PAPER

FATE OF *LISTERIA MONOCYTOGENES* DURING PRODUCTION AND STORAGE OF ARTISAN WATER BUFFALO MOZZARELLA CHEESE

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ABSTRACT

The study aim was to assess the behaviour of *Listeria monocytogenes* during the production and shelf life of artisan water buffalo mozzarella cheese (WBMC) under different storage conditions. Raw milk was deliberately contaminated by *L. monocytogenes* and the evolution of *L. monocytogenes* count during production and shelf life was monitored. In traditional WBMC production technology *L. monocytogenes* can multiply in the curd during ripening, but its growth rate expressed in log CFU/g/h is lower than the growth rate reported by theoretical predictions. Stretching proved to be a process with good repeatability and able to reduce *L. monocytogenes* contamination by about 2 Log CFU/g. The intrinsic characteristics of traditional WBMC proved to be able to obstacolate the growth of *L. monocytogenes* during storage even in the case of severe thermal abuse.

- Keywords: *Listeria monocytogenes*, water buffalo mozzarella cheese, stretching, shelf life, food safety -

INTRODUCTION

Water buffalo mozzarella cheese (WBMC) is a fresh "pasta filata" cheese produced from whole chilled buffalo milk moulded into various shapes, most commonly oval-spherical. Although pasteurization of milk and the use of defined starter cultures are recommended, traditional technology involving the use of unpasteurized milk and natural whey cultures is still employed for WBMC production in Italy. The production process was described by ADDEO and COPPOLA (1983) and VILLANI *et al.* (1996). In the artisan mozzarella cheese factory, mozzarellas are stored at room temperature in a conditioning liquid commonly composed of water resulting from stretching, acidified with whey from the previous manufacture (VILLANI *et al.* 1996) or, more recently, by tap water salted and acidified with lactic or citric acid.

Foodborne diseases associated with cheese consumption are reported worldwide but diseases associated with the consumption of cheeses produced by pasteurized milk are less common. The main concerns related to the use of unpasteurized milk for making WBMC are the pathogenic bacteria *Salmonella* spp. and *Listeria monocytogenes*, which may contaminate raw milk and, consequently, be harboured in the cheese. *Listeria monocytogenes* has been implicated in a number of food poisoning outbreaks involving milk, dairy products, meat products and vegetables. Between 1988 and 2007 dairy products accounted for 41.5% of 53 foodborne outbreaks of listeriosis reported internationally and *L. monocytogenes* was involved in 6.6% of foodborne outbreaks caused by the consumption of dairy products (GREIG and RAVEL, 2009). The notification rate in the EU in 2008, independently by the food involved, was 0.3 cases per 100,000 population (ANON, 2010). Several listeriosis outbreaks involving cheese as a vehicle have been reported in the EU in recent years (KOCH *et al.*, 2010). The incidence of listeriosis in Europe appears to increase among persons over 60 years of age (GOULET *et al.*, 2008).

Potential sources of contamination of WBMC by *L. monocytogenes* are raw milk (LOVETT *et al.*, 1987; RYSER, 1999), natural whey starter cultures, whey used as an ingredient of the conditioning liquid (VILLANI *et al.*, 1996), not sanitized surfaces or dairy tools or equipment, and handlers (D'AMICO and DONNELLY, 2009).

According to Regulation (EC) 2073/2005, ready to eat food in which *L. monocytogenes* can grow must not contain the bacterium in 25 g at the time when the food leaves the production plant and *L. monocytogenes* must not be present in levels above 100 Colony Forming Units (CFU)/g during shelf life. In addition, producers should be able to make the decision on the shelf life assigned to food, and the indication on duration and storage temperature should be based on a product-specific risk analysis, taking into account reasonable storage conditions and use by consumers. The Regulation suggests specific challenge tests to be carried out on experimentally contaminated food. Producers' instructions on the storage conditions of artisanal WBMC during shelf life differ widely: some producers claim a shelf life of five days keeping the product at room temperature, others claim storage at refrigerator temperature for up to three weeks, and yet others a shelf life of five to ten days storing the product at room temperature for one to three days and in a refrigerator thereafter.

