

PAPER

Behaviour of *Salmonella Typhimurium* during production and storage of artisan water buffalo mozzarella cheese

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Abstract

Water buffalo *mozzarella* cheese (WBMC) is a fresh *pasta filata* cheese produced from whole chilled buffalo milk. 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 aim of this study were to assess the behaviour of *Salmonella Typhimurium* during the production of artisan water buffalo *mozzarella* cheese and during its shelf life under different temperature conditions. Raw milk was inoculated with *S. Typhimurium* and the evolution of *S. Typhimurium* count during production and shelf life was monitored. In artisan WBMC production technology *S. Typhimurium* multiplied in the curd during ripening, but its growth rate expressed in log CFU/g was lower than the growth rate reported by theoretical predictions. Stretching proved to be a process with good repeatability and able to reduce *S. Typhimurium* contamination by 5.5 Log CFU/g. The intrinsic characteristics of traditional WBMC proved to be unable to obstruct the growth of *S. Typhimurium* during storage in the case of thermal abuse. Control of raw milk contamination and a proper refrigeration temperature are key factors in reducing the risk for consumers.

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.

The traditional production process of water buffalo *mozzarella* cheese includes the use of raw milk. The presence of foodborne pathogens in raw milk in Italy was reported (Giacometti *et al.*, 2012a, 2012b) and the prevalent pathogens seem to be *Listeria monocytogenes* and *Salmonella* spp. (Oliver *et al.*, 2009). Handling conditions may influence the growth and survival of foodborne pathogens in raw milk (Giacometti *et al.*, 2012c) and cheeses produced from raw milk can be less safe, in particular unripened or less ripened cheeses (Little *et al.*, 2008). In Europe, *Salmonella* spp. has been implicated in several foodborne outbreaks and was by far the most frequent (49%) among the notified foodborne diseases; however milk and milk products were involved in only 1.8% of the *Salmonella* outbreaks, being responsible for 19% of the 177 outbreaks implicating milk and milk products (De Buyser *et al.*, 2001). Since 1980, of the 60 documented milk-borne or milk product-borne disease outbreaks, 29 *Salmonella* outbreaks were identified, 14 associated with milk and 14 with cheese of which 8 were made from raw milk and 3 from unpasteurized milk (De Buyser *et al.* 2001). Since 2001, several cheese-associated outbreaks of *Salmonella* spp. have occurred (Haeghebert *et al.*, 2003, Kinde *et al.*, 2007; CDC 2007, 2008; Pastore *et al.*, 2008; Dominguez *et al.*, 2009; Van Duynhoven *et al.*, 2009; Van Caueren *et al.*, 2009). In the last years, very few *Salmonella* findings were reported both from cow's milk and cheeses (EFSA, 2011), but the presence of *Salmonella* spp. in WBMC was reported in Italy (De Carlo *et al.*, 1999).

WBMC can be contaminated by *Salmonella* spp. through contaminated milk or post pro-

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cessing by direct contact with contaminated surfaces or handlers. Producers' instructions on the storage conditions of artisan-made 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 behaviour of *S. Typhimurium* in WBMC in the case of post processing contamination was described Oliviero *et al.* (2010). The purpose of this study was therefore to assess the behaviour of *S. Typhimurium* during the production and shelf life of WBMC under four different storage conditions assuming a contamination of the product due to the presence of *S. Typhimurium* in raw milk.

Materials and methods

The experiment design consisted of four trials: three replicates of inoculated batches and one non-inoculated control batch.

Bacterial strains

The following three strains of *S. Typhimurium* were evaluated: *S. Typhimurium* ATCC 6994 and field strains *S. Typhimurium* IZSLER 2008/96908/2 and IZSLER 2008/43259/2 isolated from raw milk.

Strains were grown separately on blood agar

base (Oxoid, Basingstoke, UK) with 5% defibrinated sheep blood incubated at 37°C for 24 h; bacterial colonies were collected by washing with saline (NaCl 0.85%, VWR International, Milano, Italy) and the suspensions obtained were cultured in 1 L of brain heart infusion broth (Oxoid) incubated in continuous agitation at 37°C for 24 h, then centrifuged at 3000 g for 1 h; the pellet was resuspended in 100 mL of saline (NaCl 0.85%, VWR International). Milk was inoculated by adding equal parts of each bacterial suspension to raw milk to obtain a final concentration of 10⁷ CFU/mL of milk.

