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Molecular methods to assess *Listeria monocytogenes* route of contamination in a dairy processing plant

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Short title: *Listeria monocytogenes* in a dairy processing plant

Abstract

In this study we investigated the occurrence of *Listeria monocytogenes* in a dairy processing plant during two sampling campaigns in 2007 and 2008. Samples represented by semi-finished and finished cheeses, swabs from the equipment and brines from the salting step, were subjected to analysis by using traditional and molecular methods, represented mainly by quantitative PCR. Comparing the results obtained by the application of the two approaches used, it became evident how traditional microbiological analysis underestimated the presence of *L. monocytogenes* in the dairy plant. Especially samples of the brines and the equipment swabs were positive only with qPCR. For some equipment swabs it was possible to detect a load of 10^4-10^5 cfu/cm^2, while the modified ISO method employed gave negative results both before and after the enrichment step. The evidences collected during the first sampling year, highlighting a heavy contamination of the brines and of the equipment, lead to the implementation of specific actions that decreased the contamination in these samples during the 2008 campaign. However, no reduction in the number of *L. monocytogenes* positive final products was observed, suggesting that a more strict control is necessary to avoid the presence of the pathogen. All the isolates of *L. monocytogenes* were able to attach to abiotic surfaces, and, interestingly, considering the results obtained from their molecular characterization it became evident how strains present in the brines, were genetically connected with isolates from the equipment and from the final product, suggesting a clear route of contamination of the pathogen in the dairy plant. This study underlines the necessity to use appropriate analytical tools, such as molecular methods, to fully understand the spread and persistence of *L. monocytogenes* in food producing companies.

Keywords: *Listeria monocytogenes*; quantitative PCR; dairy processing plant; molecular characterization; biofilm.

1. Introduction

Listeria monocytogenes is a foodborne pathogen of great concern for the food producing companies. Due to its physiological characteristics, such as resistance to acidic and sodium chloride stress, ability to grow at low temperature and possibility to form biofims, it can persist and/or re-contaminate food products, thereby representing an important risk for the safety of the consumers (Olesen et al., 2009; Phan-Thanh et al., 2000; Gardan et al. 2003; Liu et al. 2003; Pan et al., 2006). The term "*Listeria* hysteria" was coined towards the end of 1980s following a series of listeriosis outbreaks due to the consumption of soft-cheese and ready-to-eat (RTE) meats in the UK. Recently, this emerged again in the large outbreaks in Canada caused by deli meats (Warriner and Namvar, 2009). The lack of decrease in the occurrence of listeriosis, reported in the community summary report on foodborne outbreaks in the European Union in 2007 (Anonymous, 2009), warns for the need of special attention to this foodborne pathogen in order to combat its presence in foodstuffs.

Several reviews have been published focusing on: the epidemiology and pathogenesis (Ramaswamy et al., 2007), the survival in adverse conditions and the adaptation mechanisms (Gandhi and Chikindas, 2007) and the methods for isolation and identification (Gasanov et al., 2005). Moreover, the data reported in a large number of research studies in the recent years, suggests that *L. monocytogenes* is not an "emerging" pathogen anymore.

L. monocytogenes, although less frequent in humans compared to campylobacters and salmonellas, shows a high mortality rate of 20 per cent, particularly amongst vulnerable groups such as the elderly. Listeriosis is also very dangerous to pregnant women as it can cause fetal infections, miscarriages and stillbirths (Rocourt and Cossart, 1997). The foods most frequently associated with the outbreaks have been identified as RTE foods, smoked fish and other fishery products, followed by meat products and cheese (Lianou and Sofos, 2007).

In the effort of combating *L. monocytogenes* in foodstuffs, several researchers focused on the development of new methods, based on molecular biology, which could detect this pathogen more rapidly and reliably with respect to traditional microbiological methods. In this context, a new group of methods, based on the polymerase chain reaction (PCR) could detect target pathogens without the need of their cultivation. Nowadays, with the second generation of PCR methods, in which a quantification of the target microorganisms is also possible, new applications become available. Several quantitative PCR (qPCR) protocols have been recently published, highlighting that this method can be advantageously used to detect and quantify *L. monocytogenes* in food (O'Grady et al., 2009; Rantsiou et al., 2008; Long et al., 2008; Pan and Breidt, 2007; Berrada et al., 2006; Rudi et al., 2005, Rodriguez-Lazaro et al., 2004).

In this study, in the frame of the 6th EU Framework program, the presence of *L. monocytogenes* was monitored in a dairy company by using traditional and molecular methods. Cheese samples, as well as equipment swabs and brines used in the salting process, were collected in two sampling campaigns in 2007 and 2008, respectively, and they were analyzed with both approaches. Lastly, isolated strains were molecularly identified and characterized and their capability to attach to abiotic surfaces was investigated.

2. Materials and Methods

2.1 Samples

The dairy company considered in this study was sampled for the presence of *L. monocytogenes* for two consecutive years (2007 and 2008) for a period of three weeks each year. More specifically, samples were collected during weeks 48, 49 and 50 in 2007, and weeks 47, 48 and 49 in 2008, spanning the last two weeks of November and the first week of December. The samples analyzed in this study were represented by semi-finished and finished fresh cheeses, brines used in the salting process and swabs from machinery and equipment in

contact with the cheeses during processing and packaging. A total of 151 and 50 samples were processed in 2007 and 2008, respectively (Tables 1 and 2). The cheese produced by the dairy company considered in this study was a soft cheese produced by pasteurized milk, added of a starter cultures (*Streptococcus thermophilus*) and salted in brine containing 19% NaCl (w/vol). Maturation is carried out for 3 to 5 days in controlled chambers with a temperature of 5 to 10°C and a relative humidity of 90%. The cheese is characterized by a final pH ranging from 5.2 to 5.6 and a salt content of about 1% (w/w).