The purpose of this study was therefore to assess the behaviour of *L. monocytogenes* during production and shelf life of WBMC under four different storage conditions assuming raw milk as the source of contamination.

MATERIALS AND METHODS

All experiments were carried out for four batches: three inoculated batches and one non-inoculated batch for control.

Bacterial strains

The following five strains of *L. monocytogenes* were evaluated: *L. monocytogenes*: ATCC strain n. 19115 (Id. Riboprinter DUP1042, ECORI 189-11-S-1); field strain IZSLER n. 2007/34985/2 isolated from cheese (Id. Riboprinter DUP 1042 ECORI 189-11-S-1); field strain IZSLER n. 2007/32929/2 isolated from cheese (Id. Riboprinter DUP 1060, ECORI 189-15-S-4); field strain IZSLER n. 2008/323272/2 isolated from raw milk (DUP 1046, ECORI 189-554-S-3); field strain IZSLER n. 2008/148454/2 isolated from raw milk (DUP 1044, ECORI 189-939-S-1). Strains were grown separately on blood agar base with 5% defibrinated sheep blood (Oxoid, Basingstoke, United Kingdom) incubated at 37°C for 24 h; bacterial colonies were collected by washing with saline (NaCl 0.85%, VWR International, Milan, Italy) and 5 mL of suspensions obtained were used to inoculate 1 L of brain heart infusion broth (Oxoid, Basingstoke, United Kingdom). The broth inoculated was incubated under stirring conditions at 37°C for 24 h. Cells were collected by centrifugation (3,000 g for 1 h) and the pellet was resuspended in 100 mL of saline (NaCl 0.85% VWR International, Milan, Italy). Milk was contaminated by adding equal parts of each bacterial suspension to obtain a final density of about 10^6 CFU/mL of milk of each bacterial strain. The contamination was performed after heating the milk to 38°-40°C and before the natural whey starter and rennet addition.

Water buffalo mozzarella cheese production

To avoid unknown contamination during processing tools used for the experimental productions were sterilized by autoclaving; the surfaces

in contact with milk, curd or cheese were sanitized by 94° ethanol and by steaming; all workers wore sterile gloves during production. Mozzarella was produced according to the traditional technology using 50 litres of unpasteurized milk and natural whey culture as starter for each batch. Briefly the raw milk was heated to 38°-40°C; the natural whey starter and rennet were added and the curd was left to ripen at 35°-38°C for about 4 hours. The curd was extracted from the whey and stretched in hot water (85°-90°C) for about 2 min. The stretched curd was then molded in the traditional round shape. Each mozzarella weighed 250 g. The conditioning liquid was prepared with tap water, salt up to 2° Bé and lactic acid 80% to a final pH of 2.79 and a titratable acidity of 5.5° SH/50 mL. Single 250 g WBMC were packaged in trays completely covered by the conditioning liquid. The temperature of the curd during production and stretching was measured by a Hobo H08-002-02 data logger.

Storage test

For each batch 60 packaged WBMC were divided into four groups (15 WBMC for each group) for the storage tests at four different temperatures (5°, 10°, 15°, and 20°C) for 12 days. The storage conditions were chosen to simulate optimal storage conditions (5°C), domestic storage (10°C, BEAUFORT *et al.*, 2008) and thermal abuse (15° and 20°C).

L. monocytogenes count

Before inoculating *L. monocytogenes,* each batch of raw milk and natural whey starter was tested as described by ISO 11290-2:1998 in order to exclude the presence of unknown strains of *L. monocytogenes*.

L. monocytogenes count for each batch was performed in duplicate for inoculated raw milk, curd at the end of ripening, curd after stretching and WBMC after 60 minutes of packaging and during shelf life at 0, 1, 3, 5, 7 and 12 days. Curd and WBMC were homogenized by a stomacher, then serially diluted and plated on Agar Listeria Ottaviani Agosti (ALOA) (Biolife, Milan, Italy). Plates were incubated aerobically at 30°C for 48 h.

