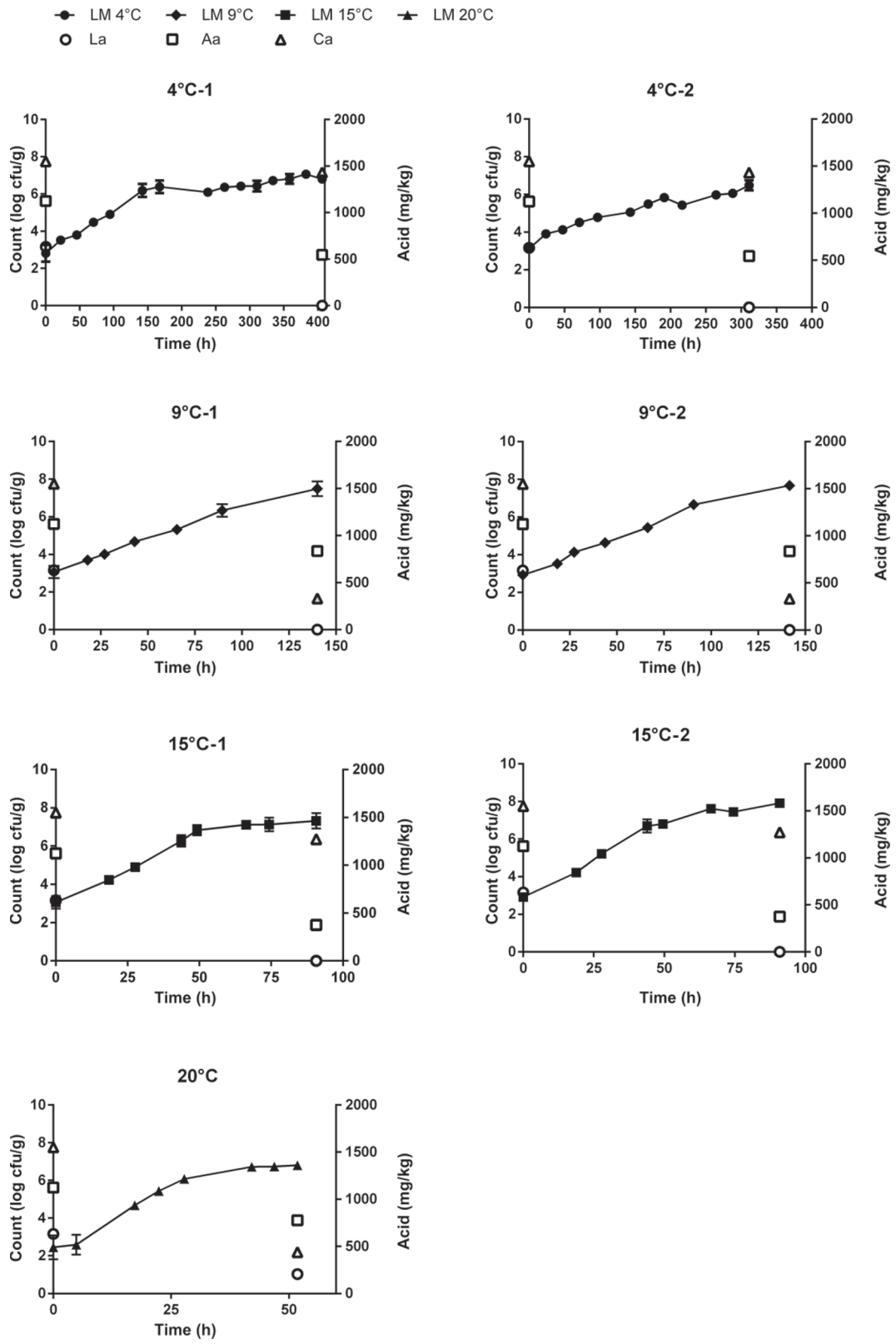

Figure 1. Growth curves of *Listeria monocytogenes* (LM) inoculated in cow mozzarella stored at 4, 9, 15, and 20°C (2 independent trials at 4, 9, and 15°C; 1 trial at 20°C). Aa = acetic acid; Ca = citric acid; La = lactic acid.

