Behaviour of *Listeria monocytogenes* in artisanal raw milk *Pecorino Umbro* cheese: a microbiological challenge test

Roberta Ortenzi,[1] Raffaella Branciari,[2] Sara Primavilla,[1] David Ranucci,[2] Andrea Valiani[1]

[1]Institute for Experimental Veterinary Medicine of Umbria and Marche, Perugia; [2]Department of Veterinary Medicine, University of Perugia, Italy

Abstract

In the present study, a microbiological challenge test in artificially contaminated raw milk *Pecorino Umbro* cheese during cheese-making was carried out. Raw ewe milk was contaminated by a suspension of particular *Listeria monocytogenes* strains. The number of *L. monocytogenes* and *L. monocytogenes* dynamic growth were evaluated during cheese-making and storage. A significant decrease of the viable count of *L. monocytogenes* was observed during ripening and *L. monocytogenes* viable count was below the limit of quantification during storage. The results show that the product is unable to support the growth of the pathogen.

Introduction

Listeria monocytogenes is a foodborne pathogen, which has been responsible for several outbreaks of listeriosis reported in Europe (Gambarin *et al*., 2012; Lunden *et al*., 2004). Some outbreaks have been linked to the consumption of food products of animal origin that did not undergo heat treatment; their contamination came from several farm sources (Kongo *et al*., 2006). In particular, the contamination of fermented dairy products made from raw milk by *L. monocytogenes* may be due to either the use of contaminated raw milk or post-processing contamination from environmental sources not directly linked to raw milk (Kells and Gilmour, 2004). According to Regulation No. 2073/2005 (European Commission, 2005), the tolerance level of 100 CFU g^{-1} of *L. monocytogenes* in ready-to-eat (RTE) products such as cheese was introduced in Europe and RTE were legislatively distinguished into those that support the growth of *L. monocytogenes* and those that do not support the growth of this species. For RTE foods that are able to support the growth of *L. monocytogenes* and which may pose a *L. monocytogenes* risk for public health, food business operators responsible for manufacturing of the product shall conduct scientific studies in order to demonstrate that the product complies with the criteria of the regulations throughout the shelf-life (Spanu *et al*., 2014). Nevertheless, food business operators hardly found studies in the literature that could be adopted for their own products, especially for artisanal ones (Dalzini *et al*., 2014).

For these reasons, the aim of the study was to evaluate the dynamic growth of *L. monocytogenes* during cheese-making and the storage of a traditional raw milk *Pecorino Umbro* cheese characterized by a shorter ripening time (40 gg) and semi-hard consistency (Branciari *et al*., 2014). The aim of this study was to propose a useful scientific tool for food manufacturers that produces cheese with similar characteristics, to demonstrate whether their product supports the growth of *L. monocytogenes* or not, according to EC Regulation No. 2073/2005 (European Commission, 2005).

Correspondence: David Ranucci, Department of Veterinary Medicine, University of Perugia, Via San Costanzo 4, 06126 Perugia (PG), Italy
Tel: +39.075.5857931 - Fax: +39.075.5857928.
E-mail: david.ranucci@unipg.it

Conflict of interest: the authors declare no potential conflict of interest.

Key words: *Listeria monocytogenes;* Microbiological challeng test; Ewe cheese.

Received for publication: 16 June 2015.
Revision received: 14 July 2015.
Accepted for publication: 14 July 2015.

Italian Journal of Food Safety 2015; 4:5370
doi:10.4081/ijfs.2015.5370

Materials and Methods

Cheesemaking of *Pecorino Umbro*

For *Pecorino Umbro* cheese making, whole sheep milk was heated at 55-58°C and a lyophilised mixed-strain starter culture was added (*Streptococcus thermophilus*, *Lactobacillus delbrueckii subsp. bulgaricus* and *Lactococcus lactis subsp. lactis e/o subsp. cremoris*, Lyofast MOT 0.82 CE, CO, Italy). The coagulation took place at 33-38°C with the addition of powder calf rennet, and was completed in about 40 min. Then the curd was cut in grain of 3-5 mm, and was cooked at 41-43°C. Afterwards, 2 kg-cheeses were placed into moulds and salting was carried out in brine (NaCl 25%) for 24 hours. Ripening was conducted in appropriate cells for 40 days at 12°C. The shelf-life of the cheese was 50 days at +4°C, as established by the manufacturer.