Determination of cell density of lactic acid bacteria, pH and a_w

The following samples were collected in duplicate from each batch: natural whey starter, raw milk after *L. monocytogenes* inoculation, milk after natural whey starter addition, curd at the end of ripening; curd after stretching, WBMC 60 min after packaging in the conditioning liquid and packed WBMC at 0, 1, 3, 5, 7 and 12 days at each storage temperature (5°, 10°, 15°, and 20°C). The following analyses were made on each sample: count of mesophilic and thermophilic lactococci by decimal dilution and inclusion in M17 agar plates (Oxoid, Basingstoke, United Kingdom) incubated aerobically at 30° and 42°C respectively for 48 hours; count of mesophilic and thermophilic lactobacilli by decimal dilution and inclusion in MRS agar plates (Oxoid, Basingstoke, United Kingdom) incubated under microaerophilic conditions at 30° and 42°C respectively for 48 hours; pH was measured by an instrument with automatic temperature compensation (Hanna Instruments HI 223); a_w was determined by AquaLab model series 3 (Decagon Devices Inc. Pullmann, Usa).

Data, including calculation of *L. monocytogenes* generationtime were calculated using programs available on www.combase.cc. Statistical analysis was performed by T-test using SPSS software 12.0.

RESULTS

L. monocytogenes behaviour

L. monocytogenes was not detected in non-inoculated raw milk or natural whey starter.

L. monocytogenes count showed a moderate but significant increase ($p < 0.01$) during curd ripening (from 7.13 to 7.39 Log CFU/g in about 4 h) (Table 1); the combase theoretical predicted growth rate was 0.45 Log/CFU/h at pH 5 and 0.79 log CFU/g/h at pH 6. A decrease (about 1 Log CFU/g) of *L. monocytogenes* count was observed at the end of curd stretching. Sixty min after packaging in the conditioning liquid a further, but not significant 1 Log CFU/g decrease of *L. monocytogenes* count was observed.

During storage test, after a moderate increase for one day at all storage temperatures considered, *L. monocytogenes* count at 5°C storage decreased by about 0.5 Log CFU/g; increasing the storage temperature enhanced the decrease of *L. monocytogenes* count (up to 1.58 Log CFU/g at 20°C storage) (Table 2). By contrast, Combase prediction reported a theoretical growth of *L. monocytogenes* at all storage temperatures ranging from a generation time of 3 days and 20 h at 5°C storage to 9 h at 20°C storage.

pH, a_w and temperature profile

From inoculation of raw milk to the end of ripening pH decreased from 6.89 to 5.18; a further decrease to 5.08 was observed in WBMC at 60 min after packaging in the conditioning liquid (Table 1). During the first days of storage test pH dropped to about 5.0, 4.8, 4.7 and 4.4 during storage at 5°, 10°, 15°, and 20°C respectively and then remained unchanged till the end of the storage test (Table 2). a_w values remained substantially unchanged during storage test at all temperatures ranging from an initial value of 0.979 to a final value of 0.974. No significant differences were observed in the pH and a_w values of the inoculated and non-inoculated batches (data not shown).

Table 1 - Evolution of pH and of *Listeria monocytogenes* and lactic acid bacteria populations count (Log CFU/g) during water buffalo mozzarella cheese traditional production process (Mean of 3 batches ± standard deviation).

Sample	*L. monocytogenes*	pH	Mesophilic lactobacilli	Thermophilic lactobacilli	Mesophilic lactococci	Thermophilic lactococci
Raw milk	n.d.[1]	6.89±0.00	n.a.[2]	n.a.	n.a.	n.a.
Natural whey starter	n.d.	4.00±0.17	8.21±0.59	8.52±0.25	9.16±0.06	9.18±0.08
Inoculated raw milk	7.13±0.06[a]	6.89±0.00	4.25±0.54	3.11±0.45	4.86±0.38	4.32±0.57
Raw milk after natural whey starter addition	n.a.	6.40±0.01	5.98±0.46 [a]	6.98±0.06 [a]	7.76±0.08 [a]	7.73±0.05 [a]
Curd at the end of ripening	7.39±0.04[b]	5.18±0.36	7.91±0.62 [b]	8.38±0.36 [b]	8.64±0.34 [b]	8.67±0.29 [b]
Curd after stretching	6.21±0.27 [c]	5.21±0.14	5.92±0.65 [c]	5.26±0.23 [c]	4.11±0.53 [c]	4.33±0.16 [c]
WBMC 60 min after packing in conditioning liquid	5.14±1.45 [c]	5.08±0.09	5.56±1.18 [c]	1.55±0.29 [d]	3.33±1.46 [d]	2.85±1.06 [d]

[1]: not detected;
[2]: not analysed.
Different letters in a column show significant differences ($p < 0.01$).