Samples were collected using sterile gloves, knifes and pipettes, and transferred in sterile plastic bags and tubes. For the swabs, 5 ml Ringer solution (Oxoid, Milan, Italy) were added in the tube containing the cotton flock at least 24 hours prior the sampling, and a surface of 10 cm^2 was wiped off. All the samples were maintained at 4 °C and transported within 12 hours in the laboratory for analyses.

2.2 Microbiological examinations

Both traditional and molecular based methods were used in this study in order to assess the presence of *L. monocytogenes* and to quantify it in the dairy plant investigated.

The ISO method 11290 (Anonymous, 1997) was followed to perform traditional microbiological examination, with the following modifications: only the first enrichment in Half Fraser was carried out and only the Oxford agar was used as isolation solid medium. Samples were examined without enrichment (T0) and after an 18 hours overnight period (T24). Solid samples (25 g) were homogenized with 225 ml of Half Fraser broth (Oxoid) and serially diluted in Ringer solution. Serial dilutions in Ringer were also prepared directly from brines and swabs. At T0, *L. monocytogenes* was counted on Oxford agar (Oxoid), by spreading the appropriate dilutions and incubating the plates at 37°C for 24-48 h. Suspected colonies of *L. monocytogenes* were first counted and then up to five colonies were streaked for isolation on Brain Heart Infusion (BHI) agar (Oxoid). Pure cultures were stored at -80°C

with 30% glycerol (vol/vol) prior to DNA extraction. For the enrichment step, one ml of the brines, as well as 1 ml of the swab solutions, was mixed with 9 ml of Half Fraser, and incubated, together with the previously prepared solid samples, at 37°C for about 18 hours. After that period, one full loop of the enriched broth was streaked on Oxford agar and incubated at 37°C for 24-48 h. If suspected colonies were present, isolation was carried out as described above. For all the samples, both at T0 and T24, 1 ml of the 1:10 dilution was transferred in a 1.5 ml eppendorf tube, and after a centrifugation at 14,000*xg* for 10 min at 4°C, the pellet was saved at -80°C for further DNA extraction.

2.3 DNA extraction from pure cultures

One ml of an 18 hours overnight culture of the isolates in BHI broth was centrifuged at max speed for 10 min, and the pellet was treated as described previously (Cocolin et al., 2002). DNA was quantified by using the Nanodrop Instrument (Celbio, Milan, Italy) and diluted to a concentration of 100 ng/ml.

2.4 DNA extraction from samples collected from the dairy company

The procedure described by Rantsiou et al. (2008) was employed in this study. More specifically, the pellets originating from the 1 ml of the ten times diluted samples, prior and after enrichment, were extracted by using the Master-Pure™ Complete DNA and RNA Purification Kit (Epicentre, Madison, WI, USA) following the manufacturer's instructions. Only samples positive at T24, were also extracted at T0 for quantification.

2.5 Quantitative PCR

A couple of primers and a Taqman probe, specific for *L. monocytogenes*, were used in this study as previously described (Rantsiou et al., 2008). Primers IGS1 (5'-GGCCTATAGCTCAGCTGGTTAGAG-3') and IGS2 (5'-GCTGAGCTAAGGCCCCGTAAAAGGG-3') were used in combination with the probe IGS (5'-FAM-ATAAGAAATACAAATAATCATACCCTTTTAC-TAMRA-3'). Amplifications

were performed in a final volume of 25 µl in the Chromo4 Real-Time PCR Detection System (Biorad, Milan, Italy). One hundred ng of DNA extracted from pure cultures, or 1 µl of DNA solution obtained from the samples, was added to a mix constituted of the Fluomix for probe kit of Euroclone (Celbio, Milan, Italy), containing 8 mM $MgCl_2$, and of the primers at final concentration of 400 nM and the probe at 250 nM. The amplification cycle was as follows: initial denaturation at 95 °C for 10 min, 95 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s. For quantification purposes, standard curves were created by amplifying DNA extracted from serially diluted cells of *L. monocytogenes* EGDe inoculated in fresh cheese or Ringer solution. More specifically two standard curves were constructed: one in fresh cheese for the quantification of *L. monocytogenes* in semifinished and finished products, a second in Ringer solution for the quantification of the pathogen in brines and swabs. The procedures followed were as described by Rantsiou et al. (2008).

2.6 Molecular identification and characterization of L. monocytogenes isolates.

Suspected colonies on the Oxford plates were subjected to qPCR as described above for identification purposes. Subsequently isolates identified as *L. monocytogenes* were subjected to molecular characterization by the means of randomly amplified polymorphic DNA (RAPD), PCR amplification of repetitive bacterial DNA elements (Rep-PCR) and Sau-PCR. Primers M13 (5'-GAGGGTGGCGGTTCT-3') and SAG1 (5'-CCGCCGCGATCAG-3') were used for RAPD and Sau-PCR analysis as previously described by Cocolin et al. (2005), while Rep-PCR was carried out with primer $(GTG)_5$ (5'-GTGGTGGTGGTGGTG-3') as reported by Gevers et al. (2001). The amplification products were analyzed by agarose gel electrophoresis (1.5% w/vol) in TAE 1X for 4 h at 120 V. After the run, gels were stained in TAE 1X containing 0.5 µg/ml for 30 min and then observed under UV illumination by using the UVI pro Platinum 1.1 Gel Software (Eppendorg, Hamburg, Germany) for the recognition of the bands present. Comparisons of the fingerprints obtained for the *L. monocytogenes*

isolates with the different techniques were performed using the pattern analysis software package, Bionumerics, Version 4.6 (Applied Maths, Kortrijk, Belgium). Calculation of similarity in the profiles of bands was based on Pearson product– moment correlation coefficient. Dendrograms were obtained by means of the Unweighted Pair Group Method using Arithmetic Average (UPGMA) clustering algorithm (Vauterin and Vauterin, 1992).