Preparation of samples

For the experiment, three batches of cheese were produced and each batch consisted in 3 experimental groups of 10 2 kg-cheese each. The first group was produced at the Institute for Experimental Veterinary Medicine of Umbria and Marche (IZSUM) and was done with contaminated milk, during the adjunct of starter, by a suspension of multi-strain mix of *L. monocytogenes* at approximatively 10^2 CFU mL^{-1} (CLm). This sample unit was used to evaluate the behaviour of *L. monocytogenes* during cheese making and storage.

The second group (CTRIZS) was produced by the food business operator and ripened/stored at the IZSUM to ensure that the same ripening condition and product evolution occurred both at manufacturer and IZSUM level. Chemical, physical and microbial analyses of the cheese were carried out to verify the ripening conditions. The unit was also used to detect and/or enumerate *L. monocytogenes* occurring naturally in this type of cheese.

The third group (CTR) was produced and ripened by the manufacturer in order to compare the products submitted to challenge testing (CLm) to the products that are routinely produced by the processor (CTR) and to evaluate the physicochemical and microbial characteristics of cheese.

In total, 81 cheeses (9 sample units/3 groups/3 batches) were sampled along the testing period and submitted to analytical determinations. After ripening, the cheese was maintained at 8°C for 7 days and 12°C for 42 days. These storage conditions were determined following the EURL Lm Technical Guidance Document - On shelf-life studies on *L. monocytogenes* in ready-to-eat foods Version 2 – November 2008.

Bacterial cultures and inoculum preparation

A multi-strain mix of *L. monocytogenes* (ATCC® 7644™, Lm2963/12 and Lm6976/13 from the IZSUM collection and derived from pecorino cheese) was used in the study. Each stock culture was lyophilised and kept at +4°C.

The cultures were regenerated into BHI and incubated at 37°C for 24 h. Aliquots of each culture were transferred into tubes containing BHI and incubated at 12±1°C, which is a temperature close to the storage condition of the product. The stationary phase was reached after 18-20 h. After centrifuging each strain at 2178 g at 10°C for 5 min, the supernatant was discarded and the pellet was resuspended in 10

mL of sterile physiological solution (H_2O with 0.9% NaCl). Counts were confirmed by serial decimal dilution and inoculation in Agar Listeria Ottaviani (ALOA Selective Supplement, ALOA Enrichment Supplement; Biolife, Italy) plates incubated at 37°C for 24-48 h. Each of the 3 strains was combined in an equal quantity at the concentration of about 7 Log CFU g^{-1}. Dilutions of the mixed cultures were made to obtain a concentration in the foodstuff that was similar to the concentration occurring naturally in the foodstuff. These second stationary phase subcultures were used for contamination.

Physicochemical analysis of the cheese

The physicochemical determinations were conducted at 0, 1, 4, 7, 15, 30, and 40 days of ripening and after 20 and 50 days of storage time in two unit samples of each batch (CTR and CTRIZS). The pH measurements were made using a puncture electrode probe connected to a portable pH meter (Mettler Toledo Inc., Columbus, OH, USA). The salt content was determined as described by Branciari *et al*. (2014). Water activity (a_w) was measured at 25°C with the a_w recorder AquaLab, series 3, Model TE (Decagon Devices Inc., Pullman, WA, USA).

Microbiological analysis of the cheese

Enumeration of viable lactic acid bacteria in cheese

The enumeration of lactic acid bacteria (LAB) was conducted at 0, 1, 4, 7, 15, 30, and 40 days of ripening and after 20 and 50 days of storage time in all unit samples of each batch. The analyses were performed using the Tempo System (automated quality indicator solution; bioMérieux, Mercy Etoile, France) and Tempo LAB tests (automated test for the enumeration of LAB microorganisms; bioMérieux). The Tempo System calculates the number of microorganisms according to a calculation based on the most probable number method. The Tempo LAB test has been demonstrated to obtain performance levels similar to the standard NF ISO 15214, 1998 (Jaworska *et al*., 2011).

