International Journal of Food Microbiology 305 (2019) 108247

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Evaluation of *Mycobacterium avium* subsp. *paratuberculosis* survival during the manufacturing process of Italian raw milk hard cheeses (Parmigiano Reggiano and Grana Padano)

G. Cammi[a], M. Ricchi[a], A. Galiero[a,*], P. Daminelli[b], E. Cosciani-Cunico[b], E. Dalzini[c], M.N. Losio[b], R. Savi[a], G. Cerutti[a], C. Garbarino[a], S. Leo[a], N. Arrigoni[a]

[a] Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, National Reference Centre for Paratuberculosis, Strada Faggiola 1, loc. Gariga - Podenzano (PC), 29027, Italy
[b] Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia "Bruno Ubertini", Food Control Department, Via Bianchi 9, 25124, Brescia, Italy
[c] Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia "Bruno Ubertini", National Reference Centre for Emerging Risks in Food Safety, Via Celoria 12, 20133, Milan, Italy

ARTICLE INFO

Keywords:
Mycobacterium avium subsp. *paratuberculosis*
Hard cheeses
Italian PDO
Challenge test
Food safety

ABSTRACT

Mycobacterium avium subsp. *paratuberculosis* (MAP), the agent of paratuberculosis in ruminants, is suspected to be involved in the aetiology of some human diseases. Notably, the consumption of milk and dairy products is considered to be the main route of human exposure to MAP because of its ability to survive during pasteurization and manufacturing processes. The aim of this study was to investigate, through a microbiological challenge test, the survival of MAP during the manufacturing and ripening period of two Italian hard cheeses, Parmigiano Reggiano and Grana Padano, made from raw bovine milk. The challenge test was performed in two different phases: the creaming phase and the manufacturing phase.

The creaming phase, which is the first step of cheese production, was reproduced in the laboratory employing raw cow's milk spiked with a MAP reference strain at a final concentration of 5.58 $\log_{10}$ CFU/mL. After the creaming at 18 °C and 27 °C for 12 h, a decrease of 0.80 $\log_{10}$ and 0.77 $\log_{10}$ was observed in partially skimmed milk, respectively.

In the second phase, two batches of raw cow's milk (1000 L each) were inoculated with MAP reference and wild strains, respectively. Then, the entire manufacturing process for Parmigiano Reggiano and Grana Padano, both of Protective Designation of Origin (PDO), was reproduced in an experimental cheese factory, starting from a concentration in milk of 5.19 ± 0.01 and 5.28 ± 0.08 $\log_{10}$ CFU/mL of MAP reference and wild strains, respectively. Heating the curd at 53 °C for 20 min did not affect MAP survival, however a significant decrease ($p < 0.05$) in MAP viability was observed during the moulding phase and after salting in brine, regarding the wild strains and the reference strain, respectively. In addition, a significant decrease was observed during the ripening period, at which time the MAP concentration dropped below the limit of detection from the second and the third month of ripening, for the wild and reference strains, respectively.

Taking into account the poor data availability about MAP survival in hard cheeses, this study may improve the knowledge regarding the effect of the cheese manufacturing process on the MAP dynamics, supporting also the safety of traditional raw milk hard cheeses.

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP), the causative agent of paratuberculosis (Johne's disease) in ruminants, is suspected to be also involved in the aetiology of some human diseases, such as Crohn's disease (Chiodini et al., 2012), Type I Diabetes, multiple sclerosis and Hashimoto's thyroiditis (Atreya et al., 2014; Cossu et al., 2011; Dow, 2012; Scanu et al., 2007; Sisto et al., 2010). This has long been investigated, but to date no final agreement about the role of MAP in the pathogenesis of these diseases has been reached (Chiodini et al.,

* Corresponding author at: Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, National Reference Centre for Paratuberculosis, Diagnostic Section of Piacenza, Strada Faggiola 1, loc. Gariga - Podenzano (PC), 29027, Italy.
E-mail address: alessia.galiero@izsler.it (A. Galiero).

https://doi.org/10.1016/j.ijfoodmicro.2019.108247
Received 17 January 2019; Received in revised form 5 June 2019; Accepted 6 June 2019
Available online 08 June 2019

2012; Waddell et al., 2015). However, measuring exposure risk for consumers to this potential zoonotic hazard seems to be a prudent approach (Collins, 2011). With reference to this, the consumption of milk and dairy products is considered to be the main route of human exposure to MAP (Gill et al., 2011). In particular, MAP cells can be directly shed into milk by clinically and subclinically infected cows at concentrations of 100 CFU/mL and 2–8 CFU/50 mL of milk, respectively (Giese and Ahrens, 2000; Sweeney et al., 1992). In addition, faecal contamination can accidentally occur during milking, leading to an increase in the MAP concentration in bulk tank milk (Atreya et al., 2014; Cocito et al., 1994; Rademaker et al., 2007; Vissers et al., 2007); this aspect is relevant, in particular when the hygienic conditions are poor, considering that faeces of a symptomatic cow can contain up to 10^{12} MAP cells/g (Cocito et al., 1994).

Furthermore, it should be considered that not only raw milk and unpasteurized derivates, but also dairy products from pasteurized milk can constitute a hazard because this process can only reduce the MAP load originally present in milk (Eltholth et al., 2009; Gill et al., 2011; Okura et al., 2012).

Notably, many studies have detected not only the presence of MAP DNA (Botsaris et al., 2010; Clark Jr. et al., 2006; de Melo et al., 2014; Faria et al., 2014; Galiero et al., 2015; Galiero et al., 2016; Galiero et al., 2017; Ikonomopoulos et al., 2005; Stephan et al., 2007), but also viable MAP cells in retail cheese, showing that MAP survival in these products depends on variable factors, particularly the thermal treatment applied, the employment of starter cultures and the length of the ripening period (Faria et al., 2014; Galiero et al., 2016; Galiero et al., 2017; Ikonomopoulos et al., 2005; Williams and Withers, 2010).

The aim of this study was to investigate MAP survival during the manufacturing process of two Italian cheeses, Parmigiano Reggiano (PR) and Grana Padano (GP), which have received the Protected Designation of Origin (PDO) recognition by the European Union. PR and GP are hard cooked cheeses characterized by a long ripening period and produced from partially skimmed raw cow's milk with natural whey cultures of lactic acid bacteria used as a starter. They are both made exclusively from Italian milk and the area of origin is the Po Valley; particularly, the production area of GP includes 32 provinces and five different regions (Piedmont, Lombardy, Veneto, Emilia Romagna and Trentino South Tyrol), while that of PR is the Emilia Romagna Region (Parma, Reggio Emilia, Modena, Bologna) and the right-hand side of the Po river in the Mantua province. Other elements which differentiate PR and GP concern the cattle nutrition and the milk collection procedures. Particularly, the employment of silage feed and the consequent use of lysozyme to prevent anomalous fermentation due to Clostridia are permitted only for GP, while are not allowed for PR. In addition, GP is made from naturally skimmed milk obtained from two milking sessions (collected together, once a day), while PR is made from naturally skimmed milk of the evening session mixed with the same volume of whole fat morning's milk (collected separately, twice a day) (https://www.granapadano.it/en-uk/grana-padano-and-parmigiano-reggiano-particular-aspects-and-specifications-v1.aspx, from data of January 2019). Regarding paratuberculosis status in the area of production (Lombardy and Emilia Romagna regions), the estimated herd-level true seroprevalence of paratuberculosis in dairy cattle is 70% for Lombardy region, but the within-herd true seroprevalence is lower (6.7%) (Pozzato et al., 2011). For Emilia-Romagna region, a recent PCR survey, carried out on bulk milk of 88.6% of total herds, showed an apparent prevalence of 26.9%, but only 3.1% of samples exceeded the MAP the LOD (1.5×10^1 MAP cells/mL) (Ricchi et al., 2016).

