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Detection of *Listeria monocytogenes* in Italian-style soft cheeses

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ABSTRACT

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Aims: A rapid detection system specific for *Listeria monocytogenes* and based on the polymerase chain reaction (PCR) was developed.

Methods and Results: Primers annealing to the coding region of the *actA* gene, critically involved in virulence and capable of discrimination between two different alleles naturally occurring in *L. monocytogenes*, have been utilized. The procedure was applied to recover *L. monocytogenes* cells in artificially contaminated fresh Italian soft cheeses (mozzarella, crescenza and ricotta). Low levels of *L. monocytogenes* were detected in mozzarella and crescenza homogenates (0·04–0·4 and 4 CFU g^{-1}, respectively) whereas in ricotta the detection limit was higher (40 CFU g^{-1}).

Conclusions: This PCR-based assay is highly specific as primers used recognize the DNA from different *L. monocytogenes* strains of clinical and food origin, while no amplification products result with any other *Listeria* spp. strains.

Significance and Impact of the Study: This study highlighted a low-cost and rapid procedure that can be appropriated for the detection in real time of low *L. monocytogenes* levels in soft cheese.

Keywords: *actA* gene, DNA detection, *Listeria monocytogenes*, polymerase chain reaction, soft cheese.

INTRODUCTION

Bacteria belonging to the genus *Listeria* are widely distributed in nature – on decaying vegetation, soils, sewage, silage and water (Farber and Peterkin 1991). Among listeriae, *Listeria monocytogenes* is the only pathogen of concern for humans associated with listeriosis, a severe foodborne disease, usually leading to meningitis and sepsis (Slutsker and Schuchat 1999), as well as gastrointestinal illness (Dalton *et al.* 1997; Hof 2001). Immunocompromised individuals are at risk of infection, and during pregnancy infection may result in abortion or dramatic septic illness of the newborn (Lorber 1996; McLauchlin 1996). Although listeriosis occurs sporadically, the high mortality rate associated with this disease makes it a serious public health problem (Schuchat *et al.* 1991).

The ability of *L. monocytogenes* to survive and grow over a wide range of temperatures, at high salt concentration and low pH (Gahan *et al.* 1996; Kim *et al.* 1998) makes it a potential hazard in milk and other dairy products, often implicated as the source of infection in severe outbreaks (De Buyser *et al.* 2001). *L. monocytogenes* is detected in about 2–5% of raw milk that can be contaminated from environmental sources, including faeces and milk from infected animals (Ryser 1999a). The heat treatment given during cheese manufacture is generally sufficient to inactivate small numbers of listeriae that might be present (Buazzi *et al.* 1992a,b; Villani *et al.* 1996), although postprocessing contamination can also occur (Stecchini *et al.* 1995). From over 15 surveys of cheeses manufactured in Italy, this pathogen was recovered in 3·1% of Italian cheeses, with a prevalence of about 6% in mozzarella, classified as 'pasta

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filata' soft cheese and of about 4% in various other soft cheeses (Castellucci *et al.* 1996; Ryser 1999b).

Different criteria or guidelines for tolerable levels of *L. monocytogenes* have been established in milk and milk-based products. Italy is one of the countries which has the most rigid policy as zero tolerance is required for soft cheese, and therefore rapid isolation and confirmation methods for *L. monocytogenes* are of importance. Significant improvements in selective enrichment and isolation procedures have been achieved over the last decade but traditional cultural methods are still time-consuming, laborious and cannot detect low *L. monocytogenes* contamination levels (Anon. 1996). An additional disadvantage is the faster growth in selective enrichment broths of *Listeria innocua* that is more often detected in foodstuffs than *L. monocytogenes* (Donnelly 1999). Recently, a number of new analytical methods for rapid detection of *L. monocytogenes* have been proposed, including immunological and nucleic acid-based procedures (Batt 1999). For polymerase chain reaction (PCR) methods a diversity of genetic elements have been selected, mainly targeting virulence genes, such as those encoding phospholipase C (*plcB*), listeriolysin O (*hly*), the transcriptional regulatory protein (*prfA*), the invasion-associated protein (*iap*) and the metalloprotease (*mpl*) (Cooray *et al.* 1994; Manzano *et al.* 1996; Scheu *et al.* 1998, 1999; Hein *et al.* 2001). However, isolation and harvesting procedures for bacterial target DNA, as well as the inhibition caused by dairy product components, may represent in some instances, a limiting factor for the reliability of these assays (Bickley *et al.* 1996; Batt 1999).