2.7 Attachment to abiotic surfaces

The strains isolated from the samples analyzed in this study were investigated for their capabilities to attach to abiotic surfaces. The method described by Djordjevic et al. (2002), was used with some modifications. *L. monocytogenes* were grown for 18-24 hours in 10 ml of BHI broth at 37°C. Then, 0.1 ml of the cultures were transferred in 10 ml of fresh BHI broth and transferred into polyvinyl chloride (PVC) microtiter plates (Biolog, Hayward, CA, USA). Eight wells per strain were filled up with 100 µL of culture. Plates were previously rinsed with 70 % ethanol and air dried. Plates were incubated at 37°C for 24 h. Each plate included eight wells of BHI broth without *L. monocytogenes*, as negative control. *L. monocytogenes* EGDe and ScottA were used as reference strains, able to produce biofilms (Chavant et al., 2007; Moltz and Martin, 2005). After the incubation period, spent BHI broth was removed from wells and the microtiter plates were washed five times with sterile distilled water, in order to remove loosely associated bacteria. Plates were air dried for 45 min and each well was stained with 150 µL of 1% crystal violet solution in water for 45 min. After staining, plates were washed five times with sterile distilled water. The attachment was determined by adding 200 µL of 95% ethanol to de-stain the wells. One hundred microliters from each well were transferred to a new microtiter plate and the optical density at 590 nm was measured by using a Biolog MicroStation (Biolog) microtiter plate reader. Three independent measurements were made for each strain.

3. Results

3.1 2007 sampling

The results obtained from the 2007 campaign are shown in Table 1. A total of 54, 74 and 23 samples were collected in weeks 48, 49 and 50, respectively. In week 48, by traditional microbiological analysis using the modified ISO method, none of the samples analyzed was positive for the presence of *L. monocytogenes*, while applying qPCR, 2 semifinished products, 1 brine and 5 swabs from equipment resulted to be contaminated. After quantification, the counts spanned from 10^3 to 10^4 cfu/ml or cm^2 in brines and swabs, respectively, and reached 10^5 cfu/g in the semi-finished products (Table 3). No final products contained *L. monocytogenes*. Considering the second week of sampling, an increase in the positives was observed. At T24, four semifinished products, as well as 1 swab, showed *Listeria* colonies on the Oxford agar, which were subsequently identified as *L. monocytogenes*. At T0, the same swab showed high counts of *Listeria* spp. ($3x10^5$ cfu/cm^2) and also a sample of brine gave a count of 10 cfu/ml, while for the semifinished products, no colonies developed on Oxford (data not shown). Also in these two cases of the swab and the brine at T0, isolates were confirmed to belong to *L. monocytogenes* species. A different picture was obtained by applying qPCR. All brines and 10 of the 18 swabs collected were positive after enrichment. The loads of *L. monocytogenes* in brines were in the order of 10^3-10^4 cfu/ml, while the equipment resulted to be contaminated with 10^3-10^4 cfu/cm^2 (Table 3). Lastly, in week 50, *L. monocytogenes* was detected in 2 final products and 3 equipment swabs after enrichment. At T0, only the swabs presented suspected colonies of *L. monocytogenes*, subsequently confirmed to belong to the species, with counts of 10^2-10^3 cfu/cm^2 (data not shown). Also in this case a significant difference was found applying molecular methods. Almost all the brines (5 of 6) and the swabs (4 of 5) gave visible qPCR signals after enrichment and *L. monocytogenes* at T0 were determined to be 10^3-10^4 cfu/ml or cm^2 (Table 3).

3.2 2008 sampling

The results of the 2008 campaign are reported in Table 2. Also in this case three weeks were considered including 14, 24 and 12 samples, respectively. As shown in Table 2, for all of them, no suspected colonies of *L. monocytogenes* were observed on the Oxford agar, either before or after the enrichment step. However, several samples resulted to contain *L. monocytogenes* as determined by qPCR. In week 47, 4 of 9 final products and 4 of 5 equipment swabs were positive at T24. *L. monocytogenes* could be quantified in one final product and resulted to be $1.88x10^4$ cfu/g (Table 3). The same trend was observed also in week 48, when the majority of the final products (3 of 5) showed presence of *L. monocytogenes* after enrichment. By quantification at T0, it was determined that samples contained from $1.01x10^3$ cfu/g to $2.35x10^5$ cfu/g (Table 3). Considering the equipment swabs and the brines, *L. monocytogenes* was detected in 1 of 7 and 3 of 12, respectively, after enrichment. None of these samples could be quantified by qPCR. Finally, in the last week of sampling, only two samples (one final product and one swab) gave positive signals in qPCR after enrichment, but the load of *L. monocytogenes* at T0 was below the quantification limit.