2. Materials and methods

2.1. Experimental design

Two different challenge tests were carried out in our study. Firstly, the effect of natural milk creaming (first step of the PR and GP manufacturing process) on MAP survival dynamics was evaluated, reproducing this phase on laboratory scale. Then, a second challenge test was carried out on two batches of raw milk (1000 L each), contaminated with the ATCC 19698 MAP strain (Batch A) and a mixture of wild strains (Batch B), respectively. A total of four cheeses, according to the manufacturer's instructions, was produced and the MAP survival was evaluated during the first 12 months of ripening. Notably, the milk used for each experiment was previously tested by IS900-qPCR (Ricchi et al., 2016), in order to verify the absence of natural MAP contamination.

2.2. Mycobacterium avium paratuberculosis strains and inocula preparation

MAP ATCC 19698 (type C strain, isolated from cow faeces) (Hsu et al., 2011) and three type C wild strains (MAP 706/2011, MAP 15/2012 and MAP 755/2012, isolated from faeces of naturally infected cows from herds in Emilia Romagna, Northern Italy), were used in order to spike raw cow milk during the challenge tests. All the strains belonged to the National Reference Centre for Paratuberculosis's collection (IZSLER, Piacenza, Italy). Each strain was cultured in Middlebrook 7H9 broth medium, containing 10% (vol/vol) Middlebrook OADC Enrichment (Becton Dickinson, USA) and Mycobactin J (2 mg/L) (IDvet Innovative Diagnostics, Montpellier, France) for four to six weeks at 37 °C in a shaker incubator (Excella E24 Incubator Shaker Series, New Brunswick Scientific, NJ, USA). At the end of the incubation period, each culture was centrifuged at $2135 \times g$ for 30 min and the pellets were resuspended in the same volume of growth broth (previously described) supplemented with 5% of glycerol. Then, the suspensions were frozen at −80 °C. Before the challenge tests, each suspension was thawed to a temperature of 4 °C, centrifuged at $2135 \times g$ for 30 min and the sediments were resuspended in the same volume of phosphate buffered saline (PBS). The suspensions were de-clumped by vortexing for 45 s, in the presence of 5 mm diameter glass beads (Sigma Aldrich, Milan, Italy).

To study the MAP survival dynamics during the creaming phase, the ATCC strain was employed to spike the milk: an inoculum of 15 mL of milk containing 8 $\log_{10}$ CFU/mL was added to 4 L of milk to reach an initial contamination level of 5.58 $\log_{10}$ CFU/mL.

To evaluate the different survival dynamics of MAP in the entire process, the milk (1000 L) deputed to the production of two cheese wheels was spiked with the ATCC strain (1 L containing approximately 8.3 $\log_{10}$ CFU/mL). The final concentration of MAP in milk was 5.19 ± 0.01 $\log_{10}$ CFU/mL. Another bulk of milk, dedicated to the production of other two cheese wheels, was spiked instead with a mixture (1 L containing 8.4 $\log_{10}$ CFU/mL) approximately of equal proportions of three wild strain suspensions to reach an initial contamination level of 5.28 ± 0.08 $\log_{10}$ CFU/mL CFU/mL of milk.

2.3. Challenge tests

2.3.1. Creaming phase

To study the influence of the creaming phase on MAP survival dynamics, 4 L of raw cow's milk (2 L for two different temperatures) provided from a local farm, were spiked with the ATCC 19698 reference strain. Then, the spiked milk was stored at 18 °C and at 27 °C (temperatures representing the extreme conditions previously recorded in working conditions in five different facilities) for 12 h. At the end of this phase, two fractions were obtained, partially skimmed milk (94.5% of the total volume) and cream (5.5%).

2.3.2. Manufacturing process

To study the effect of technological process and of physicochemical changes on MAP survival during the manufacturing process of PR and GP cheeses, a second challenge test was performed.

Cheese production was carried out according to the specifications stated in the “Disciplinare Grana Padano PDO” (www.granapadano.it)