In the present study a detection procedure consisting of the amplification of the virulence *actA* gene, encoding ActA, a transmembranal protein exclusively present in *L. monocytogenes* species, was developed. During intracellular life ActA protein induces actin polymerization, allowing bacterial directional intracellular cytoplasm mobility and spread to neighbouring cells (Tilney and Portnoy 1989; Vazquez-Boland *et al.* 2001). Two *actA* alleles were identified, differing solely by the presence or absence of a 105 nucleotide direct repeat encoding a proline-rich repeat structure (Wiedmann *et al.* 1997).

This PCR method was applied to exported Italian-style fresh soft cheeses: mozzarella, crescenza and ricotta, artificially contaminated with different concentrations of *L. monocytogenes* cells. A detection limit ranging from 0·04 to 40 CFU g^{-1} of cheese was reached. This specific method could also be particularly suitable to monitor the different distributions of two allelic forms. In fact, the use of *actA* gene as a target for amplification of a 300- or 400-bp fragment can give information about strains on the allelic polymorphism of this gene, which in turn has been associated with differences in the pathogenic potential (Wiedmann *et al.* 1997).

MATERIALS AND METHODS

Bacterial strains and culture conditions

Bacteria used in this study are listed in Table 1. Bacterial strains included strains from American Type Culture Collection (ATCC), National Collection of Type Cultures (NCTC) and Culture Collection of the University of Gőteborg (Sweden), strains isolated in the Microbiology Laboratory of the Department of Public Health Sciences of Rome University La Sapienza (Italy), strains kindly provided by Dr P. Valenti of Naples II University (Italy) and by Dr C. Rees of Nottingham University (UK). *Listeria innocua* was kindly provided by Dr P. Aureli of Public Health Institute of Rome (Italy). Bacteria were routinely grown in brain heart infusion broth (BHI, Oxoid), pH 7·2 and maintained by serial passages onto tryptone soy agar (Oxoid). *Listeria monocytogenes* strain ATCC 7644, a haemolytic wild-type strain capable of invading and multiplying in cultured cells (Conte *et al.* 1994), was used for artificial contamination of cheese samples.

Sample preparation

The cheese samples used in this study (mozzarella, crescenza and ricotta from bovine milk) were obtained from a local retail store. Twenty-five grams of fresh cheese (within 48 h after manufacture) was weighed in a sterile stomacher bag, 225 ml of BHI (Oxoid) was added and the mixture was homogenized for 2 min in a Bagmixer Stomacher 400 (Interscience, St Norm, France). In some experiments the cheese homogenates were autoclaved at 121°C for 20 min. Then, 10 ml of the sample was transferred in a 15 ml centrifuge tube and centrifuged for 10 min at $150 \times g$ to remove large particulate matter. The cheese sample homogenates were artificially contaminated with *L. monocytogenes* ATCC 7644 by directly adding appropriate volumes of serial dilutions of overnight cultures in phosphate-buffered saline (PBS) pH 7·2 to obtain inoculum levels of *ca.* 1, 10^1, 10^2, 10^3, 10^4 and 10^5 CFU ml^{-1}. The exact inoculum level was controlled by plating the inocula diluted in PBS on Oxford agar (Oxoid) in duplicate. Colonies were enumerated after 24 h incubation at 37°C.

To verify the outcome of *L. monocytogenes* after 7-day storage at 4°C and the sensitivity of the above described sample preparation protocol for the recovery of sub-lethally injured bacteria, 25 g of mozzarella, crescenza and ricotta cheeses were inoculated prior to the homogenization step with *L. monocytogenes* and kept for 7 days at 4°C. After incubation, inoculated cheese samples were put in a sterile stomacher bag, 225 ml of Listeria enrichment broth base (Oxoid) was added and the mixture was homogenized for 2 min. The homogenates were centrifuged for 10 min at $150 \times g$ to remove large particulate matter and immediately submitted to DNA

Table 1 *Listeria* strains used for specificity tests for primers L1 (*actA*-F) and L2 (*actA*-R)

Species	Source
L. monocytogenes NCTC 7973 serotype 1	National Collection of Type Cultures (UK)
L. monocytogenes ATCC 7644 serotype 1	American Type Culture Collection (USA)
L. monocytogenes LM1 serotype 4 *L. monocytogenes* LM2 serotype 4	Clinical isolates, University of Rome La Sapienza (Italy)
L. monocytogenes Scott A serotype 4 *L. monocytogenes* LM24 serotype 1 *L. monocytogenes* LM25 serotype 4	Clinical isolates, University of Nottingham (UK)
L. monocytogenes LM27 serotype 4 *L. monocytogenes* CAL serotype 1 *L. monocytogenes* SA serotype 4 *L. monocytogenes* OH serotype 4	Clinical isolates, II University of Naples (Italy)
L. monocytogenes LGP serotype 4	Food isolate, University of Rome La Sapienza (Italy)
L. monocytogenes LM7 serotype 1 *L. monocytogenes* LM29 serotype 4 *L. monocytogenes* LM32 serotype 4 *L. monocytogenes* LM6 serotype 4	Food isolates, University of Nottingham (UK)
L. innocua	Public Health Institute of Rome (Italy)
L. welshimeri 15529 *L. seeligeri* 15530 *L. ivanovii* 15528 *L. grayi/murrayi* 4984 *L. grayi/murrayi* 5118	Culture Collection University of Goteborg (Sweden)