Listeria monocytogenes detection and enumeration

The detection and enumeration of *L. monocytogenes* were conducted at 0, 1, 4, 7, 15, 30, and 40 days of ripening and after 20 and 50 days of storage time in all unit samples of each batch according to Annex I of Regulation No. 2073/2005 (European Commission, 2005), using the validated method AFNOR BIO 12/11-03/04 (AFNOR BIO, 2010) for detection and EN ISO 11290-2 (ISO, 1996), the reference method for enumeration.

Results

The physicochemical and microbial characteristics of the cheese during ripening and storage are reported in Table 1. No difference in pH, a_w and NaCl were detected among batches, or between the two different sample units during the ripening and storage time. Both CTR and CTRIZS cheese samples showed a rapid decrease in pH in the early stage of ripening (first days). The decrease was about 1.2 U (from 6.40 to 5.20). During ripening pH values undergo only a slight fall reaching a final value of 5.04-5.05 for CTR and CTRIZS respectively. At the beginning of the shelf life, the average value of pH was 4.96 and 4.99 for CTR and CTRIZS respectively. At the end of shelf life the pH value slightly increased. A reduction of a_w values during cheese ripening occurred gradually and the value 0.989 and 0.988 for CTR and CTRIZS, respectively, in the curd reached the values 0.960 and 0.959, respectively, at the end of ripening. During the first stage of storage, the a_w value was 0.956 and 0.954 for CTR and CTRIZS, respectively, and reached values of 0.949 and 0.952, respectively, at the end of storage. The NaCl value, as expected, increased with ripening time and during storage.

The results of the behaviour of LAB during ripening and storage performed on CTR and CTRIZS also showed no difference among batches and between CTR and CTRIZS samples. A significant increase of LAB was observed in the first two week of cheese ripening and a small decline of LAB was observed at the end of the shelf life. The results of the behaviour of *L. monocytogenes* are reported in Figure 1.

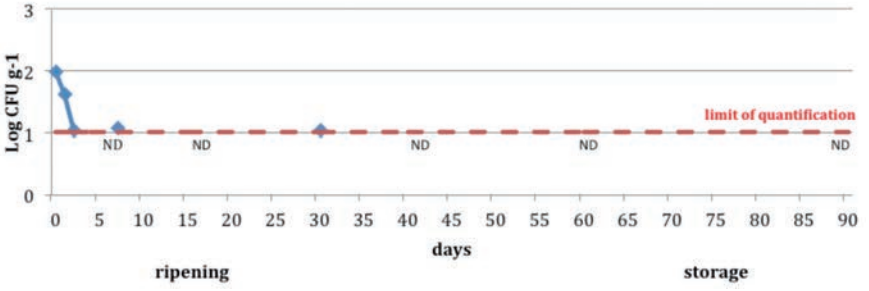

Figure 1. Behaviour of *Listeria monocytogenes* (Log $CFUg^{-1}$) during cheese ripening and storage in *Pecorino Umbro* cheese (data represent the average values±standard deviation of three replicates samples for three cheesemaking replicates).

Table 1. Physicochemical and microbial characteristics in curd and *Pecorino Umbro* cheese during cheese ripening and storage. Data represent the average values±standard deviation of three replicates samples for three cheese-making replicates.

		Curd	Cheese ripening						Storage	
		0	1	4	7	15	30	40	20	50
CTR	pH	6.40±0.10	5.20±0.02	5.19±0.01	5.24±0.17	5.19±0.08	5.18±0.09	5.04±0.13	4.96±0.16	5.05±0.13
	a_w	0.989±0.003	0.985±0.002	0.982±0.006	0.973±0.010	0.977±0.002	0.965 ±0.004	0.960 ±0.004	0.956±0.004	0.949±0.008
	NaCl %	0.163±0.004	0.328±0.134	0.543±0.130	0.780±0.051	1.371±0.133	1.753±0.339	1.994±0.314	2.318±0.212	2.508±0.281
	LAB (Log_{10} CFU g^{-1})	5.90±0.69	6.57±0.68	7.30±0.60	7.83±0.27	8.28±0.52	8.43±0.15	8.64±0.33	8.28±0.07	8.20±0.17
CTRIZS	pH	6.40±0.10	5.20±0.01	5.14±0.11	5.22±0.14	5.19±0.07	5.17±0.09	5.05±0.10	4.99±0.15	5.07±0.14
	a_w	0.988±0.003	0.988±0.001	0.986±0.001	0.979±0.009	0.976±0.002	0.959±0.003	0.959±0.006	0.954±0.007	0.952±0.003
	NaCl %	0.156±0.008	0.383±0.127	0.637±0.028	0.876±0.107	1.331±0.167	1.873±0.215	2.052±0.297	2.288±0.180	2.452±0.229
	LAB (Log_{10} CFU g^{-1})	5.90±0.69	6.72±0.62	7.19±0.68	7.86±0.32	8.34±0.52	8.53±0.24	8.38±0.25	8.27±0.14	8.02±0.09