Table 4. Initial cell concentration, lag time, maximum specific growth rate, and maximum population density determined by fitting a primary model to growth kinetics obtained from challenge tests at different temperatures[1]

	4°C		9°C		15°C		20°C
Item	a[2]	b[2]	a	b	a	b	a
Initial cell concentration (log cfu/g)	2.81	3.12	3.06	2.92	3.06	2.92	2.45
Lag time (h)	—	—	—	2.4	3.4	6.5	4.4
Maximum specific growth rate (h^{-1})	0.060	0.053	0.084	0.096	0.21	0.21	0.39
Maximum population density (log cfu/g)	6.80	6.48	7.49	7.67	7.32	7.91	NA

[1]Dash indicates not present; NA = not applicable for the short storage time.
[2]a, b = duplicate samples.

production and good keeping quality, as already reported (Wong, 1974).

Citric acid concentration in mozzarella cheese accounted for 45.5% of the total organic acid content; this was expected because this acid is added during manufacturing for technological reasons. The concentration showed a tendency to decrease during the period considered (this trend was marked at 9 and 20°C and slighter at the other storage temperatures). These findings were in agreement with those reported by Lombardi et al. (1994) for Reggianito cheese. This decrease could be due to the metabolism of the natural or added microflora, with citrate involved as a substrate for the Krebs cycle (Adda et al., 1982).

Acetic acid concentration initially (T0) accounted for about 35.9% of the total organic acid content; during the storage period, a gradual decrease was constantly observed in the samples at all temperatures considered. Lactic acid concentration initially (T0) accounted for about 18.6% of the total organic acid content, contrary to that found in mozzarella by Califano and Bevilacqua (1999), who reported that this acid accounted for about 79.3% of the total content after 3 d. The difference obtained in this substrate could be explained by the different mozzarella analyzed, as Califano and Bevilacqua (1999) analyzed a commercial brand of low-moisture mozzarella, whereas in the present study we focused on a high-moisture mozzarella typology. These results highlight the influence of different cheese-making processes on the amount and distribution of acids present.

Lactic acid is of particular importance because the main purpose of added dairy starter culture is to produce this acid from lactose. As described for other organic acids, ripening time also affects lactic acid content (Upreti et al., 2006). As reported by Akalin et al. (2002) and in agreement with our data (Figure 1), young or fresh cheeses, such as mozzarella cheese, between d 0 and the first month are characterized by a decrease in lactic, citric, and pyruvic acid concentrations and by an increase in propionic, butyric, and formic acid contents.

CONCLUSIONS

We evaluated a wide variety of mozzarella brands on the market, in terms of chemical-physical and microbiological characteristics, in the present study. Considering the same production technology (mozzarella produced with cow milk by adding citric acid or starter culture, mozzarella for pizza, and mozzarella produced with buffalo milk), a vast heterogeneity was found in chemical-physical parameters, especially in organic acid concentrations and in the composition of indigenous microflora. The organic acid concentrations detected in all product typologies were lower than the MIC reported in the literature for *L. monocytogenes*, and thus were insufficient to prevent *Listeria* growth. This finding was confirmed by simulating postproduction contamination by the pathogen; very fast growth was evidenced at all storage temperatures applied (from 4 to 20°C) and was not inhibited by the microflora present or by the environmental conditions (organic acids, pH) present in the food matrix. Safety of mozzarella is thought to be maintained while the package is sealed, and *L. monocytogenes* may be a risk once the package is opened and contamination occurs. Correct handling and management, especially during domestic storage, is suggested to minimize the potential for the replication of pathogenic bacteria such as *L. monocytogenes* that were able to grow in different storage conditions.

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