Table 2 - Evolution of pH and of *Listeria monocytogenes* and mesophilic lactobacilli count (Log CFU/g) during water buffalo mozzarella cheese storage test at 5°, 10°, 15°, and 20°C (Mean of 3 batches ± standard deviation).

	Storage temperature 5°C			Storage temperature 10°C		
Days after packaging	*L. monocytogenes*[a]	pH	Mesophilic lactobacilli[b]	*L. monocytogenes*[c]	pH	Mesophilic lactobacilli[c]
0	5.14±1.45	5.08±0.14	5.56±1.18	5.14±1.45	5.08±0.14	5.56±1.18
1	5.51±0.41	5.14±0.08	6.95±0.34	5.53±0.76	5.07±0.11	6.89±0.40
3	5.04±0.42	5.09±0.11	7.40±0.28	5.22±1.36	4.88±0.15	6.76±1.05
5	5.02±0.92	4.99±0.11	6.63±0.37	5.19±1.06	4.83±0.16	7.14±0.13
7	5.01±1.49	4.97±0.13	6.60±1.25	4.33±0.93	4.83±0.15	7.73±0.11
12	4.97±1.46	4.83±0.12	6.56±0.60	3.46±1.61	4.82±0.15	7.66±0.13
	Storage temperature 15°C			**Storage temperature 20°C**		
Days after packaging	*L. monocytogenes*[c]	pH	Mesophilic lactobacilli[c]	*L. monocytogenes*[c]	pH	Mesophilic lactobacilli[c]
0	5.14±1.45	5.08±0.14	5.56±1.18	5.14±1.45	5.08±0.14	5.56±1.18
1	5.29±1.13	4.93±0.11	6.87±0.96	5.95±0.47	4.81±0.09	7.41±0.31
3	5.19±1.00	4.72±0.09	6.86±0.66	5.22±0.95	4.42±0.11	7.53±0.40
5	4.34±0.89	4.71±0.13	7.76±0.24	3.48±1.42	4.41±0.17	7.91±0.47
7	5.26±0.26	4.70±0.11	7.67±0.38	3.23±3.21	4.40±0.18	8.06±0.22
12	3.33±2.14	4.71±0.12	7.87±0.23	3.56±1.89	4.41±0.17	8.18±0.09

[a]: a non significant reduction in *L. monocytogenes* count was shown by T test ($p > 0.01$);
[b]: a non significant increase in mesophilic lactobacilli count was shown by T test ($p > 0.01$);
[c]: a significant increase was shown by T test ($p < 0.01$).

The temperature reached by the curd during stretching of inoculated curds is reported in Fig. 1. The maximum temperature reached during curd stretching was 71.6°C±1.6. Curd temperature during stretching remained over 65°C for 3 min in all four batches (data not shown).

Evolution of lactic acid population

During curd ripening an increase of 4-5 Log CFU/g was observed in all lactic acid bacterial populations. The heat treatment of stretching reduced the counts of the different lactic acid bacteria populations by about 1.99 to 4.5 Log CFU/g (Table 1). During the storage test thermophilic lactobacilli and thermophilic lactococci counts remained substantially unchanged at regardless of the temperature (data not shown). Mesophilic lactococci count decreased by 0.96 Log CFU/g when WBMC was stored at 5°C but was unaffected by higher storage temperatures (data not shown). Mesophilic lactobacilli count showed a not significant increase ($p > 0.01$) during storage at 5°C, but a significant increase ($p < 0.01$) was observed at 10°, 15°, and 20°C (Table 2). No significant differences were observed in the evolution of lactic acid bacteria populations of the inoculated and non-inoculated batches (data not shown).