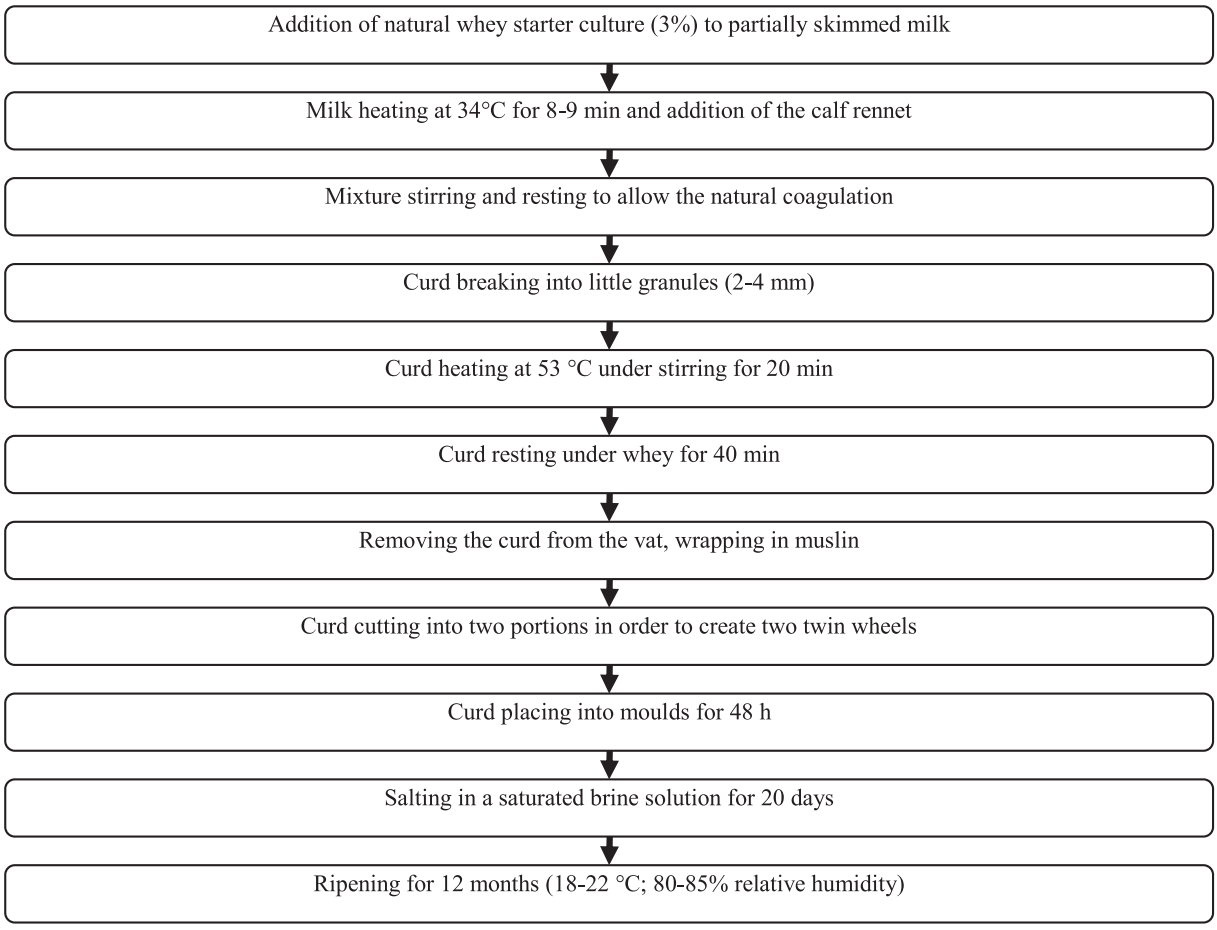

Fig. 1. Cheese manufacturing steps according to the specifications stated in the "Disciplinare Grana Padano DOP" and in the "Disciplinare Parmigiano Reggiano DOP".

and in the "Disciplinare Parmigiano Reggiano PDO" (www.parmigianoreggiano.com), using the most permissive parameters (times and temperatures) to MAP survival, among those previously recorded in working conditions in five different facilities.

The lysozyme addition to the raw milk, only permitted for GP, was omitted in order to provide the opportunity for maximal MAP survival.

Two batches of raw cow's milk (1000 L each), provided from a local dairy farm, were separately spiked with MAP reference strain (Batch A) and with MAP wild strains (Batch B), and the cheese manufacturing process was reproduced in an experimental cheese factory, in order to obtain four cheese wheels (two for each batch), following the steps described in Fig. 1.

Cheeses were ripened for 12 months in climatic chambers (TRH Climatic System, Perugia, Italy) under controlled conditions of temperature (18–22 °C) and air moisture (80–85% relative humidity) according to the manufacturer's instructions.

2.4. Sampling

Sampling procedures for microbiological analyses were carried out according to ISO 707:2008 (IDF 50: 2008) (ISO, 2008), taking every possible precaution to avoid cross-contamination.

During the first challenge test, 10 mL of milk (before the creaming phase) and 100 mL of partially skimmed milk (after the creaming) were collected under sterile conditions. MAP concentration was determined on both samples; fat/casein ratio was determined on the second sample.

During the second challenge test, the sampling was carried out on 50 mL of milk and 25 g of curd [(curd, cooked curd, curd under whey, curd in mould (1 h), curd in mould (24 h), curd in mould (48 h)] and cheese [cheese after salting (20 days), cheese during ripening (2, 3, 4, 5, 6, 8, 10 and 12 months)]. All the samples were taken in triplicate. In the moulding phase, the curd samples were taken, under sterile conditions and with a sterile knife, from the curd's top surface, where the temperature decreases more rapidly and consequently it is more likely that MAP could survive.

Regarding cheese, core samples were aseptically removed from both wheels of each batch, using a sterile cheese trier; the holes were filled with a mixture of sterile paraffin/vaseline oil (2,1 ratio).

MAP and Lactic Acid Bacteria (LAB) count, pH and a_w were determined as described below.

2.5. Microbiological and physicochemical analyses

The microbiological analyses were performed on milk through direct plate count. For curd and cheese samples, 10 g of each sample were homogenised using 90 mL of cheese diluent [casitone 1% (wt/vol) (Becton Dickinson, USA), sodium citrate 2% (wt/vol) (Carlo Erba, Milan, Italy), sodium chloride 0.5% (wt/vol) (VWR Prolabo, Fontenay Sous Bois, France)], employing a Stomacher 400 blender (Seward Medical, London, UK) (260 rpm for 3 min); then, the suspensions were warmed in water bath at 37 °C for 40 min. The samples were homogenised again for 3 min and 1 mL of homogenate was taken and serially tenfold diluted in Buffered Peptone Water (BPW). MAP count was determined by plating 0.1 mL of the homogenised cheese samples or milk and of the corresponding ten-fold dilutions into Petri dishes (9 cm of diameter) containing Herrold's egg yolk medium supplemented with Mycobactin J (2 mg/L) (IDvet Innovative Diagnostics, Montpellier, France), 0.4% (wt/vol) sodium pyruvate (Merk, Germany), 0.01% (wt/

vol) Malachite Green (Sigma-Aldrich, Milan, Italy), 2.4% (vol/vol) MGIT PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin) (Becton Dickinson and Company, USA), 0.1% (wt/vol) Nisin (VGP Pharmachem, Spain) and 100 IU/mL Penicillin G sodium salt (Sigma-Aldrich, Milan, Italy) (HEYM-PPN: Herrold Egg Yolk, Mycobactin, PANTA, Penicillin, Nisin). Petri dishes were incubated, after tape sealing, for three months at 37 °C.

Starting from the beginning of the ripening phase, the volume of the homogenised samples plated was raised to 0.5 mL in quadruplicate (total volume 2 mL), in order to maximize the assay sensitivity. For the same reason, from the third to the twelfth month of ripening, the homogenised sample volume plated was raised to 10 mL (20 plates, each inoculated with 0.5 mL of suspension).

LAB concentration was determined according to ISO 15214:1998 (ISO, 1998).

Regarding physicochemical analyses, during the creaming phase the fat/casein ratio was determined using MilkoScan 4000 (Foss, Padua, Italy), while, during all the manufacturing process, the pH was measured using a HI 223 Calibration check™ Microprocessor pH meter (Hanna Instrument, USA). Water activity (a_w) was measured at 25 °C with the a_w recorder AquaLab, series 3, Model TE (Decagon Devices, Inc., Pullman, USA), according to ISO 21807:2004 (ISO, 2004) and the temperature profile was constantly monitored and registered using a Thermo Button 22 L data logger (Astori Tecnica s.n.c., Italy).

2.6. Determination of limit of detection (LOD) for standard cultural analysis

The MAP ATCC 19698 reference strain was used to determine the limit of detection (LOD) of the culture test. Briefly, colonies from solid cultures were harvested and resuspended in PBS with 5 mm diameter glass beads (Sigma Aldrich, Milan, Italy), and mixed by vortexing for 45 s. The optical density at 600 nm was adjusted to 0.7 and, subsequently, the suspensions were forced through a syringe (needle 26 G) several times and then filtered through a sterile 5 µm diameter filter. Then, the suspension was examined in a Bürker chamber to grossly count the number of MAP cells. The initial suspensions were ten-fold serially diluted in PBS, using tubes containing 5 mm diameter glass beads (Sigma Aldrich, Milan, Italy) and mixed by vortexing for 20 s between each dilution step.

One hundred microliters of each dilution were plated (in duplicate) on Petri plates (diameter of 9 cm) containing HEYM-PPN.