extraction or added with selective supplement SR141E (Oxoid), poured in a 500 ml receptacle and incubated for 18 h at 30°C to allow the growth of listeria cells. A non-inoculated portion in each cheese matrix was analysed for any possible natural presence of *L. monocytogenes*.

Nucleic acid extraction

DNA extraction from *Listeria* strains (Table 1) was performed by boiling 1 ml of overnight cultures at 100°C for 12 min in the presence of PBS-0·05% Tween 20. Cell debris was removed by centrifugation and 1 μl of the supernatant fluid used in the PCR.

DNA extraction from cheeses artificially contaminated with *L. monocytogenes* was performed by submitting 1 ml of homogenate to centrifugation for 10 min at 6500 × *g* at 4°C. The supernatant was then discarded, the pellet washed twice in PBS at 1600 × *g* for 5 min at 4°C, boiled at 100°C for 12 min in the presence of 100 μl PBS-0·05% Tween 20 and put in ice. Cell debris was removed by centrifugation and 20 μl of the supernatant fluid used in the PCR.

Polymerase chain reaction primer design

Two *L. monocytogenes*-specific in-house oligonucleotide pairs based on the sequence of *actA* virulence gene were designed on the basis of the reported nucleotide sequence of the *actA* gene (Vazquez-Boland *et al.* 1992):

Primer	GenBank accession no.	Sequence (nt position)	Product size (bp)
L1: *actA*-F	AF281897	5′-GTGATAAAATC GACGAAAATCC-3′ (679–701)	400 or 300
L2: *actA*-R		5′-CTTGTAAAACTA GAATCTAGCG-3′ (1057–1079)	

Primers were supplied by M-Medical Genenco (Florence, Italy). The primer sequences allowed amplification of a 300- or 400-bp fragment internal to *actA* gene.

Amplification and PCR product detection

A 20-μl DNA sample was subjected to PCR, using the two oligonucleotide primers reported above. The PCR was performed in a Thermal Cycler 480 (Perkin-Elmer, Norwalk, CT, USA). The positive control contained *L. monocytogenes* ATCC 7644 strain as template DNA. The annealing temperatures were 58°C. Amplification reactions were carried out in 50 μl containing 1x PCR

buffer II (50 mmol l^{-1} KCl, 10 mmol l^{-1} Tris–HCl, pH 8·3) (Perkin-Elmer), 2 mmol l^{-1} $MgCl_2$, 0·2 mmol l^{-1} each deoxynucleotide triphosphate, 25 pmol l^{-1} each primer, 2·5 U *Taq* polymerase (Perkin-Elmer). The thermal programme consisted of an initial denaturation step at 95°C for 5 min, then a total of 35 cycles were performed, each cycle comprising 45 s at 94°C, 1·5 min at the annealing temperature, and 2 min at 72°C. A no-template negative control was included in all experiments. The PCR products were analysed by gel electrophoresis using 2% agarose gels in (Tris–Borate–EDTA) TBE buffer (89 mmol l^{-1} Tris-base, 89 mmol l^{-1} boric acid, 2 mmol l^{-1} EDTA, pH 8·0). Gels were run for 75 min at 100 V in TBE buffer containing 0·5 μg ml^{-1} ethidium bromide to enable visualization of the PCR products by UV transillumination. Molecular weight markers were included on each gel (100-bp DNA ladder; M-Medical Genenco, Florence, Italy). The length of amplified fragments was 400 or 300 bp.

Reproducibility and confirmation of *L. monocytogenes* detection procedure

Each detection test was run in triplicate. The official standard ISO 11290-1/ISO 11290-2 procedures (Anon. 1996) were conducted in parallel with the rapid detection procedure in the examination of contaminated cheese samples.

RESULTS

Determination of PCR specificity

Specificity of our PCR detection system was tested by amplifying DNA isolated from an extensive range of *L. monocytogenes* isolates of different origin and from four other *Listeria* species (Fig. 1). In Fig. 1a the *actA* PCR products obtained for *L. monocytogenes* strains are reported. A size difference (400 or 300 bp) of PCR products is shown, consistent with the fact that the two different alleles were present in *L. monocytogenes* strains. In Fig. 1b gel electrophoresis indicates that no amplification products were detected for strains other than those of *L. monocytogenes*.