3.3 Molecular identification and characterization of the L. monocytogenes isolates

From each Oxford plate presenting suspected colonies of *L. monocytogenes*, if possible, 5 colonies were isolated. A total of 87 isolates, all coming from samples collected during the 2007 campaign, were gathered and subjected to molecular identification. Twenty five isolates were confirmed to belong to *L. monocytogenes*. These *L. monocytogenes* strains were subsequently characterized by using three fingerprinting techniques, namely RAPD, Rep-PCR and Sau-PCR. The results of the molecular characterization are shown in Figure 1A. Combining the results of the three methods, a dendrogram comprised of three clusters could be obtained. In cluster 1, 12 isolates were included. All of them came from the packaging room during samplings in weeks 49 and 50. More specifically, 8 were isolated from the

conveyor belt, 3 from the portioning knife and 1 from the surface of the packaging machine. Cluster 2 contained only 3 isolates all from semifinished products during week 49. Lastly, cluster 3 grouped 10 isolates from samples collected in week 50, apart from one coming from a sample of brine from week 49. Interestingly, the cluster was composed by the following isolates: 3 from final products, 1 from semifinished products, 2 from the portioning knife in the packaging room, 3 from the surface of the packaging machine and 1 from brines.

3.4 Ability to attach to PVC microplates of the L. monocytogenes isolates

The strains of *L. monocytogenes* previously characterized by molecular methods, were tested for their ability to attach to abiotic surfaces. For this purpose, a colorimetric method in PVC microtiter plates was exploited. The results obtained are presented in Figure 1B. Results reported are the means and standard deviations of three independent experiments in which 8 wells were inoculated with the tested strain. Also two strains from international collections (EGDe and ScottA) were included in the analysis. As shown, all isolates investigated were able to attach and 11of them showed rather high capability to do so (O.D.> 0.2).

4. Discussion

L. monocytogenes is a foodborne pathogen representing an important concern for food producing companies. Based on current EU legislation, food business operators have to guarantee the absence of this pathogen in the case of RTE products if they are destined for risk categories, or they have to respect the limit of 100 cfu/g or ml at the end of the shelf life, when they support its growth, or at the moment they leave the control of the producing FBO, if they do not support the growth of *L. monocytogenes* (EU Reg. 2073/2005) (Anonymous, 2005). Based on this regulation, there must be a strict control of possible *L. monocytogenes* contamination in order to comply with the law. Especially in food producing plants where raw materials are processed, thereby eliminating the risk associated with the presence of *L.*

monocytogenes, recontamination routes are the main cause of concern. For this reason, FBOs have to implement severe control strategies to assess its spread in the producing environment, including equipment, ingredients and final products.

One of the features that *L. monocytogenes* possess, which is of particular interest in this context, is its capability to form biofilms. These organized three-dimensional structures are a continuous source of contamination for food products and it has been repeatedly demonstrated that in this form *L. monocytogenes* is more resistant to stress and sanitizing agents than the planktonic cells (Pan et al., 2006).

In the last decade, traditional microbiological methods have been supported by modern techniques, based on molecular biology, such as conventional and quantitative PCR (qPCR), able to complement the results obtained by plate counting and isolation, thereby allowing a better understanding of the presence and spread of pathogens in food. Especially the application of qPCR opened a new field of investigation related to the direct quantification of microorganisms in food, without a need for their cultivation, thereby avoiding the biases inherent to the use of microbiological media.

In this paper we describe the use of a molecular approach to identify and control the route of contamination of *L. monocytogenes* in a dairy company. Samples were analyzed in two consecutive years (2007 and 2008), both with traditional and molecular methods and isolated colonies of *L. monocytogenes* were subsequently molecularly characterized in order to better understand the *L. monocytogenes* intraspecies diversity in the specific dairy plant. Analyzing the results obtained in the two campaigns, in terms of positive and negative samples, it is evident that molecular methods were able to detect a higher number of samples that were contaminated with the pathogen, when compared to the modified ISO method used in this study (Fig. 2). Especially in 2008, no sample resulted to be positive with traditional methods, when molecular methods were able to detect up to 60% positives in week 47. This is an

important aspect that should be taken into consideration: if only traditional methods are used to control the presence of *L. monocytogenes* false negative results can be generated. This evidence is directly connected with the risk of allowing on the market food products that are contaminated with the pathogen. Moreover, the altered picture obtained by using only conventional methods, does not allow the FBO to really understand the level of the contamination, thereby not permitting the implementation of strategies able to eliminate the pathogen from the processing plant.

In this study, the samples that more often gave false negative results were represented by brines and equipment swabs collected in 2007. As it is presented in Table 1, a significant difference in the response was found: especially in week 49, out of 8 brines and 18 swabs, only with molecular methods it was possible to define the *L. monocytogenes* presence, that was subsequently quantified. In some cases, counts up to 10^5 cfu/cm^2 were found in swabs, underlining a heavy contamination of the plant equipment. To our knowledge, this is the first time qPCR was applied in order to quantify populations of *L. monocytogenes* on the surface of the equipment in a food producing plant. This contamination was, however, resolved, if the results of 2008, presented in Table 2, are analyzed. The majority of the swabs and of the brines gave negative results, with both methods. Only in week 47, almost all of the swabs resulted positive after enrichment, however, at T0 they could not be quantified. This highlights that the load was below the quantification limit of the method determined to be 500 cfu/cm^2 (Rantsiou et al., 2008). No persistent strains in the processing plant were isolated. These results represent an evident improvement in the plant. As a matter of fact, based on the results of the 2007 sampling, several actions were taken by the company in order to eliminate the contamination. In this respect, for example, a new brine pasteurizer was purchased because it was discovered that the old used, presented cracks in the exchange plates, allowing a recontamination of the brine already subjected to the heat treatment. The results presented in

this study are in agreement with previously published papers, where it was once more underlined, how equipment in dairy farms and processing plants represent a continuous source of *L. monocytogenes* contamination and that a regular at- and on-line hygiene monitoring is essential to guarantee low risk of contamination (Fox et al., 2009; Latorre et al., 2009; Pappelbaum et al., 2008; Ho et al., 2007; Kells and Gilmour, 2004).