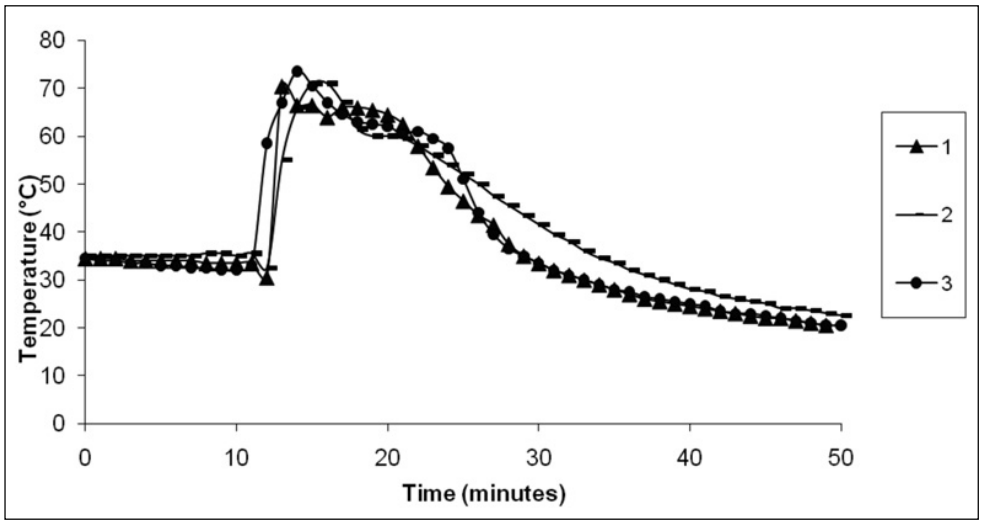

Fig. 1 - Evolution of the temperature during stretching of experimentally contaminated water buffalo mozzarella cheese (3 batches).

DISCUSSION

The results of this study show that if contamination levels are high, stretching cannot ensure the complete destruction of *L. monocytogenes* in traditional WBMC. Many authors have argued that although stretching is capable of destroying pathogenic bacteria, the variability of factors such as temperature, time and initial level of milk contamination do not allow to consider the stretching process as a substitute of the pasteurization (ADDEO and COPPOLA, 1983; KIM *et al.*, 1998; MURRU *et al.*, 1999a). Stretching, however, is a very important stress factor for *L. monocytogenes*: a study conducted by BUAZZI *et al.* (1992) proved that the stretching phase in mozzarella cheese is able to eliminate *L. monocytogenes* contamination if the curd retains a temperature of 77°C for three to four minutes. MURRU *et al.* (1999a) claim that a stretching phase run at a temperature between 65°C and 69°C for five minutes cannot eliminate *L. monocytogenes* contamination in WBMC if the initial contamination level of the pathogen is higher than 4 Log CFU/g. The contamination of raw milk, if present, is assumed to be low because the few available studies on water buffalo raw milk contamination reported the absence of *L. monocytogenes* (HAN *et al.* 2007; MURRU *et al.* 2009b); nevertheless it must be evaluated that *Listeria* spp., including outbreak strains of *L. monocytogenes*, are regularly isolated from dairy processing and cheese-making environments (D'AMICO and DONNELLY, 2009) and the ability of *L. monocytogenes* to adhere to many materials found in food-processing environments (BERESFORD *et al.* 2001) may represent a significant source of contamination increasing the natural contamination of milk or of the curd in non-experimental conditions. In our study the temperature of the curd reached a maximum of 71.6°C and the temperature remained above 65°C for three minutes. The decrease of 2 Log CFU/g of *L. monocytogenes* (1 Log CFU/mL during stretching and 1 Log in the following 60 min) is in agreement with the data reported by VILLANI *et al.* (1998). Although according to Regulation (EC) 2073/2005, WBMC could be considered a ready to eat food able to support the growth of *L. monocytogenes* (pH < 4.4 or a_w < 0.92 or pH <5.0 and a_w < 0.94), under our experimental conditions *Listeria monocytogenes* was not able to grow during storage of WBMC, even in the case of severe thermal abuse. *L. monocytogenes* is known to grow over a wide range of temperatures (-1.5° to 45°C) and pH (4.3 to 9.4) (TE GIEFFEL and ZWIETERING, 1999). MILLET *et al.* (2006) reported a pH growth/no growth limit in Saint-Nectaire-type cheese of 5.2. Other authors reported a pH growth limit of 4.6 in cottage cheese and in feta cheese (PEARSON and MARTH, 1990). The ability to grow at different temperatures was strictly correlated with the pH of the cheeses. A decrease of pH value coincides with an inhibition of *L. monocytogenes* growth as reported by ROGGA *et al.* (2005) and UHLICH *et al.* (2006) in Queso Blanco, whereas an increase in pH leads to an increase of *L. monocytogenes* cell density (BACK *et al.*, 1993; MENDOZA-YEPES *et al.*, 1999; GENIGEORGIS *et al.*, 1991); RAMSARAN *et al.* (1998) observed an increased number of *L. monocytogenes* during initial ripening of feta cheese followed by a growth arrest after 24 h when the pH value dropped down.