Water buffalo mozzarella cheese production

Mozzarella was produced according to the traditional technology using 50 L of unpasteurized milk and natural whey culture as starter for each batch. Briefly the raw milk inoculated with *S. Typhimurium* was heated to 38°C-40°C; the whey and rennet were added and the curd was left to ripen at 35-38°C for about 4 h. The curd was extracted from the whey and stretched in hot water (85-90°C). 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 5.5°SH (Soxhlet-Henckel)/50 mL. Single 250 g WBMC were packed in conditioning liquid. The temperature during production and stretching was measured by a Hobo H08-002-02 data logger.

Storage test

For each batch 60 packed WBMC were divided into four groups (15 WBMC for each group) for the storage tests at four different temperatures (5°C, 10°C, 15°C 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).

S. Typhimurium count

Before inoculating *S. Typhimurium*, each batch of raw milk and natural whey starter and conditioning liquid was tested as described by ISO 6579:2002.

S. Typhimurium 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 min of packing and during shelf life at 0, 1, 3, 5, 7 and 12 days. Each sample was stored at 4±2°C and processed within 20 mins after sampling. Curd and WBMC were homogenized by a stomacher; *S. Typhimurium* was counted by milk and homogenates by decimal dilution and direct plating on Hektoen enteric agar plates (Oxoid) incubated aerobically at 37°C for 24 h in duplicate.

Typical colonies were counted and a selection of 10 colonies for each plate was confirmed by rapid agglutination with Salmonella O Antiserum Group Poly A-I and Vi (Becton Dickinson, Franklin Lakes, NJ, USA).

Lactic acid bacteria count, pH and a_w determination

The following samples were collected in duplicate from each batch: natural whey starter, raw milk after *S. Typhimurium* inoculation, milk after natural whey starter addition, curd at the end of ripening; curd after stretching, WBMC 60 min after packing in the conditioning liquid and packed WBMC at 0, 1, 3, 5, 7 and 12 days at each storage temperature (5°C, 10°C, 15°C and 20°C). The following analyses were made on each sample: count of mesophilic and thermophilic lactic acid bacteria (LAB) by decimal dilution and inclusion in M17 agar plates (Oxoid) incubated aerobically at 30° and 42°C respectively for 48 h; count of mesophilic and thermophilic LAB by decimal dilution and inclusion in MRS agar plates (Oxoid) incubated under microaerophilic conditions at 30° and 42°C respectively for 48 h;

pH was measured by an instrument with automatic temperature compensation (Hanna Instruments HI 223); a_w was determined by AquaLab model series 3.

Data, including calculation of *S. Typhimurium* generation time were calculated using ComBase Predictor programs (Dmfit) available on www.combase.cc and based on Baranyi model (Baranyi and Roberts, 1994; Baranyi and Tamplin, 2004). Statistical analysis was performed by T-test using SPSS software 12.0.

Results

Details of the evolution of pH, LAB and *S. Typhimurium* population during production and storage at different temperatures are reported in Tables 1 and 2.

S. Typhimurium behaviour

S. Typhimurium was not detected in non-inoculated raw milk, natural whey starter and conditioning liquid.

S. Typhimurium count showed a moderate increase (P<0.01) during curd ripening (from 7.43 Log CFU/g to 8.03 Log CFU/g in about 4 h): at similar conditions of temperature and a_w, the combase theoretical predicted growth rate was 1.58 Log/CFU/h at pH 5 and 2.03 log CFU/g/h at pH 6. A reduction (about 4.3 Log CFU/g) of *S. Typhimurium* count was observed at the end of curd stretching. Sixty min after packing in the conditioning liquid a further 1.2 Log CFU/g reduction of *S. Typhimurium* count was observed.