In order to determine the MAP load in cheese (expressed as CFU/g), aliquots (10 g) of GP cheese, previously testing negative to IS900-qPCR (Ricchi et al., 2016), were spiked in six replicates with 100 µL of the previously described suspensions. Then, they were homogenised with 90 mL of cheese diluent in a stomacher plastic bag, using Stomacher 400 Circulator (260 rpm for 3 min) and warmed in water bath at 37 °C for 40 min. The samples were homogenised again for 3 min, then 2 mL (0.5 mL in four plates) of the undiluted homogenate and 0.1 mL of each diluted suspension were plated on HEYM-PPN plates. The plates were incubated at 37 °C for 90 days.

2.7. Statistical analysis

The results of the microbiological plate counts were expressed as colony forming unit (CFU) per mL or g. Microbial counts were reported in terms of $\log_{10}$ CFU/g or mL. The means and standard deviations of microbiological and physicochemical results were determined on the basis of the average of three samples. The data was statistically analysed using the R statistical software version 2.7.0 (R Development Core Team, 2008). Differences between mean values were detected through the HSD Tukey's test and evaluations were based on a confidence interval of 95%.

3. Results

3.1. Limit of detection (LOD) for standard cultural analysis

The LOD of the traditional cultural method on HEYM-PPN was 1 $\log_{10}$ CFU MAP/g of cheese.

3.2. Creaming phase

After 12 h of natural creaming phase, the fat/casein ratios in skimmed milk were 0.94 and 0.99 at 18 °C and 27 °C, respectively. The values were in line with those reported by the “Disciplinare Grana Padano D.O.P.” (limits 0.80–1.05). In partially skimmed milk a MAP reduction ($p < 0.05$) of 0.77 $\log_{10}$ was observed at 27 °C (from 5.58 ± 0.11 $\log_{10}$ CFU/mL to 4.80 ± 0.07 $\log_{10}$ CFU/mL), while the observed reduction at 18 °C was 0.80 $\log_{10}$ (from 5.58 ± 0.11 $\log_{10}$ CFU/mL to 4.78 ± 0.10 $\log_{10}$ CFU/mL). On the contrary, the two temperature conditions were not statistically different.

3.3. Manufacturing process

During the cheesemaking process, starting from milk at 11.8 °C, the temperature raised gradually to 34 °C, when the coagulation occurred in around 10 min. Then, the temperature raised to 53 °C in about 10 min, remaining constant for 20 min during the curd heating. After this phase, the curd dropped to the bottom of the vat, resting under whey and maintaining a temperature around 52 °C for a further 40 min (Fig. 2). Then, the curd was extracted and put in a mould, where a Thermo Button 22 L data logger (Astori Tecnica s.n.c., Italy), located a few centimetres below the cheese surface, registered a temperature of over 50 °C for at least the following 8 h. A progressive cooling was observed in cheese wheels during the moulding phase and the salting phase, in which the temperature reached 20 °C after three days (Fig. 3). During the ripening period, the cheese wheels were kept in climatic chambers (TRH Climatic System, Perugia, Italy) under controlled conditions of temperature (18–22 °C) and air moisture (80–85% relative humidity), according to the manufacturer's instructions.

The main changes in microbiological and physicochemical properties of cheese were recorded during the first phases of the manufacturing process (Table 1).

Particularly, LAB concentration in milk was 3.48 ± 0.04 and 3.27 ± 0.06 $\log_{10}$ CFU/mL in Batch A and Batch B, respectively, and raised during the cheesemaking and the moulding phase. During the ripening, the LAB concentration was almost constant until the fifth month, decreasing ($p < 0.05$) from the sixth month, and no changes were observed until the end of the ripening period ($p > 0.05$) (Table 1).

The LAB growth caused a rapid drop of the pH which decreased during the cheesemaking: starting from 6.67 ± 0.01 and 6.68 ± 0.01 in milk, the pH decreased ($p < 0.05$) to 5.31 ± 0.16 and 5.25 ± 0.06 in curd under whey in Batch A and Batch B, respectively. Then, no significant pH changes were observed during the ripening until the twelfth month, when a significant increase ($p > 0.05$) was observed in both the tested batches.

An a_w decrease was observed during all the manufacturing process, reaching values of 0.909 ± 0.004 and 0.900 ± 0.004 in Batch A and Batch B, respectively, at the end of the ripening period (Table 1).

Notably, MAP concentration did not change significantly during the processing of the cheese in the vat, while a significant decrease ($p < 0.05$) was observed during the moulding and after the salting in brine, regarding the wild strains and the reference strain, respectively (Fig. 4).

During the ripening phase, the viable MAP concentration showed a time-dependent decrease for both spiked cheese wheels. Particularly, in the Batch A, spiked with the reference ATCC strain, no viable MAP cells were detectable from the third month of ripening, while in the Batch B,

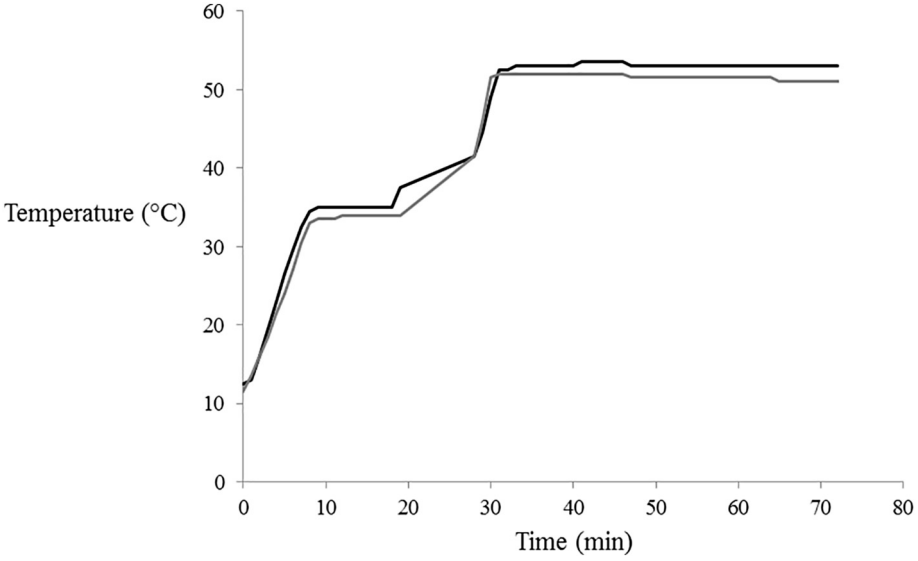

Fig. 2. Time-temperature profile registered during the first 72 min of the cheese manufacturing in Batch A (reference strain) (black line) and in Batch B (wild strains) (gray line).

spiked with the mixture of wild strains, no viable MAP were detectable from the second month of ripening (Fig. 4).

4. Discussion

This is the first paper reporting data about MAP survival dynamics concerning the Italian hard cheeses, Parmigiano Reggiano and Grana Padano. Our study clearly demonstrated how the different manufacturing phases of these products affect MAP viability. In particular, the creaming phase was able to reduce the MAP load in milk by nearly one $\log_{10}$. This could be due to the fact that fat micelles are able to entrap MAP during this phase, reducing its load, similarly to what has been observed for other bacteria, like Clostridia (D'Incecco et al., 2015; D'Incecco et al., 2018).

