

***Listeria monocytogenes* contamination in dairy plants: evaluation of *Listeria monocytogenes* environmental contamination in two cheese-making plants using sheeps milk**

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Abstract

Listeria monocytogenes harbouring niches established in the processing plant support post-process contamination of dairy products made from pasteurised or thermised milk. The present study investigated *L. monocytogenes* environmental contamination in two sheep's milk cheese-making plants. Persistence of contamination in the area at higher risk was also investigated. During a one-year survey 7 samplings were carried out in each dairy plant, along the production lines of *Pecorino Romano* and *ricotta salata* cheese. A total of 613 environmental samples collected from food contact and non-food contact surfaces were analysed according to ISO 11290-1:2005 standard method. Identification of the isolated strains was carried out by polymerase chain reaction. *L. monocytogenes* prevalence was 23.2% in dairy A and 13.1% in dairy B, respectively. The higher prevalence rate was found in the following areas: salting, products washing, packaging, *ricotta salata* storage and *Pecorino Romano* ripening rooms. *L. monocytogenes* was never found in the cheese-making area. The probability of observing samples positive for the presence of *L. monocytogenes* was associated with dairy plant, sampling area and the period of cheese-making ($P < 0.001$). The greater persistence of contamination over time was observed in the washing, salting, and *Pecorino Romano* ripening areas. The control of persistent environmental contamination relies on the identification of *L. monocytogenes* niches within the processing environment and the prevention of harborage sites formation. The importance of strict cleaning and sanitising procedure in controlling *L. monocytogenes* environmental contamination is confirmed by the lower level of contamination observed after these procedures were correctly implemented.

Introduction

Raw milk contaminated with *Listeria monocytogenes* could represent a potential source for the introduction of the pathogen in dairy products processing plant environment. The prevalence of positive raw milk samples ranges between 3.0 and 6.5% (Vitas *et al.*, 2004; Al-Tahiri *et al.*, 2008), with estimated most probable concentration of 0.1 colony forming units/mL (Meyer-Broseta *et al.*, 2003). The application of thermisation and pasteurisation processes in sheep milk cheese-making plants results in a 3 to 6 log reduction of the number of viable *L. monocytogenes* cells present in raw milk (ICMSF, 1996; Pearce *et al.*, 2012). Therefore, the presence of *L. monocytogenes* in dairy products is mainly ascribed to a post-process contamination originating from the processing environment (Tompkin, 2002; Unnerstad *et al.*, 1996). Contamination is generally associated with the presence of resident strains that colonise specific niches in processing plant environments (Blackman and Frank, 1996). Niches are represented by areas with favorable conditions for *L. monocytogenes* growth and persistence, such as moisture and condenses, low temperatures and high NaCl concentrations (Tompkin, 2002). Moreover, the ability of *L. monocytogenes* to adhere on surfaces and form biofilms allows the microorganism to adapt and resist against detergents and sanitisers (Chavant *et al.*, 2004; Pan *et al.*, 2006). The evaluation of *L. monocytogenes* environmental contamination is essential, especially in cheese-making plants producing foods able to support *L. monocytogenes* growth or that are exposed to the environment after lethality treatments are applied. (European Commission, 2005; USDA/FSIS, 2006; Health Canada, 2011). In Sardinia milk production and cheese-making are seasonal and gathered during the winter-spring period, which is referred to as cheese-making season. The aim of the present research was to evaluate the prevalence of *L. monocytogenes* environmental contamination over the cheese-making season in two Sardinian sheep's milk cheese-making plants. The relationship between *L. monocytogenes* persistence niches and dairy products contamination during the dairy season was also assessed.

Materials and Methods

Environmental samples were collected from processing areas and equipment in two sheep milk cheese-making plants located in Sardinia. In order to take into account seasonal variations each dairy plant was visited 7 times during the cheese-making season. Three visits

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were conducted during the peak of production (January-June), 2 visits during the break in cheese-making production (September-October) and the other 2 at the beginning of cheese-making season (November-December). Overall 613 environmental samples were collected. During each visit environmental samples were collected along production lines of *ricotta* cheese, *Pecorino Romano* and in environments shared by these products, such as areas of coagulation, *ricotta* production, molding, salting, washing, ripening and packaging. Environmental sampling included food contact surfaces (tables, carts, shelves, molds, machinery) and non-food contact surfaces (outer surfaces of equipment, walls, floors and floor drains). Sampling was conducted during processing operation using sterile sponges pre-moistened with neutralising buffer (3M, St. Paul, MN, USA). In order to increase the likelihood of detecting *L. monocytogenes* no delimitation of sampling area was defined. After sampling each sponge was placed into a sterile bag and kept refrigerated until analysis. The detection of *L. monocytogenes* was performed according to standard method ISO 11290-1/A1 (ISO, 2005). In the present study a persistent contamination site was defined as the recovery of *L. monocytogenes* from a defined site, in two or more sampling time during the observation period. All the isolates were identified as *L. monocytogenes* by PCR and major serotype were also detected using primers *lmo0737*, *lmo1118*, *ORF2819* and *ORF2110* (Doumith *et al.*, 2004). Differences in *L. monocytogenes* prevalence between the two cheese-making plants were compared using chi-square test. The probability of recovering *L. monocytogenes* was investigated using logistic regression (Statgraphics