Independently from the structural improvements that were carried out in the dairy plant analyzed in this study, it is important to underline that, as shown in Figure 2, no reduction of the contaminated food samples can be observed. On the contrary, and increase of the % of positive samples for *L. monocytogenes* was recorded the first week of the 2008 campaign. However, it should be pointed out that the contamination levels found in this second sampling period are undoubtedly lower than the ones determined in 2007. This evidence is supported by the impossibility to quantify samples that resulted positive after the 18 hours overnight enrichment. The majority of the T24 positive samples did not give any signals in the qPCR when analyzed at T0, thereby demonstrating the low contamination level. The only exceptions were the 3 positive final products collected at week 48 (Table 2). Therefore, major efforts have to be dedicated in order to completely solve the issue of contamination of the final products.

The molecular characterization of the isolated *L. monocytogenes* gave results that could be interpreted to comprehend the route of contamination of this pathogen in the plant. As shown in Figure 1, clusters grouped strains from different samples and from different locations indicating a certain degree of spread of a specific biotype in the dairy environment. While in cluster 1, isolates colonizing specific equipments were detected, cluster 3 presented *L. monocytogenes* that were isolated from brines, equipment and final product. It can be speculated that strains highly contaminating brine samples, could form biofilms on the equipment, which subsequently were responsible for contamination of the final product. This

hypothesis is also supported by the evidence that practically all the isolated strains, especially the ones isolated from the brines and from the equipment, had strong abilities to attach to abiotic surfaces.

In conclusion, the results of this study highlight once more how it is vital for food producing companies to have control of the contamination routes of foodborne pathogens in their plants. This can be achieved with the implementation of correct sampling schemes and adequate cleaning and disinfection procedures. In addition, the choice of the analytical procedure should also be considered as a relevant decision. As demonstrated in this study, the modified ISO method only partially can monitor the presence of *L. monocytogenes* in the processing plant. Especially when environmental conditions influence the fitness and behavior of the pathogen, such as in the brines (with 15-20% salt) or in the equipment swabs (where cells have to respond to several stresses such as disinfectants, starvation and dried conditions), molecular methods should be used to properly detect it. It has been previously proven that *L. monocytogenes* undergoing stresses enters a viable but not culturable state (VBNC) that makes impossible its recovery by culture dependent methods (Rowan, 2004). The application of molecular methods can result in a better comprehension of the spread of a specific pathogen in a processing plant, thereby allowing the implementation of corrective actions to eliminate or decrease the risk associated with its presence in the final product.

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References.

Anonymous, 1997. International Organization for Standardization. Microbiology – General guidance on methods for the detection of *Listeria monocytogenes*. Draft International Standard ISO/DIS 11290.

Anonymous, 2005. Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs.

Anonymous, 2009. The Community report on trends and sources of zoonoses and zoonotic agents on the European Union in 2007. The EFSA Journal 223.

Berrada, H., Soriano, J. M., Picó, Y., Mañes, J., 2006. Quantification of *Listeria monocytogenes* in salads by real time quantitative PCR. International Journal of Food Microbiology 107, 202-206.

Chavant, P., Gaillard-Martinie, B., Talon, R., Hébraud, M., Bernardi, T., 2007. A new device for rapid evaluation of biofilm formation potential by bacteria. Journal of Microbiological Methods 68, 605–612.

Cocolin, L., Rantsiou, K., Iacumin, L., Cantoni, C., Comi, G., 2002. Direct identification in food samples of *Listeria* spp. and *Listeria monocytogenes* by molecular methods. Applied and Environmental Microbiology 68, 6273-6282.

Cocolin, L., Stella, S., Nappi, R., Bozzetta, E., Cantoni, C., Comi, G., 2005. Analysis of PCR-based methods for characterization of *Listeria monocytogenes* strains isolated from different sources. International Journal of Food Microbiology 103, 167-178.

Djordjevic, D., Wiedmann, M., McLandsborough, L.A., 2002. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. Applied and Environmental Microbiology 68, 2950-2958.

Fox, E., O'Mahony, T., Clancy, M., Dempsey, R., O'Brien, M., Jordan, K., 2009. *Listeria monocytogenes* in the Irish dairy farm environment. Journal of Food Protection 72, 1450-1456.

Gandhi, M., Chikindas, M., L., 2007. *Listeria*: a foodborne pathogen that knows how to survive. International Journal of Food Microbiology 113, 1-15.

Gardan, R., Cossart, P., The European *Listeria* Genome Consortium, Labadie, J., 2003. Identification of *Listeria monocytogenes* genes involved in salt and alkaline-pH tolerance. Applied and Environmental Microbiology 69, 3137-3143.

Gasanov, U., Hughes, D., Hansbro, P. M., 2005. Methods for the isolation and identification of *Listeria* spp. and *Listeria monocytogenes*: a review. FEMS Microbiology Reviews 29, 851-875.

Ho, A. J., Lappi, V. R., Wiedmann, M., 2007. Longitudinal monitoring of *Listeria monocytogenes* contamination patterns in a farmstead dairy processing facility. Journal of Dairy Science 90, 2517-2524.

Kells, J., Gilmour, A., 2004. Incidence of *Listeria monocytogenes* in two milk processing environments and assessment of *Listeria monocytogenes* blood agar for isolation. International Journal of Food Microbiology 91, 167-174.

Latorre, A. A., Van Kessel, J. A. S., Karns, J. S., Zurakowski, M. J., Pradhan, A. K., Zadoks, R. N., Boor, K. J., Schukken, Y. H., 2009. Molecular ecology of *Listeria monocytogenes*: evidence for a reservoir in milking equipment on a dairy farm. Applied and Environmental Microbiology 75, 1315-1323.