Notably, regarding PR production, the benefit of the creaming phase on MAP load is reduced by nearly 50% because it is made from naturally skimmed milk of the evening session mixed with the same volume of whole fat milk of the morning.

Moreover, our data suggests that the curd cooking at 53 °C for 20 min does not kill MAP, contrary to the lethal effects reported in PR and GP for other non-sporulating bacteria (*Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* Typhimurium, and *Staphylococcus aureus*) (Ercolini et al., 2005; Nocetti, 2006; Panari et al., 2004). This is in agreement with a previous study which highlighted that the curd-cooking temperature of 53 °C for 45 min had a minimal effect on MAP survival in the hard cheese Swiss Emmentaler (Spahr and Schafroth, 2001). It is possible, however, that the long permanence (about 9 h) at over 50 °C could have caused sub-lethal damage to MAP cells which,

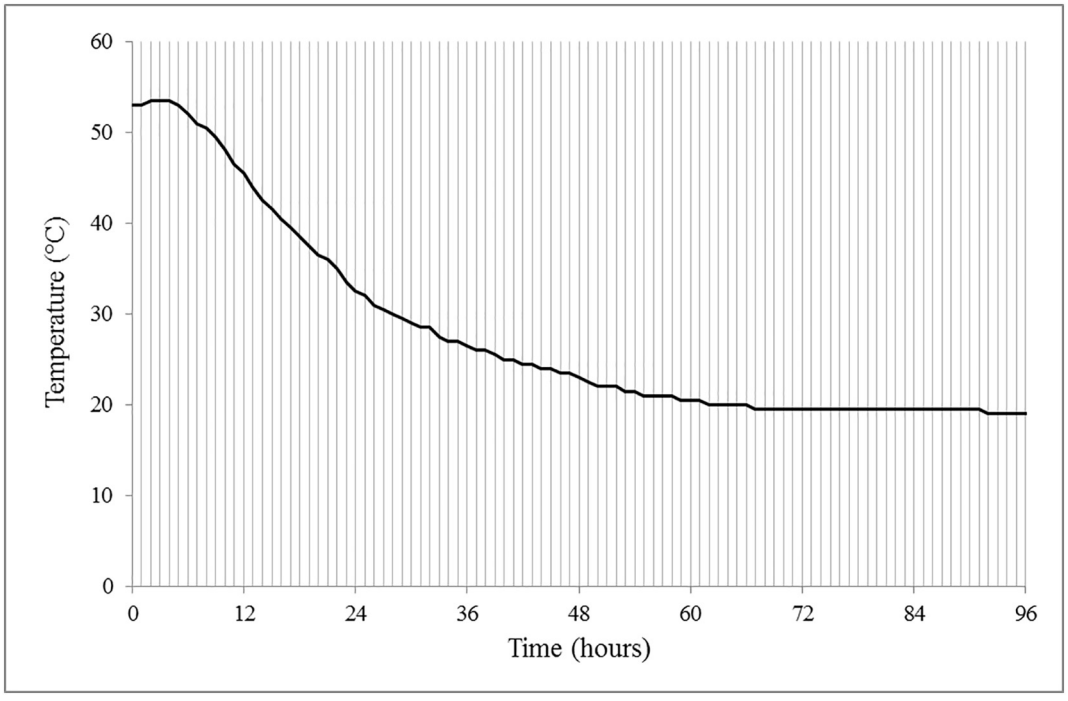

Fig. 3. Time-temperature profile registered from the moulding phase until the first 96 h of the cheese manufacturing.

Table 1
Changes of pH, a_w and Lactic acid bacteria (LAB) during the cheese manufacturing process. Data represent the average values ± standard deviation of three replicates samples. For each parameter, means with different uppercase letters within a column are significantly different ($p < 0.05$) during the process, means with different lowercase letters within a row are significantly different ($p < 0.05$) between the batches.

Manufacturing process	Product	pH		a_w		LAB[b]	
		Batch A	Batch B	Batch A	Batch B	Batch A	Batch B
Cheese making	Milk	6.67 ± 0.01 Aa	6.68 ± 0.01 Aa	ND[a]	ND[a]	3.48 ± 0.04 Aa	3.27 ± 0.06 Ab
	Curd	6.33 ± 0.03 Ba	6.26 ± 0.05 Ba	0.992 ± 0.001 Aa	0.993 ± 0.003 Aa	5.66 ± 0.17 Ba	5.77 ± 0.18 Ba
	Cooked curd	6.02 ± 0.06 Ca	5.81 ± 0.09 Cb	0.992 ± 0.002 Aa	0.991 ± 0.002 Aa	5.38 ± 0.08 Ba	5.57 ± 0.03 Bb
	Curd under whey	5.31 ± 0.16 DEa	5.25 ± 0.06 Da	0.988 ± 0.001 Aa	0.987 ± 0.001 Aa	6.18 ± 0.18 Ca	6.35 ± 0.3 Ca
Moulding	Curd in mould (1 h)	5.34 ± 0.02 Da	5.34 ± 0.02 Da	0.987 ± 0.001 Aa	0.986 ± 0.001 Aa	6.92 ± 0.11 Da	6.74 ± 0.04 CEa
	Curd in mould (24 h)	5.22 ± 0.02 Da	5.21 ± 0.02 Da	0.984 ± 0.002 Aa	0.986 ± 0.001 Aa	6.82 ± 0.26 Da	6.49 ± 0.29 Ca
	Cheese in mould (48 h)	5.3 ± 0.05 Da	5.26 ± 0.08 Da	0.981 ± 0.006 Aa	0.985 ± 0.001 Aa	5.88 ± 0.08 BCa	5.48 ± 0.36 Ba
Salting in brine	Cheese after salting (20 days)	5.36 ± 0.01 Da	5.32 ± 0.03 Da	0.972 ± 0.003 Aa	0.963 ± 0.018 Ba	7.73 ± 0.06 Ea	7.67 ± 0.07 Da
Ripening	Cheese (2 months)	5.22 ± 0.08 Da	5.27 ± 0.04 Da	0.949 ± 0.018 Ba	0.945 ± 0.003 Ba	7.86 ± 0.14 Ea	7.94 ± 0.12 Da
	Cheese (3 months)	5.31 ± 0.01 Da	5.30 ± 0.01 Da	0.945 ± 0.007 Ba	0.938 ± 0.005 BCa	7.72 ± 0.1 Ea	7.83 ± 0.07 Da
	Cheese (4 months)	5.36 ± 0.05 Da	5.32 ± 0.02 Da	0.914 ± 0.005 Ca	0.923 ± 0.013 CDa	7.34 ± 0.16 Ea	7.39 ± 0.04 Da
	Cheese (5 months)	5.33 ± 0.02 Da	5.27 ± 0.02 Da	0.894 ± 0.009 Ca	0.902 ± 0.011 Da	7.12 ± 0.07 Ea	7.05 ± 0.02 Ea
	Cheese (6 months)	5.29 ± 0.04 Da	5.26 ± 0.02 Da	0.905 ± 0.011 Ca	0.891 ± 0.005 Da	6.28 ± 0.02 Ca	5.91 ± 0.12 Bb
	Cheese (8 months)	5.27 ± 0.01 Da	5.3 ± 0.02 Da	0.900 ± 0.002 Ca	0.882 ± 0.008 Db	5.71 ± 0.34 Ba	5.45 ± 0.18 Ba
	Cheese (10 months)	5.3 ± 0.04 Da	5.32 ± 0.03 Da	0.898 ± 0.010 Ca	0.901 ± 0.009 Da	5.33 ± 0.15 Ba	5.36 ± 0.11 Ba
	Cheese (12 months)	5.48 ± 0.06 Ea	5.45 ± 0.07 Ea	0.909 ± 0.004 Ca	0.900 ± 0.004 Da	5.48 ± 0.07 Ba	5.53 ± 0.05 Ba