During the storage test a decrease of *S. Typhimurium* count was observed in WBMC stored at 5°C; on the contrary when WBMC were stored at 10°C, 15°C and 20°C an increase of *S. Typhimurium* count was observed; increasing the storage temperature,

Table 1. Evolution of pH and of *Salmonella Typhimurium* and lactic acid bacteria count (Log CFU/g) during water buffalo mozzarella cheese traditional production process (mean of 3 batches ± SD).

Production phase	<i>S. Typhimurium</i>	pH	Mesophilic LAB (MRS Agar)	Thermophilic LAB (MRS Agar)	Mesophilic LAB (M17 Agar)	Thermophilic LAB (M17 Agar)
Raw milk	nd	6.89±0.00	na	na	na	na
Natural whey starter	nd	4.00±0.17	8.21±0.59	8.52±0.25	9.16±0.06	9.18±0.08
Inoculated raw milk	7.43±0.04 ^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	na	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	8.03±0.06 ^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	3.73±1.91 ^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	2.49±0.22 ^d	5.08±0.09	5.56±1.18 ^c	1.55±0.29 ^d	3.33±1.46 ^d	2.85±1.06 ^d

LAB, lactic acid bacteria; WBMC, water buffalo *mozzarella* cheese; nd, not detected; na, not analyzed; ^{a-d}different letters in a column show significant differences (P<0.01).

the generation time of *S. Typhimurium* decreased (from 19 h at 10°C to 5 h at 20°C storage).

Profile of pH, a_w and temperature

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. During the first days of storage test, pH dropped to about 5.0, 4.8, 4.7 and 4.4 during storage at 5°C, 10°C, 15°C and 20°C respectively and then remained unchanged till the end of the storage test (Table 2). The 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).

The stretching temperature profile of inoculated curds is reported in Figure 1. The maximum temperature reached during curd stretching was $71.6^\circ\text{C} \pm 1.6$ standard deviation (mean of three batches). Curd temperature during stretching remained over 65°C for 3 min in all three batches.

Lactic acid bacteria behaviour

During curd ripening an increase of 4-5 Log CFU/g was observed in all LAB populations (Table 1). The heat treatment of stretching reduced the counts of the different LAB popu-

lations by about 1.99 to 4.5 Log CFU/g (Table 1). During the storage test thermophilic LAB (both on MRS agar plates and on M17 agar plates) counts remained substantially unchanged at all storage temperatures (data not shown). Mesophilic LAB count (on M17 agar plates) 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 LAB count (on MRS Agar plates) showed a not significant increase ($P > 0.01$) during storage at 5°C, but a significant increase ($P < 0.01$) was observed at 10°C, 15°C and 20°C storage temperatures (Table

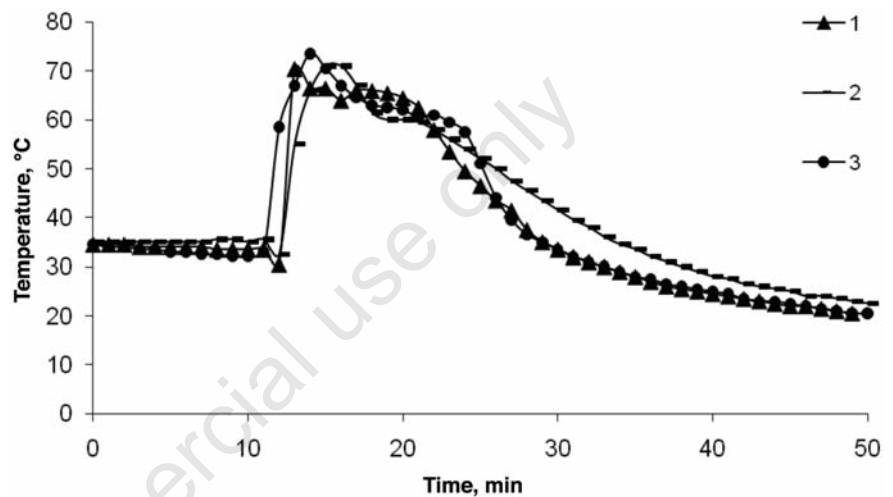


Figure 1. Evolution of the temperature during stretching of experimentally contaminated water buffalo mozzarella cheese (3 batches).