Plus software ver. 16.0.09). The probability of observing samples positive for *L. monocytogenes* was the binary response variable (presence, absence). The effect of the dairy (A and B), production phase (peak, break and resume of production), production line (*Pecorino Romano*, *ricotta*, common line), areas (cheese curdling, whey floating, salting, seasoning *Pecorino Romano*, *ricotta* storage, cheese washing, packaging, other), sampling site (shelves, carts, tables, cheeses washing machines, floors, drains, molds, other), type of surface (food contact and non-food contact) were included in the model.

Results

L. monocytogenes was detected in 109 (17.8%) out of the 613 collected samples. Overall the prevalence was 23.2% for dairy A and 13.1% for dairy B. In dairy A *L. monocytogenes* was detected in 29 (26.4%) out of 110 food contact surfaces and in 37 (21.1%) out of 175 non-food contact surfaces. In dairy B *L. monocytogenes* was detected in 15 (10.9%) out of 138 food contact surfaces and in 28 (14.7%) out of 190 non-food contact surfaces.

L. monocytogenes prevalence reported for the two cheese-making plants in the three main production phases are showed in Table 1. In Table 2 is reported the prevalence of *L.*

monocytogenes along the production lines of *Pecorino Romano*, *ricotta salata* and in the common production areas. The production period influenced *L. monocytogenes* prevalence only for *Pecorino Romano*'s ripening rooms. The probability (odds ratio) to detect *L. monocytogenes* in *Pecorino Romano*'s ripening rooms was ca. 6 times lower in the resume production period as compared to the peak of production and break periods (Table 3). Comparison between the cheese-making plants showed that the probability of contamination was 2 times greater in dairy A than in dairy B ($P < 0.01$). The highest rate of contamination was observed for cheese washing rooms (44.5%), packaging area (36.8%), salting room (33.3%), *ricotta* storage room (25.6%) and *Pecorino Romano* ripening area (13.9%). *L. monocytogenes* was detected in 3.3% of the samples collected from whey floating area, while, in curdling area the microorganism was never detected. In the areas common to the two main production lines, *L. monocytogenes* prevalence was 5.9%. Logistic regression analysis showed that the probability of detecting *L. monocytogenes* in samples was significantly affected by the following factors: cheese making plant, production phases and production areas ($P < 0.001$). No significant effect of production line, site and surface type was observed. *L. monocytogenes* persistent contamination was detected in cheese washing rooms of both cheese-making plants, in the following

sites: washing machine, floors and drains. *Pecorino Romano* production line was characterised by persistent contamination in the ripening rooms (shelves, drains and floors) and salting room (drains). *Ricotta salata* storage rooms showed persistent contamination of drains and floors. Polymerase chain reaction results on serotyping showed that all the 43 strains isolated from dairy B were serotype 1/2a. On the other hand, dairy A showed a wide strains variability with 17 isolates (25.8%) belonging to serotype 1/2a, 27 (40.9%) and 22 (33.0%) to serotypes 1/2b and 4b, respectively.

Discussion and Conclusions

The present study was aimed to investigate the prevalence and the persistence of *L. monocytogenes* environmental contamination in two sheep milk cheese-making plants. The probability of detecting *L. monocytogenes* positive samples was significantly affected by the plants, processing areas and production phases during the cheese-making season ($P < 0.001$). *L. monocytogenes* environmental contamination was twice as much in dairy A as compared to dairy B. However, great part of the difference in *L. monocytogenes* prevalence was accountable to contamination observed along the *Pecorino Romano* production line. Little differences between cheese-

Table 1. *Listeria monocytogenes* prevalence on surfaces in relation to the period of dairies activity (A, B).

Dairy	Cheese-making period								
	n	Peak* Lm+	%	n	Break° Lm+	%	n	Resuming# Lm+	%
A	79	29	36.7	76	21	27.6	83	16	19.3
B	94	30	31.9	94	8	8.5	111	5	4.5
Total	173	38	34.3	170	29	17.1	194	21	10.8

Lm, *Listeria monocytogenes*. *Peak of production in January, March, and June; °break of production in September and October; #resuming of production in November and December.

Table 2. *Listeria monocytogenes* prevalence in different areas of the two dairies.