[a] ND: not determined.
[b] Values are means $\log_{10}$ CFU/g ± standard deviation.

combined with the unfavourable environmental conditions, in particular low pH values recorded during the ripening period, could explain the subsequent decrease in the MAP load. With reference to this, Sung and Collins (2000) observed that the ripening period and low pH values are more effective in killing MAP if a heat treatment is previously applied.

In addition, our study demonstrated that the ripening phase inactivates MAP in these typical Italian products, largely before the end of the minimum legal ripening period (nine months for GP and twelve months for PR); in fact, this bacterium became undetectable by traditional cultural test after two (reference strain) and three months (wild strains) of ripening. Furthermore, another relevant factor affecting MAP survival in our study could be the high LAB concentration which could have overwhelmed the other bacterial populations, including MAP, not being affected by the low pH values recorded. This is in agreement with a recent paper which showed that in ultra-filtered white cheese, made with MAP spiked milk added with a starter culture, MAP load dropped below the detection limit after 40 days of ripening, while in the same cheese, made without the employment of the starter culture, MAP survived up to 60 days (Hanifian, 2014). LAB cause nutrient depletion and produce inhibitory substances, which could have a synergistic detrimental effect on MAP survival, as hypothesized by previous researches (Alemdar and Ağaoğlu, 2010; Hanifian and Khani, 2012; Mohammadi et al., 2009). In this regard, LAB can produce a wide

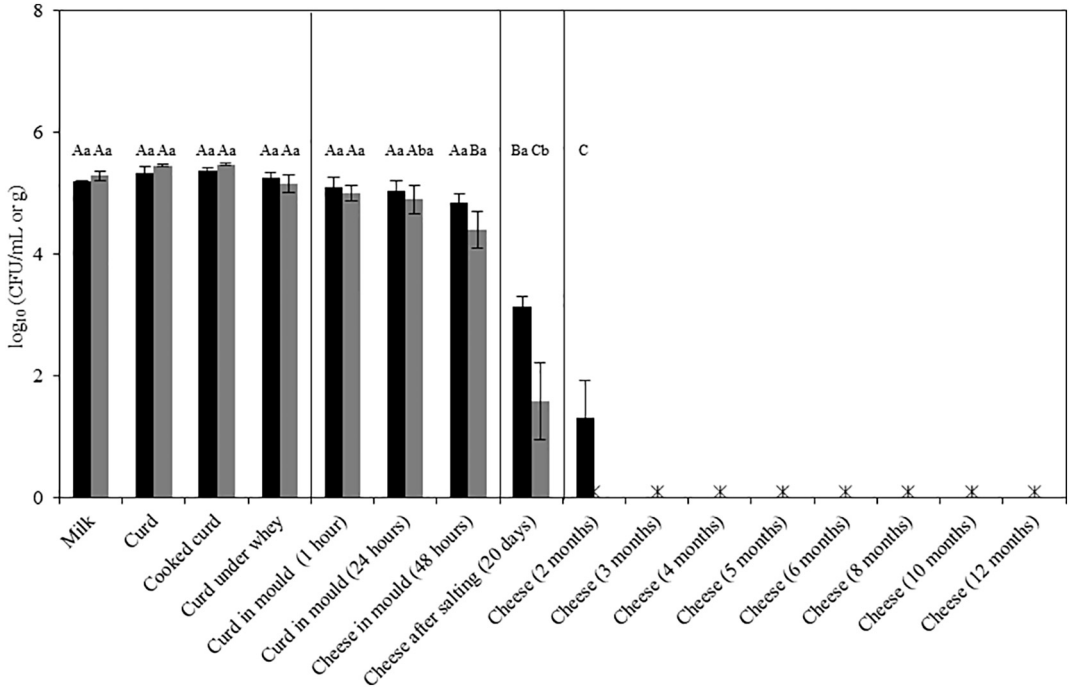

Fig. 4. Variation of Map concentration (Log CFU/mL-g) throughout the cheese manufacturing and ripening: wild strain mixture (IZSLER 706/2011, IZSLER 55/2012, IZSLER 755/2012) (gray bars) and ATCC 19698 strain (black bars). Bars with different uppercase letters are significantly different ($p < 0.05$) during the process, bars with different lowercase letters are significantly different ($p < 0.05$) between the batches.

spectrum of bacteriocins, which are complex compounds with the ability to kill other bacteria, especially under stressful conditions, like overcrowding (Carroll et al., 2010).

Notably, the reference ATCC 19698 strain and the wild strain mixture employed in our study showed a slightly different behaviour during the ripening period, being the latter no more detectable after the second month of ripening *versus* the third month for the ATCC strain. This could be due to the fact that the laboratory-adapted strain ATCC 19698 has a higher thermal tolerance than that of the wild strains, as previously demonstrated by Sung and Collins (1998).

In addition, we excluded the possibility that MAP was no more detectable by culture during the ripening phase because of the inhibitory effect of HEYM-PPN on MAP growth. In this regard, a preliminary study (data not shown) assessed that the selectivity for MAP of HEYM-PPN, tested on spiked milk in parallel with HEYM-VAN (a standard medium, largely used in the diagnostics of MAP) (Whittington, 2010), was not significantly different. On the contrary, HEYM-PPN proved to be very effective in inhibiting the growth of indigenous and technological flora and its use for MAP detection in cheeses was recommended by Donaghy and Rowe (2009).

In conclusion, our study demonstrates that the long ripening period in which a high load of microbiota and low pH levels are recorded, can ensure the total MAP inactivation in these products, which is relevant considering that these cheeses are entirely made by raw milk. In addition, it should be considered that the probability of MAP survival is correlated with the MAP contamination level of the milk employed for the cheese manufacturing (Donaghy et al., 2004). In this regard, a recent study, carried out on bulk tank milk of 2934 herds of Emilia Romagna Region (Italy) (Ricchi et al., 2016), where the milk is mainly destined for the PR and GP production, proved that MAP was either absent or present at a concentration below 15 cells/mL, corresponding to 0.15–1.5 CFU/mL, according to previous studies (Herthnek et al., 2008; Kralik et al., 2012; Ricchi et al., 2017), in 97% of the samples tested (Ricchi et al., 2016). Instead, out of the positive samples, the maximum MAP load observed was 1424 MAP cells/mL, corresponding to 14–142 CFU/mL (1.15–2.15 $\log_{10}$ CFU/mL) (Herthnek et al., 2008; Kralik et al., 2012; Ricchi et al., 2017), at least three $\log_{10}$ lower than the contamination level applied in this study (5 $\log_{10}$ CFU/mL of milk), without considering the effect of the creaming phase.