Table 2. Evolution of pH and of *S. Typhimurium* and mesophylic lactic acid bacteria on MRS Agar (Log CFU/g) during water buffalo mozzarella cheese storage test at 5°C, 10°C, 15°C and 20°C (mean of 3 batches \pm SD).

Days after packing	Storage temperature 5°C			Storage temperature 10°C		
	<i>S. Typhimurium</i> ^o	pH	Mesophilic [#] LAB (MRS Agar)	<i>S. Typhimurium</i> ^s	pH	Mesophilic ^s LAB (MRS Agar)
0	2.49 \pm 0.22	5.08 \pm 0.14	5.56 \pm 1.18	2.49 \pm 0.22	5.08 \pm 0.14	5.56 \pm 1.18
1	2.20 \pm 0.41	5.14 \pm 0.08	6.95 \pm 0.34	2.70 \pm 0.76	5.07 \pm 0.11	6.89 \pm 0.40
3	1.90 \pm 0.46	5.09 \pm 0.11	7.40 \pm 0.28	2.94 \pm 0.26	4.88 \pm 0.15	6.76 \pm 1.05
5	1.69 \pm 0.62	4.99 \pm 0.11	6.63 \pm 0.37	3.72 \pm 0.30	4.83 \pm 0.16	7.14 \pm 0.13
7	1.49 \pm 1.00	4.97 \pm 0.13	6.60 \pm 1.25	3.79 \pm 0.42	4.83 \pm 0.15	7.73 \pm 0.11
12	0.99 \pm 0.54	4.83 \pm 0.12	6.56 \pm 0.60	4.04 \pm 0.19	4.82 \pm 0.15	7.66 \pm 0.13
Days after packing	Storage temperature 15°C			Storage temperature 20°C		
	<i>S. Typhimurium</i> ^o	pH	Mesophilic [#] LAB (MRS Agar)	<i>S. Typhimurium</i> ^s	pH	Mesophilic ^s LAB (MRS Agar)
0	2.49 \pm 0.22	5.08 \pm 0.14	5.56 \pm 1.18	2.49 \pm 0.22	5.08 \pm 0.14	5.56 \pm 1.18
1	2.58 \pm 0.37	4.93 \pm 0.11	6.87 \pm 0.96	3.58 \pm 0.12	4.81 \pm 0.09	7.41 \pm 0.31
3	3.97 \pm 0.23	4.72 \pm 0.09	6.86 \pm 0.66	4.77 \pm 0.45	4.42 \pm 0.11	7.53 \pm 0.40
5	4.27 \pm 0.14	4.71 \pm 0.13	7.76 \pm 0.24	5.88 \pm 0.63	4.41 \pm 0.17	7.91 \pm 0.47
7	4.43 \pm 0.44	4.70 \pm 0.11	7.67 \pm 0.38	5.03 \pm 0.41	4.40 \pm 0.18	8.06 \pm 0.22
12	4.58 \pm 0.27	4.71 \pm 0.12	7.87 \pm 0.23	5.57 \pm 0.90	4.41 \pm 0.17	8.18 \pm 0.09

LAB, lactic acid bacteria; ^oa significant reduction in *S. Typhimurium* count was shown by T-test ($P < 0.01$); [#]a non significant increase in mesophilic LAB count was shown by T-test ($P > 0.01$); ^sa significant increase was shown by T-test ($P < 0.01$).

2). No significant differences were observed in the evolution of LAB populations of the inoculated and non-inoculated batches (data not shown).

Discussion

The results of this study show that if contamination levels of raw milk are high stretching as described cannot ensure the complete destruction of *S. Typhimurium* 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 prevent the stretching process being considered a replacement for pasteurization (Addeo and Coppola, 1983; Kim et al., 1998; Murru et al., 1999). However, the effect of stretching on *S. Typhimurium* is comparable to the effect on *Salmonella senftenberg* observed in WBMC (Cortesi et al., 1998) and to that reported for *Salmonella javiana* in mozzarella cheese (Eckner et al., 1990). The temperature reached by the curd during stretching is critical for the organoleptic features of traditional WBMC and the concordance of the results on the effect on *S. Typhimurium* (present work) and *S. senftenberg* (Cortesi et al., 1998) let us assume that stretching is a process with a good reproducibility and able to reduce significantly the *Salmonella* count of curd.