CTR, third group produced and ripened by the manufacturer in order to compare the products submitted to challenge testing to the products that are routinely produced by the processor; CTRIZS, second group produced by the food business operator and ripened/stored at the Institute for Experimental Veterinary Medicine of Umbria and Marche; CFU, colony forming units.

A significant decrease of 1 Log unit of the viable count of *L. monocytogenes* was observed before ripening. During 30 days of ripening, the average pathogen count was around 1 Log CFU g^{-1}. After 30 days of ripening and during storage, the *L. monocytogenes* viable count was below the limit of quantification with the method EN ISO 112090-2. Using the method AFNOR BIO 12/11-03/04, it was possible to reveal the presence of *L. monocytogenes* during storage, even when it was constantly below 1 Log CFU g^{-1}. No *L. monocytogenes* was found in the control sample.

Discussion

Results from the current study demonstrate the inhibitory effect of the process for *L. monocytogenes* growth, even though the cheese showed a pH and a_w value during storage at which *L. monocytogenes* could grow (EUCRL, 2008). The presence of a certain % of NaCl was one method used to control the growth of *L. monocytogenes,* but the concentration used in the experiment is not demonstrated to limit *L. monocytogenes* growth (Faleiro *et al*., 2003). The main injury against *L. monocytogenes* suppressing its growth probably occurred due to the Lactic acid bacteria; several studies have shown that the inoculation of food with a strain of LAB can inhibit the growth of *L. monocytogenes*. Several studies show microbial antagonism and refer to the inhibition of other microorganisms by the competition for nutrients or by the production of microbial metabolites (Holzapfel, *et al*., 1995; Hugas, 1998; Hurst, 1973; Amezquita and Brashears, 2002; Leroy and Devuyst 2004; Skalina and Nikolajeva, 2010; Reis *et al*., 2012). In addition to lactic acid and other organic acids, LAB produce other metabolites with antimicrobial activity. The inhibitory activity of LAB has been displayed towards sensitive food spoilage or pathogenic bacterial strains such as *Clostridium botulinum, Staphylococcus aureus*, and *L. monocytogenes*. This inhibitory activity towards pathogens has been documented for dairy products (Benkerroum *et al*., 2002; Foulquie Moreno *et al*., 2003; Giraffa, 1995; Leroy and Devuyst 2004; Reis *et al*., 2012). The decrease in *L. monocytogenes* counts could be due to the combined inhibitory effect of pH and the activity of lactic starters. The rapid decrease in the pH values of cheeses and the high number of LAB when the level of contamination of *L. monocytogenes* is 10^2 CFU mL^{-1} create an unfavourable environmental condition for the pathogen.

Conclusions

In cheese-making, the use of raw milk permits the manufacture of high-value traditional artisan varieties, but brings about safety risks, *e.g.* the development of *L. monocytogenes*. The control strategy of using an appropriate starter culture in the manufacturing process of raw milk pecorino cheese, as demonstrated in the present study, may be considered as a significant hurdle that creates an unfavourable environment, causing *L. monocytogenes* to be reduced to a level that no longer represents a risk to the consumer.

References

AFNOR BIO, 2010. VIDAS Listeria monocytogenes II (VIDAS LMO2). Ref. 30704 Enrichment Stage at 37°C, 12/11-03/04. Available from: http://nf-validation.afnor.org/en/wp-content/uploads/sites/2/2014/03/Synt-BIO-12-09-07-02_en.pdf

Amezquita A, Brashears MM, 2002. Competitive inhibition of Listeria monocytogenes in ready-to-eat meat products by lactic acid bacteria. J Food Protect 65:316-25.