Therefore, considering the low natural MAP contamination level of raw milk and the effect of the production process registered in this challenge study, we can conclude that the likelihood of MAP survival in PR and GP could be considered negligible.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgments

This work was supported by Public Health Service of the Ministry of Health, Italy (grant E87G11000090001-PRF 2009202).

References

Alemdar, S., Ağaoğlu, S., 2010. Survival of *Salmonella* Typhimurium during the ripening of herby cheese (Otlu Peynir). J. Food Saf. 30, 526–536.

Atreya, R., Bülte, M., Gerlach, G.F., Goethe, R., Hornef, M.W., Köhler, H., Meens, J., Möbius, P., Roeb, E., Weiss, S., 2014. Facts, myths and hypotheses on the zoonotic nature of *Mycobacterium avium* subspecies *paratuberculosis*. Int. J. Med. Microbiol. 304 (7), 858–867.

Botsaris, G., Slana, I., Liapi, M., Dodd, C., Economides, C., Rees, C., Pavlik, I., 2010. Rapid detection methods for viable *Mycobacterium avium* subspecies *paratuberculosis* in milk and cheese. Int. J. Food Microbiol. 141, S87–S90.

Carroll, J., Draper, L.A., O'Connor, P.M., Coffey, A., Hill, C., Ross, R.P., Cotter, P.D., O'Mahony, J., 2010. Comparison of the activities of the lantibiotics nisin and lacticin 3147 against clinically significant mycobacteria. Int. J. Antimicrob. Agents 36 (2), 132–136.

Chiodini, R.J., Chamberlin, W.M., Sarosiek, J., McCallum, R.W., 2012. Crohn's disease and the mycobacterioses: a quarter century later. Causation or simple association? Crit. Rev. Microbiol. 38, 52–93.

Clark Jr., D.L., Anderson, J.L., Koziczkowski, J.J., Ellingson, J.L.E., 2006. Detection of *Mycobacterium avium* subspecies *paratuberculosis* genetic components in retail cheese curds purchased in Wisconsin and Minnesota by PCR. Mol. Cell. Probes 20 (3–4), 197–202.

Cocito, C., Gilot, P., Coene, M., de Kesel, M., Poupart, P., Vannuffel, P., 1994. Paratuberculosis. Clin. Microbiol. Rev. 7 (3), 328–345.

Collins, M.T., 2011. Food safety concerns regarding paratuberculosis. Vet. Clin. North Am. Food. Anim. Pract. 27 (3), 631–636.

Cossu, D., Cocco, E., Paccagnini, D., Masala, S., Ahmed, N., Frau, J., Marrosu, M.G., Sechi, L.A., 2011. Association of *Mycobacterium avium* subsp. *paratuberculosis* with multiple sclerosis in Sardinian patients. PLoS One 6 (4), e18482.

D'Incecco, P., Faoro, F., Silvetti, T., Schrader, K., Pellegrino, L., 2015. Mechanisms of *Clostridium tyrobutyricum* removal through natural creaming of milk: a microscopy study. J. Dairy Sci. 98 (8), 5164–5172.

D'Incecco, P., Ong, L., Pellegrino, L., Faoro, F., Barbiroli, A., Gras, S., 2018. Effect of temperature on the microstructure of fat globules and the immunoglobulin-mediated interactions between fat and bacteria in natural raw milk creaming. J. Dairy Sci. 101 (4), 2984–2997.

Donaghy, J.A., Rowe, M., 2009. The EU ParaTBTools project – thematic area 3: the inactivation of *Mycobacterium avium* subsp. *paratuberculosis* in milk and dairy products. In: Proceedings of the 10th International Colloquium on Paratuberculosis, August 9–15, 2009, Minneapolis, Minnesota, pp. 207.

Donaghy, J.A., Totton, N.L., Rowe, M.T., 2004. Persistence of *Mycobacterium paratuberculosis* during manufacture and ripening of cheddar cheese. Appl. Environ. Microbiol. 70 (8), 4899–4905.

Dow, C.T., 2012. *M. paratuberculosis* heat shock protein 65 and human diseases: bridging infection and autoimmunity. Autoimmune Dis 2012, 6.

Eltholth, M.M., Marsh, V.R., Van Winden, S., Guitian, F.J., 2009. Contamination of food products with *Mycobacterium avium paratuberculosis*: a systematic review. J. Appl. Microbiol. 107 (4), 1061–1071.

Ercolini, D., Fusco, V., Blaiotta, G., Sarghini, F., Coppola, S., 2005. Response of *Escherichia coli* O157: H7, *Listeria monocytogenes*, *Salmonella* Typhimurium, and *Staphylococcus aureus* to the thermal stress occurring in model manufactures of Grana Padano cheese. J. Dairy Sci. 88 (11), 3818–3825.

Faria, A.C.S., Schwarz, D.G.G., Carvalho, I.A., Rocha, B.B., De Carvalho Castro, K.N., Silva, M.R., Moreira, M.A.S., 2014. Short communication: viable *Mycobacterium avium* subspecies *paratuberculosis* in retail artisanal Coalho cheese from northeastern Brazil. J. Dairy Sci. 97 (7), 4111–4114.

Galiero, A., Fratini, F., Turchi, B., Colombani, G., Nuvoloni, R., Cerri, D., 2015. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in a sheep flock in Tuscany. Trop. Anim. Health Prod. 47 (8), 1567–1571.

Galiero, A., Fratini, F., Mataragka, A., Turchi, B., Nuvoloni, R., Ikonomopoulos, J., Cerri, D., 2016. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in cheeses from small ruminants in Tuscany. Int. J. Food Microbiol. 217, 195–199.

Galiero, A., Turchi, B., Pedonese, F., Nuvoloni, R., Cantile, C., Colombani, G., Forzan, M., Cerri, D., Bandecchi, P., Fratini, F., 2017. Serological, culture and molecular survey of *Mycobacterium avium paratuberculosis* in a goat flock in Tuscany. Folia Microbiol 62 (6), 471–477.

Giese, S.B., Ahrens, P., 2000. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk from clinically affected cows by PCR and culture. Vet. Microbiol. 77 (3–4), 291–297.

Gill, C.O., Saucier, L., Meadus, W.J., 2011. *Mycobacterium avium* subsp. *paratuberculosis* in dairy products, meat, and drinking water. J. Food Prot. 74 (3), 480–499.

Hanifian, S., 2014. Survival of *Mycobacterium avium* subsp. *paratuberculosis* in ultra-filtered white cheese. Lett. Appl. Microbiol. 58 (5), 466–471.

Hanifian, S., Khani, S., 2012. Fate of *Yersinia enterocolitica* during manufacture, ripening and storage of Lighvan cheese. Int. J. Food Microbiol. 156, 141–146.