Lianou, A., Sofos, J. N., 2007. A review of the incidence and transmission of *Listeria monocytogenes* in ready-to-eat products in retail and food service environments. Journal of Food Protection 70, 2172-2198.

Liu, S., Graham, J.E., Bigelow, L., Morse, P.D., Wilkinson, B.J., 2002. Identification of *Listeria monocytogenes* genes expressed in response to growth at low temperature. Applied and Environmental Microbiology 68, 1697-1705.

Long, F., Zhu, X. N., Zhang, Z. M., Shi, X. M., 2008. Development of a quantitative polymerase chain reaction method using a live bacterium as internal control for the detection of *Listeria monocytogenes*. Diagnostic Microbiology and Infectious Disease 62, 374-381.

Moltz, A.G., Martin, S.E., 2005. Formation of biofilms by *Listeria monocytogenes* under various growth conditions**.** Journal of Food Protection, 68, 92-97

O'Grady, J., Ruttledge, M., Sedano-Balbás, S., Smith, T. J., Barry, T., Maher, M., 2009. Rapid detection of *Listeria monocytogenes* in food using culture enrichment combined with real-time PCR. Food Microbiology 26, 4-7.

Olesen, I., Vogensen, F.V., Jespersen, L., 2009. Gene tanscription and virulence potential of *Listeria monocytogenes* strains after exposure to acidic and NaCl stress. Foodborne Pathogens and Disease 6, 669-680.

Pan, Y., Breidt Jr, F., 2007. Enumeration of viable *Listeria monocytogenes* cells by real-time PCR with propidium monoazide and ethidium monoazide in the presence of dead cells. Applied and Environmental Microbiology 73, 8028-8031.

Pan, Y., Breidt Jr, F., Kathariou, S., 2006. Resistance of *Listeria monocytogenes* biofilms to sanitizing agents in a simulated food processing environment. Applied and Environmental Microbiology 72, 7711-7717.

Pappelbaum, K., Grif, K., Heller, I., Wüirzner, R., Hein, I., Ellerbroek, L., Wagner, M., 2008. Monitoring hygiene on- and at-line is critical for controlling *Listeria monocytogenes* during produce processing. Journal of Food Protection 71, 735-741.

Phan-Thanh, L., Mahouin, F., Aligé, S., 2000. Acid responses of *Listeria monocytogenes*. International Journal of Food Microbiology 55,121-126.

Ramaswamy, V., Cresence, V. M., Rejitha, J. S., Lekshmi, M. U., Dharsana, K. S., Prasad, S. P., Vijila, H. M., 2007. *Listeria* - review of epidemiology and pathogenesis. Journal of Microbiology, Immunology and Infection 40, 4-13.

Rantsiou, K., Alessandria, V., Urso, R., Dolci, P., Cocolin, L., 2008. Detection, quantification and vitality of *Listeria monocytogenes* in food as determined by quantitative PCR. International Journal of Food Microbiology 121, 99-105.

Rocourt, J., Cossart, P. 1997. *Listeria monocytogenes*. In: Doyle, M. P., Beuchat, L. R., Montville, T. J., (Eds), Food Microbiology - Fundamentals and Frontiers, American Society for Microbiology (ASM), Washington, pp. 337-352.

Rodríguez-Lázaro, D., Jofré, A., Aymerich, T., Hugas, M., Pla, M., 2004. Rapid quantitative detection of *Listeria monocytogenes* in meat products by real-time PCR. Applied and Environmental Microbiology 70, 6299-6301.

Rowan, N.J. 2004. Viable but non culturable forms of food and waterborne bacteria: Quo Vadis? Trends in Food Science and Technology 15, 462–467

Rudi, K., Naterstad, K., Drömtorp, S. M., Holo, H., 2005. Detection of viable and dead *Listeria monocytogenes* on gouda-like cheeses by real-time PCR. Letters in Applied Microbiology 40, 301-306.

Vauterin, L., Vauterin, P. 1992. Computer-aided objective comparison of electrophoretic patterns for grouping and identification of microorganisms. European Journal of Clinical Microbiology, 33, 633-641.

Warriner, K., Namvar, A. 2009. What is the hysteria with *Listeria*? Trends in Food Science and Technology 20, 245-254.

Figure legends.

Figure 1. Panel A, Compositive dendrogram of the *Listeria monocytogenes* isolates analyzed by RAPD, Rep and SAU-PCR. Clusters are indicated with numerals. The strains are coded by indicating the day of isolation and the specification of the sample: numbers 10, 11 and 12 refer to specific equipment in the production line, numbers 1 and 2 represent semifinished products, number 8 stands for a brine sample and ST is used for a finished cheese. The E in from of the sample code indicates that the strains was isolated after enrichment. **Panel B**, ability of the isolated *L. monocytogenes* to attach to PVC surfaces. This was assessed by staining the attached cells with crystal violet and measuring the OD at 590 nm of the ethanol solution used to de-stained them. Means and standard deviations of three independent experiments, measuring 8 wells for each strain, are presented. A non-inoculated well (negative control, NC) and two strains coming from International collections (Scott A and EGDe), able to form biofilms, were also included for comparison reasons.

Figure 2. Comparison of the percentage of the positive samples for *Listeria monocytogenes* presence by molecular and traditional methods in the two sampling periods considered in this study.