Benkerroum N, Ghouati Y, Ghalfi H, Elmejdoub T, Roblain D, Jacques P, Thonart P, 2002. Biocontrol of Listeria monocytogenes in a model cultured milk (lben) by in situ bacteriocin production from Lactococcus lactis ssp. Lactis. Int J Dairy Technol 55:145-51.

Branciari R, Mughetti L, Ranucci D, Miraglia D, Valiani A, Acuti G, Trabalza-Marinucci M, 2014. Influence of manufacturing procedure on the compositional and sensory properties of n-3 fatty acid-enriched pecorino cheese. J Dairy Res 81:455-61.

Dalzini E, Cosciani-Cunico E, Pavoni E, Bertasi B, Daminelli P, Finazzi G, 2014. Study of growth potential of Listeria monocytogenes in low fat Salami: an innovative Italian meat product. Ital J Food Safety 3:40-43.

EUCRL, 2008. Technical guidance document on shelf-life studies for Listeria monocytogenes in ready-to-eat foods. European Union Community Reference Laboratory for Listeria monocytogenes, French Food Safety Agency ed., Maisons-Alfort, France.

European Commission, 2005. Commission Regulation of 15 November 2005 on microbiological criteria for foodstuffs, 2073/2005/EC. In: Official Journal, L 338, 22.12.2005.

Foulquie Moreno MR, Rea MC, Cogan TM, De Vuyst L, 2003. Applicability of a bacteriocin-producing Enterococcus faecium as a co-culture in Cheddar cheese manufacture. Int J Food Microbiol 81:73-84.

Gambarin P, Magnabosco C, Losio MN, Pavoni E, Gattuso A, Arcangeli G, Favretti M, 2012. Listeria monocytogenes in ready-to-eat seafood and potential hazards for the consumers. Int J Microbiol 2012:497635.

Giraffa G, 1995. Enterococcal bacteriocins: their potential as anti-Listeria factors in dairy technology. Food Microbiol 12:291-9.

Holzapfel WH, Geisen R, Schillinger U, 1995. Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. Int J Food Microbiol 24:343-62

Hugas M, 1998. Bacteriocinogenic lactic acid bacteria for the biopreservation of meat and meat products. Meat Sci 49:139-50.

Hurst A, 1973. Microbial antagonism in foods. Can Inst Food Sci Technol J 6:80-90.

ISO, 1996. Microbiology of food and animal feeding stuffs: horizontal method for the detection and enumeration of Listeria monocytogenes. Part 1: detection method. ISO Norm 11290-1:1996. International Organization for Standardization, Geneva, Switzerland.

Jaworska D, Neffe K, Kolozyn-Krajewska D, Dolatowski Z, 2011. Survival during storage and sensory effect of potential probiotic lactic acid bacteria Lactobacillus acidophilus Bauer and Lactobacillus casei Bif3/IV in dry fermented pork loins. Int J Food Sci Technol 46:2491-7.

Kells J, Gilmour A, 2004. Incidence of Listeria monocytogenes in two milk processing environments, and assessment of Listeria monocytogenes blood agar for isolation. Int J Food Microbiol 91:167-74.

Kongo M, Malcata FX, Ho AJ, Wiedmann M, 2006. Detection and characterization of Listeria monocytogenes in São Jorge (Portugal) cheese production. J Dairy Sci 89:4456-61.

Leroy F, De Vuyst L, 2004. Lactic acid bacteria as functional starter cultures for the food fermentation Industry. Trends Food Sci Technol 15:67-78.

Lunden J, Tolvanen R, Korkeala H, 2004. Human listeriosis outbreaks linked to dairy products in Europe. J Dairy Sci 87:6-11.

Reis JA, Paula AT, Casarotti SN, Penna ALB, 2012. Lactic acid bacteria antimicrobial compounds: characteristics and applications. Food Engineering Rev 4:124-40.

Skalina L, Nikolajeva V, 2010. Growth potential of Listeria monocytogenes strains in mixed ready-to-eat salads. Int J Food Microbiol 144:317-21.

Spanu C, Scarano C, Ibba M, Pala C, Spanu V, De Santis EPL, 2014. Microbiological challenge testing for Listeria monocytogenes in ready-to-eat food: a practical approach. Ital J Food Safety 3:231-7.