Herthnek, D., Nielsen, S.S., Lindberg, A., Bölske, G., 2008. A robust method for bacterial lysis and DNA purification to be used with real-time PCR for detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk. J. Microbiol. Methods 75 (2), 335–340.

Hsu, C.Y., Wu, C.W., Talaat, A.M., 2011. Genome-wide sequence variation among *Mycobacterium avium* subspecies *paratuberculosis* isolates: a better understanding of Johne's disease transmission dynamics. Front. Microbiol. 2, 236.

Ikonomopoulos, J., Pavlik, I., Bartos, M., Svastova, P., Ayele, W.Y., Roubal, P., Lukas, J., Cook, N., Gazouli, M., 2005. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in retail cheeses from Greece and the Czech Republic. Appl. Environ. Microbiol. 71 (12), 8934–8936.

ISO, E, 1998. ISO 15214: 1998, microbiology of food and animal feeding stuffs–horizontal method for the enumeration of mesophilic lactic acid bacteria–Colony-count technique at 30 degrees C. In: ICS. vol. 7. pp. 300.

ISO, E, 2004. ISO 21807: 2004, Microbiology of Food and Animal Feeding Stuffs, Determination of Water Activity.

ISO, E, 2008. ISO 707: 2008 (IDF 50: 2008), Milk and Milk Products-Guidance on Sampling.

Kralik, P., Beran, V., Pavlik, I., 2012. Enumeration of *Mycobacterium avium* subsp. *paratuberculosis* by quantitative real-time PCR, culture on solid media and optical densitometry. BMC Res. Notes 5. https://doi.org/10.1186/1756-0500-5-114.

de Melo, C.B., de Sá, M.E.P., dos Reis Souza, A., de Oliveira, A.M., Moacyr, P., Mota, P.C., Campani, P.R., Luna, J.O., Pinto, S.C., Schwingel, F.F., McManus, C., Seixas, L., 2014. Bacteria in dairy products in baggage of incoming travelers, Brazil. Emerging Infect. Dis. 20 (11), 1933–1935.

Mohammadi, K., Karim, G., Razavilar, V., Hanifian, S., 2009. Study on the growth and survival of *Escherichia coli* O157:H7 during the manufacture and storage of Iranian

white cheese in brine. Iran J. Vet. Res. 10, 346–351.
Nocetti, M., 2006. Sicurezza alimentare del Parmigiano-Reggiano. Acta Biomed 77 (Quaderno 3), 00-00.
Okura, H., Toft, N., Nielsen, S.S., 2012. Occurrence of *Mycobacterium avium* subsp. *paratuberculosis* in milk at dairy cattle farms: a systematic review and meta-analysis. Vet. Microbiol. 157 (3–4), 253–263.
Panari, G., Pecorari, M., Merialdi, G., Dottori, M., 2004. The behaviour of potentially pathogenic bacteria in the production of Parmigiano-Reggiano cheese [Emilia-Romagna; Lombardy]. Scienza e Tecnica Lattiero Casearia 55 (2), 137–146.
Pozzato, N., Capello, K., Comin, A., Toft, N., Nielsen, S.S., Vicenzoni, G., Arrigoni, N., 2011. Prevalence of paratuberculosis infection in dairy cattle in northern Italy. Prev. Vet. Med. 102 (1), 83–86.
R Development Core Team, 2008. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
Rademaker, J.L., Vissers, M.M., Te Giffel, M.C., 2007. Effective heat inactivation of *Mycobacterium avium* subsp. *paratuberculosis* in raw milk contaminated with naturally infected feces. Appl. Environ. Microbiol. 73 (13), 4185–4190.
Ricchi, M., Savi, R., Bolzoni, L., Pongolini, S., Grant, I.R., De Cicco, C., Cerutti, G., Cammi, G., Garbarino, C.A., Arrigoni, N., 2016. Estimation of *Mycobacterium avium* subsp. *paratuberculosis* load in raw bulk tank milk in Emilia-Romagna region (Italy) by qPCR. Microbiologyopen 5 (4), 551–559.
Ricchi, M., Bertasio, C., Boniotti, M.B., Vicari, N., Russo, S., Tilola, M., Bellotti, M.A., Bertasi, B., 2017. Comparison among the quantification of bacterial pathogens by qPCR, dPCR, and cultural methods. Front. Microbiol 8 (JUN). https://doi.org/10.3389/fmicb.2017.01174.
Scanu, A.M., Bull, T.J., Cannas, S., Sanderson, J.D., Sechi, L.A., Dettori, G., Zanetti, S., Hermon-Taylor, J., 2007. *Mycobacterium avium* subspecies *paratuberculosis* infection in cases of irritable bowel syndrome and comparison with Crohn's disease and Johne's disease: common neural and immune pathogenicities. J. Clin. Microbiol. 45 (12), 3883–3890.
Sisto, M., Cucci, L., D'Amore, M., Dow, T.C., Mitolo, V., Lisi, S., 2010. Proposing a relationship between *Mycobacterium avium* subspecies *paratuberculosis* infection and Hashimoto's thyroiditis. Scand. J. Infect. Dis. 42 (10), 787–790.
Spahr, U., Schafroth, K., 2001. Fate of *Mycobacterium avium* subsp. *paratuberculosis* in Swiss hard and semihard cheese manufactured from raw milk. Appl. Environ. Microbiol. 67 (9), 4199–4205.
Stephan, R., Schumacher, S., Tasara, T., Grant, I.R., 2007. Prevalence of *Mycobacterium avium* subspecies *paratuberculosis* in Swiss raw milk cheeses collected at the retail level. J. Dairy Sci. 90 (8), 3590–3595.
Sung, N., Collins, M.T., 1998. Thermal tolerance of *Mycobacterium paratuberculosis*. Appl. Environ. Microbiol. 64 (3), 999–1005.
Sung, N., Collins, M.T., 2000. Effect of three factors in cheese production (pH, salt, and heat) on *Mycobacterium avium* subsp. *paratuberculosis* viability. Appl. Environ. Microbiol. 66 (4), 1334–1339.
Sweeney, R.W., Whitlock, R.H., Rosenberger, A.E., 1992. *Mycobacterium paratuberculosis* cultured from milk and supramammary lymph nodes of infected asymptomatic cows. J. Clin. Microbiol. 30 (1), 166–171.
Vissers, M.M., Driehuis, F., Te Giffel, M.C., De Jong, P., Lankveld, J.M., 2007. Short communication: quantification of the transmission of microorganisms to milk via dirt attached to the exterior of teats. J. Dairy Sci. 90 (8), 3579–3582.
Waddell, L.A., Rajić, A., Stärk, K.D.C., McEwen, S.A., 2015. The zoonotic potential of *Mycobacterium avium* ssp. *paratuberculosis*: a systematic review and meta-analyses of the evidence. Epidemiol. Infect. 143 (15), 3135–3157.
Whittington, R., 2010. Cultivation of *Mycobacterium avium* subsp. *paratuberculosis*. . In: In: Paratuberculosis: Organism, Disease, Control, pp. 244–266 .
Williams, A.G., Withers, S.E., 2010. Microbiological characterisation of artisanal farmhouse cheeses manufactured in Scotland. Int. J. Dairy Technol. 63 (3), 356–369.