Figure 1

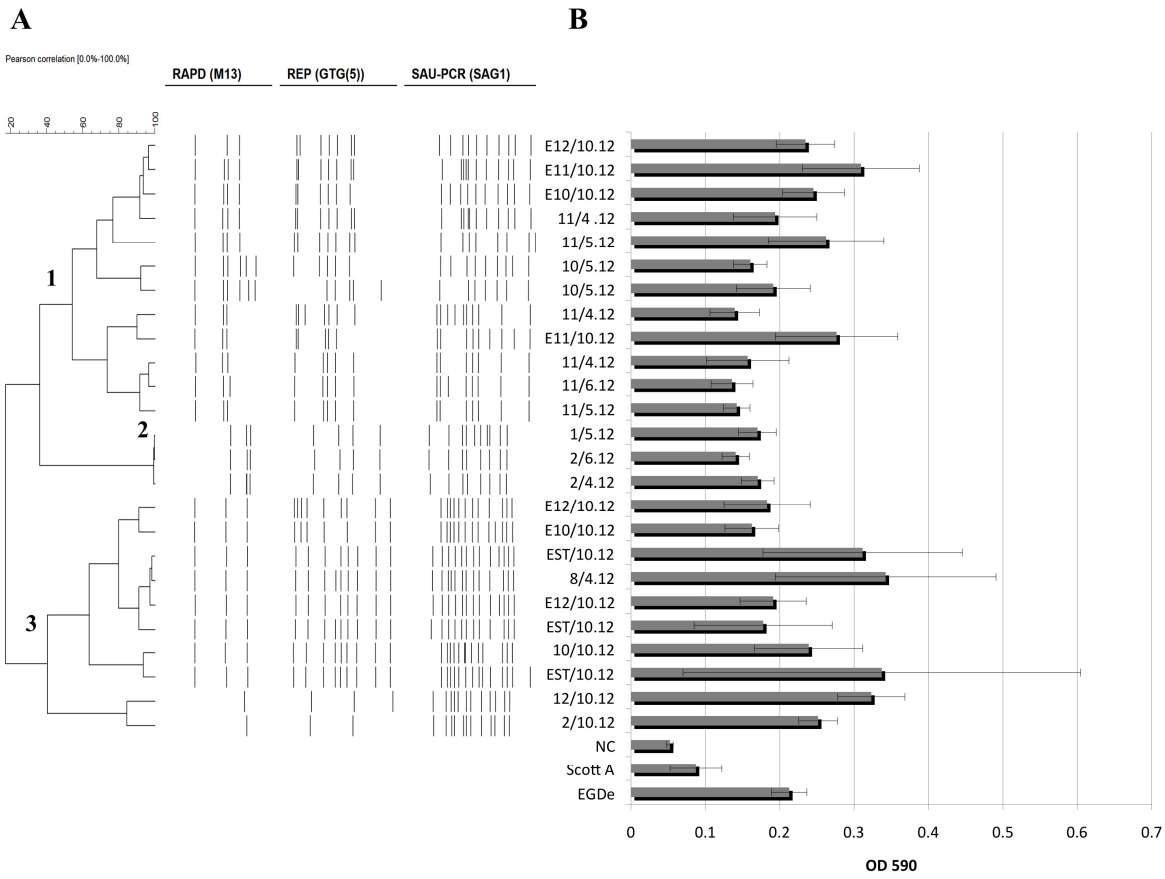
A
B
Pearson correlation [0.0%-100.0%]
RAPD (M13)
REP (GTG(5))
SAU-PCR (SAG1)
20
40
60
80
100
1
2
3
E12/10.12
E11/10.12
E10/10.12
11/4 .12
11/5.12
10/5.12
10/5.12
11/4.12
E11/10.12
11/4.12
11/6.12
11/5.12
1/5.12
2/6.12
2/4.12
E12/10.12
E10/10.12
EST/10.12
8/4.12
E12/10.12
EST/10.12
10/10.12
EST/10.12
12/10.12
2/10.12
NC
Scott A
EGDe
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
OD 590

Figure 2

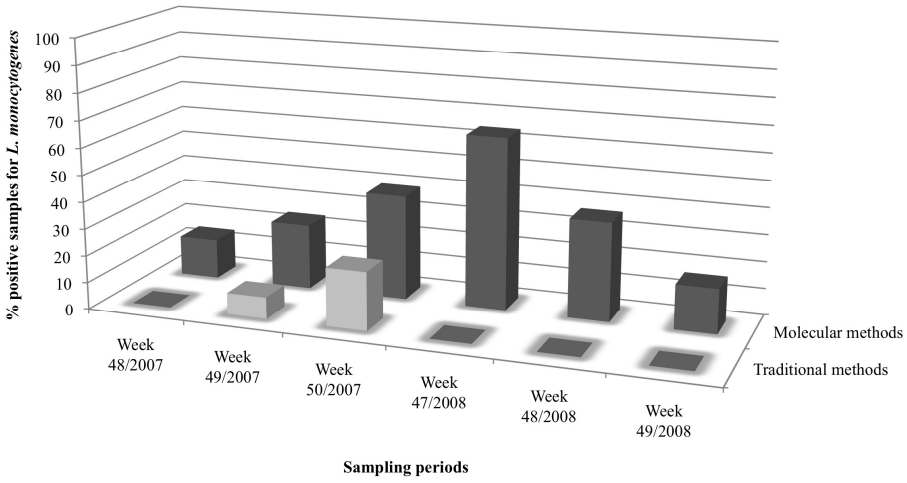
% positive samples for L. monocytogenes
100
90
80
70
60
50
40
30
20
10
0
Week 48/2007
Week 49/2007
Week 50/2007
Week 47/2008
Week 48/2008
Week 49/2008
Molecular methods
Traditional methods
Sampling periods

Table 1. Results obtained by traditional and molecular methods for the detection and quantification of *Listeria monocytogenes* in the samples obtained from the dairy company in the 2007 campaign, before (T0) and after (T24) an 18 hours overnight enrichment. The number of positive samples obtained by applying the specific method (for details see the materials and methods) is reported.