Line/room	n	Dairy						P value	Odds ratio
		A			B				
	n	Lm+	%	n	Lm+	%			
<i>Pecorino Romano</i>									
Cheese-making	28	0	0.0	33	0	0.0	-	-	
Ripening	102	20	19.6	99	8	8.1	<0.05	2.8	
<i>Ricotta salata</i>									
<i>Ricotta</i> processing	42	1	2.4	48	2	4.2	NS	0.6	
<i>Ricotta</i> storage	17	8	47.1	26	3	11.5	<0.05	6.8	
Common areas									
Product washing	68	30	44.1	42	19	45.2	NS	0.9	
Packaging	7	4	57.1	12	3	25.0	NS	4.0	
Salting	-	-	-	21	7	33.3	-	-	
Other	21	3	14.3	47	1	2.1	<0.05	7.7	
Total	285	66	23.2	328	43	13.1	<0.01	2.0	

Lm, *Listeria monocytogenes*; NS, not significant.

making plants were observed for *ricotta salata* and common production areas. Environmental conditions, pre-operational sanitation and the adoption of good manufacturing practice reduce the risk of colonization in cheese curdling and whey floating areas (Pilo *et al.*, 2007). Therefore, contamination of the products in these areas should be considered as a sporadic event. Instead, *L. monocytogenes* prevalence observed in washing, drying and packaging rooms was higher, resulting in an increased risk for products contamination (FSIS, 2010). The higher prevalence could be explained by specific condition observed in these areas, such as low temperatures, high salt concentration, presence of environmental surfaces difficult to clean and sanitise (*i.e.* wood). Cheese cleaning and ripening rooms are areas shared between *Pecorino Romano* and *ricotta salata*, therefore they may represent a potential source of cross-contamination between the two production lines. With regard to the

production phases the probability of contamination in ripening rooms was about 6 times lower during the resuming of production as compared to other phases. This could be explained by the effectiveness of cleaning and sanitizing procedures conducted during the break production period. Differences in *L. monocytogenes* prevalence were observed between the two cheese-making plants based on the sampling period. In dairy B a significant decrease in the contamination prevalence was observed in the break and resuming production phases, while in dairy A the reduction was limited. Moreover, in dairy A the contamination of food contact surfaces was higher than in dairy B. Dairy A was also characterised by a greater strains variability, with isolates belonging to *L. monocytogenes* lineage I (serotypes 1/2b and 4b) and II (serotype 1/2a), while in dairy B all strains belonged to lineage II. Persistent contamination was observed in sites with favorable conditions for microorganism survival. In cheese

washing machine the increased risk was represented by the presence of organic residues and the poor hygienic design of the equipment which limit the cleaning and sanitization effectiveness. Other sites, such as floors, drains, mainly in salting ripening and storage area, served as niches for *L. monocytogenes*. Persistent contamination seem to be related to the inability to remove and inactivate strains from niche sites rather than specific strain properties (Carpentier and Cerf, 2011). The regular implementation of environmental sampling programs is strongly recommended in order to monitor the level of *L. monocytogenes* contamination and the presence of persistent harborage sites (trend analysis). The application of strict cleaning and sanitizing procedures is essential in order to prevent on one hand the re-introduction of *L. monocytogenes* into the plant environment and on the other hand the establishment of niches which serve as a potential source of product contamination.

Table 3. Comparison of *Listeria monocytogenes* prevalence in different areas in the three production periods.

Areas*	n	Lm+	%	P value	Odds ratio
Cheese-making					
Peak	25	0	0.0	-	-
Break	18	0	0.0	-	-
Resuming	18	0	0.0	-	-
Packaging					
Peak	7	3	42.9	NS	1.5
Break	6	2	33.3	-	1
Resuming	6	2	33.3	-	1
Washing products					
Peak	46	25	54.4	NS	2.2
Break	30	12	40.0	NS	1.2
Resuming	34	12	35.3	-	1
<i>Ricotta</i> processing					
Peak	37	2	5.4	NS	1.5
Break	26	0	0.0	-	-
Resuming	27	1	3.7	-	1
Salting					
Peak	9	4	44.4	-	1
Break	6	3	50.0	NS	1.2
Resuming	6	0	0.0	-	-
Ripening					
Peak	90	16	17.8	0.01	6.1
Break	53	10	18.9	0.01	6.5
Resuming	58	2	3.5	-	1
<i>Ricotta salata</i> storage					
Peak	20	7	35.0	NS	1.1
Break	11	0	0.0	-	-
Resuming	12	4	33.3	-	1
Other					
Peak	25	2	8.0	-	1
Break	20	2	10.0	NS	1.3
Resuming	23	0	0.0	-	-

Lm, *Listeria monocytogenes*; NS, not significant. *Note that peak of production occurred in January, March, and June, break of production in September and October, and resuming of production in November and December.

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