During WBMC storage we observed a growth of mesophylic lactobacilli accompanied by a drop in pH value more enhanced at higher storage temperatures. The growth/no growth interfaces of *Listeria* reported by LE MARC *et al.* (2002) are

5.50, 4.6-4.7, 4.50 and 4.4-4.5 respectively at 5°, 10°, 15°, and 20°C. In our study these no growth pH limits were reached after 3 days of storage at 20°C, whereas they were never reached at 10° and 15°C. Therefore an increase in *L. monocytogenes* count rather than a decrease should be observed, as calculated by Combase prediction. SCHVARTZMAN *et al.* (2010) demonstrated that the growth limits of *L. monocytogenes* are matrix-dependent and that solid foods may limit the diffusion rate of organisms throughout space in food and this can be an additional factor limiting the growth of *L. monocytogenes* in WBMC. The literature reports additional factors influencing the growth of *L. monocytogenes* in milk and dairy products. The inhibitory effect of natural microbiota, namely lactic acid bacteria, lead to a longer lag phase and higher generation time of *L. monocytogenes* in pasteurized milk with respect to UHT milk or autoclaved milk (ROSENOW and MARTH, 1987; WALKER *et al.*, 1990; BOVILL *et al.*, 2000). The addition of organic acids or starter cultures in cheeses increased the minimal growth temperature of *L. monocytogenes* or negatively affected its survival (EL-SHENAWY and MARTH, 1990; GLASS *et al.*, 1995; MENDOZA-YEPES *et al.*, 1999). Additional hurdles, due to the effect of lactic acid added to the conditioning liquid and to the growth of lactic acid bacteria (competition for carbohydrates, acids and bacteriocins production) may be hypothesized, but are difficult to evaluate in a product made with raw milk and natural whey cultures. Lactic acid bacteria are known to inhibit different pathogens (NUÑEZ *et al.*, 1997; RODRIGUEZ *et al.*, 2005) and a study conducted by GAY and AMGAR (2005) found that lactic acid bacteria have a twofold higher inhibitory activity towards pathogenic microorganisms in products derived from raw milk (like artisan WBMC) than in products derived from pasteurized milk. However, because the inhibitory activity of lactic acid bacteria in dairy products depends on the biotype and cell density of lactic acid bacteria, this feature should be further investigated in WBMC.

CONCLUSIONS

In traditional WBMC production technology *L. monocytogenes* can multiply during curd ripening, but its growth rate is lower than the growth rate reported by the theoretical predictions. Therefore, it is expected that, if the contamination level of milk is low, during curd ripening *L. monocytogenes* will not reach values of cell density so high to represent a risk for consumers' health. Under our experimental conditions, curd stretching proved to be a process with good repeatability and able to decrease *L. monocytogenes* cell density of curd by about 2 Log CFU/g. Because the intrinsic characteristics of traditional WBMC were able to limit the growth of *L. monocytogenes* during storage (even in the case of severe thermal abuse), it may be issued that the key factor for reducing the risk for consumers' health is the implementation of appropriate hygiene measures to minimize the contamination of raw milk and post processing contamination.

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