	Week 48					Week 49					Week 50				
	N° of samples	Traditional methods		Quantitative PCR		N° of samples	Traditional methods		Quantitative PCR		N° of samples	Traditional methods		Quantitative PCR	
		T0[a]	T24[b]	T0[c]	T24		T0	T24	T0	T24		T0	T24	T0	T24
Semi products	6	0	0	2	2	8	0	4	0	0	2	0	0	0	0
Final products	30	0	0	0	0	40	0	0	0	0	10	0	2	0	0
Brines	3	0	0	1	1	8	1	0	8	8	6	0	0	5	5
Equipment swabs	15	0	0	5	5	18	1	1	9[d]	10	5	3	3	4	4
Totals	54	0	0	8	8	74	2	5	17	18	23	3	5	9	9

[a]Positive at T0 means counts of *L. monocytogenes* above the detection limit of the method (for food samples 100 cfu/g, for brines 10 cfu/ml and for swabs 50 cfu/cm^2) and confirmed by molecular identification of randomly selected colonies.
[b]Positive at T24 means presence of suspected colonies of *L. monocytogenes* subsequently confirmed by molecular methods.
[c]Samples for which it was possible to obtain a quantification by using the appropriate standard curves constructed as described in the materials and methods.
[d]Not all the samples positive after enrichment could be quantified at T0 since the loads of *L. monocytogenes* were below the quantification limit (for food samples 10^3 cfu/g, for brines 100 cfu/ml and for swabs 500 cfu/ cm^2) (Rantsiou et al., 2008).

Table 2. Results obtained by traditional and molecular methods for the detection and quantification of *Listeria monocytogenes* in the samples obtained from the dairy company in the 2008 campaign, before (T0) and after (T24) an 18 hours overnight enrichment. The number of positive samples obtained by applying the specific method (for details see the materials and methods) is reported.

	Week 47					Week 48					Week 49				
	N° of samples	Traditional methods		Quantitative PCR		N° of samples	Traditional methods		Quantitative PCR		N° of samples	Traditional methods		Quantitative PCR	
		T0[a]	T24[b]	T0[c]	T24		T0	T24	T0	T24		T0	T24	T0	T24
Final products	9	0	0	1[d]	4	5	0	0	3	3	6	0	0	0[d]	1
Equipment swabs	5	0	0	0[d]	4	7	0	0	0[d]	1	6	0	0	0[d]	1
Brines	--[e]	--	--	--	-	12	0	0	0[d]	3	--	--	--	--	--
Totals	14	0	0	1	8	24	0	0	3	7	12	0	0	0	2

[a]Positive at T0 means counts of *L. monocytogenes* above the detection limit of the method (for food samples 100 cfu/g, for brines 10 cfu/ml and for swabs 50 cfu/cm^2) and confirmed by molecular identification of randomly selected colonies.
[b]Positive at T24 means presence of suspected colonies of *L. monocytogenes* subsequently confirmed by molecular methods.
[c]Samples for which it was possible to obtain a quantification by using the appropriate standard curves constructed as described in the materials and methods.
[d]Not all the samples positive after enrichment could be quantified at T0 since the loads of *L. monocytogenes* were below the quantification limit (for food samples 10^3 cfu/g, for brines 100 cfu/ml and for swabs 500 cfu/ cm^2) (Rantsiou et al., 2008).
[e]Samples were not collected in the specific week.

Table 3. Results of the quantification of *Listeria monocytogenes* by qPCR in the samples collected in 2007 and 2008. Counts are expressed in colony forming units (cfu)/g for semi and final products, cfu/ml for the brines and cfu/cm^2 for the equipment swabs.

	2007						2008			
	Week 48		Week 49		Week 50		Week 47		Week 48	
	Positive at T24	Counts at T0	Positive at T24	Counts at T0	Positive at T24	Counts at T0	Positive at T24	Counts at T0	Positive at T24	Counts at T0
Semi products	2	$1.80x10^5$ $8.10x10^4$	0	n.a[a]	0	n.a	0	n.a.	0	n.a
Final products	0	n.a	0	n.a	0	n.a	1	$1.88x10^4$	4[b]	$1.01x10^3$ $2.01x10^3$ $2.35x10^5$
Brines	1	$7.72x10^3$	8	$1.09x10^4$ $1.33x10^4$ $8.90x10^3$ $7.41x10^3$ $1.10x10^4$ $6.39x10^3$ $5.51x10^3$ $8.24x10^3$	5	$6.35x10^3$ $1.08x10^4$ $1.23x10^4$ $1.81x10^4$ $8.85x10^3$	0	n.a	0	n.a
Equipment swabs	5	$1.89x10^4$ $3.54x10^4$ $3.87x10^4$ $1.03x10^3$ $5.77x10^3$	10[b]	$8.29x10^3$ $7.19x10^3$ $8.33x10^3$ $1.67x10^4$ $2.10x10^4$ $8.59x10^3$ $1.00x10^4$ $1.86x10^4$ $3.74x10^4$	4	$5.98x10^3$ $1.79x10^4$ $6.39x10^3$ $4.78x10^3$	0	n.a	0	n.a

[a]Not applicable

[b]Not all the samples positive after enrichment could be quantified at T0 since the loads of *L. monocytogenes* were below the quantification limit (for food samples 10^3 cfu/g, for brines 100 cfu/ml and for swabs 500 cfu/ cm^2) (Rantsiou et al., 2008).