The contamination of raw milk, if present, is assumed to be low because of the few available studies on water buffalo raw milk contamination. 3 reported an absence of *Salmonella* spp. (Braun and Preuss, 2007; Martuciello et al., 2008; Buzi et al., 2009) in all samples examined and one reported a positive sample out of 10 examined (De Carlo et al., 1999). In the best studied cow's milk, Van Kessel et al. (2004) estimated the contamination by *Salmonella* in 22 positive samples out of 861 bulk tank samples examined at levels of 1 to 40 CFU/10 mL. Cortesi and Murru (2007) assumed that a 10^2 Log CFU/g may be considered a high contamination level for naturally contaminated water buffalo milk: at these level of initial natural contamination and given the low replication rate of *S. Typhimurium* observed during ripening, stretching could be considered an effective treatment to destroy *S. Typhimurium*.

Maintenance of low temperature seems to be critical to counteract the multiplication of *S. Typhimurium* during storage. *Salmonellae* have been reported to grow at temperatures lower than 5°C (D'Aoust, 1991), but the growth

of most *Salmonellae* is prevented at temperatures below 7°C (Fares, 2007). The reduction of *S. Typhimurium* count we observed during storage at 5°C was not highlighted in a previous work (Cortesi et al., 1998) in which WBMC was stored at 6°C for 7 days. At the other storage temperatures an increase in LAB and reduction of pH was observed concomitant with an increase in *S. Typhimurium* count to a stationary phase reached after 3 to 5 days depending by temperature of storage (Table 2). The ability of *Salmonella* to grow at different temperatures was correlated with the pH of the medium and dependent on the type of acidulant involved. In traditional WBMC (made from raw milk and with natural whey starter) the amount of each acid present is difficult to evaluate but the predominant acid is assumed to be lactic acid (produced by LAB and added to conditioning liquid). In our work, pH values under 4.5 were reached only after 3 days of storage at 20°C and never at the other storage temperatures (Table 2): at 10°C and 15°C storage a pH under 4.7 was never recorded. These data are in agreement with literature: in BHI broth an acidification to pH 4.5 performed with lactic acid suppressed the growth of *S. Typhimurium* at 10°C, 25°C and 37°C; acidification of skim milk with lactic acid to a pH of 4.9 did not prevent *S. Typhimurium* growth even in the presence of 3% NaCl at 37°C (Goepfert et al., 1968). In cheeses Thesys et al. (2009) reported that a pH of 4.75 is not sufficient to prevent *S. Typhimurium* growth at 20°C; in a previous report the ability of *S. Typhimurium* to replicate in WBMC conditioning liquid was reported only at pH values higher than 4.4 (Oliviero et al., 2010). In the case of contamination, the replication of lactic acid bacteria and the drop in pH is not sufficiently rapid and intense to stop *S. Typhimurium* growth if WBMC is not properly stored at refrigeration temperature.

Conclusions

In traditional WBMC production technology *S. Typhimurium* can multiply during curd ripening, but its growth rate expressed in log CFU/g/h is lower than reported theoretical predictions under comparable conditions of temperature, pH and a_w . Therefore, because the natural contamination of milk is assumed to be low, *S. Typhimurium* will not reach high levels of contamination during curd ripening. In experimental productions, curd stretching proved to be a process with good repeatability and able to reduce *S. Typhimurium* contamina-

tion of raw milk by about 5.5 Log CFU/g. The intrinsic characteristics of traditional WBMC proved to be unable to obstruct the growth of *S. Typhimurium* during storage in the case of thermal abuse. The key factors to reduce the risk for consumer health posed by *S. Typhimurium* contamination are the implementation of appropriate hygiene measures to minimize the contamination of raw milk, and proper storage of WBMC at refrigeration temperature.

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