Detection of *L. monocytogenes* in artificially contaminated soft cheeses

The cheese sample preparation protocol consisted of a 10-fold dilution, homogenization and a centrifugation step to precipitate large food particles (see 'Materials and Methods'). Then, serial concentrations of *L. monocytogenes* (1, 10^1, 10^2, 10^3, 10^4, and 10^5 CFU ml^{-1}) were inoculated in fresh cheese homogenates. To determine the effect of non-*L. monocytogenes* flora on the sensitivity of the method, homogenates were analysed untreated or sterilized at 121°C

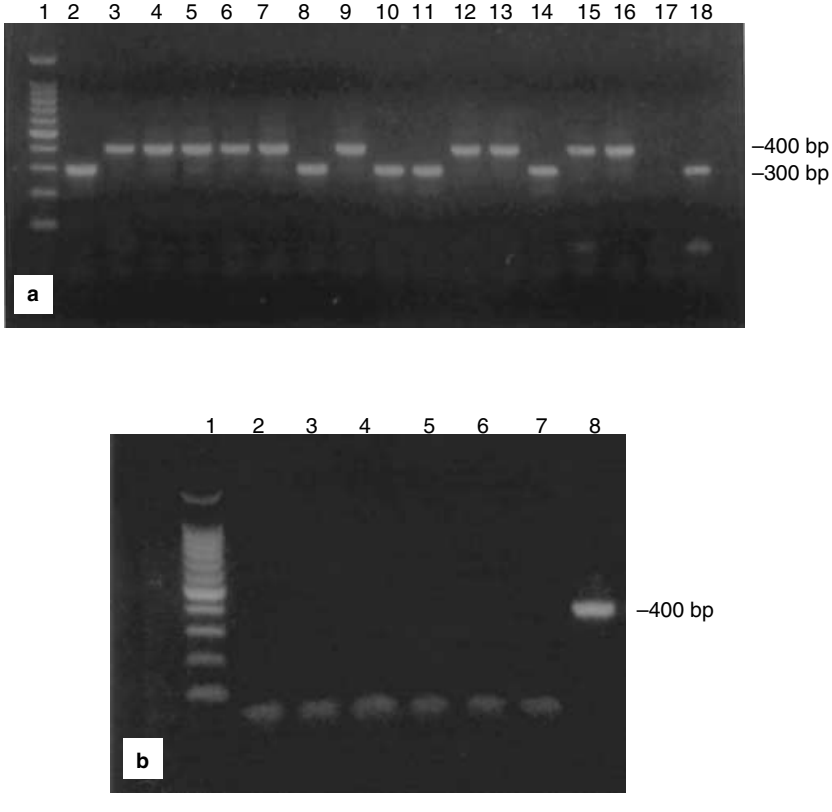

Fig. 1 Ethidium bromide-stained agarose gel with the PCR products of the *actA* gene of *Listeria monocytogenes*. (a) Lanes: 1, molecular weight markers; 2, *L. monocytogenes* Scott A; 3, *L. monocytogenes* LGP; 4, *L. monocytogenes* LM2; 5, *L. monocytogenes* LM1; 6, *L. monocytogenes* NCTC 7973; 7, *L. monocytogenes* LM24; 8, *L. monocytogenes* LM7; 9, *L. monocytogenes* LM25; 10, *L. monocytogenes* LM29; 11, *L. monocytogenes* LM27; 12, *L. monocytogenes* LM32; 13, *L. monocytogenes* CAL; 14, *L. monocytogenes* LM6; 15, *L. monocytogenes* SA; 16 *L. monocytogenes* ATCC 7644; 17, *L. innocua*; 18, *L. monocytogenes* OH. (b) Lanes: 1, molecular weight markers; 2, *L. innocua*; 3, *L. welshimeri*; 4, *L. seeligeri;* 5, *L. ivanovii*; 6–7, *L. grayi/murrayi*; 8, *L. monocytogenes* ATCC 7644.

Table 2 Detection of PCR products from untreated (a) and autoclaved (b) cheese homogenates artificially contaminated with *Listeria monocytogenes*

	Band intensity at the following concentrations of bacteria (CFU ml^{-1})*											
	1		1×10^1		1×10^2		1×10^3		1×10^4		1×10^5	
Cheese sample homogenates	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Mozzarella	+	+	++	++	++	++	+++	+++	+++	+++	++++	++++
Crescenza	–	–	–	Trace	++	++	++	++	++	++	+++	+++
Ricotta	–	–	–	–	Trace	Trace	++	++	+++	+++	++++	++++

*The band intensity was calculated using as reference the intensity (++++) shown by amplicons obtained in homogenates inoculated with the highest concentration of bacteria (1×10^5 CFU ml^{-1}).

for 20 min. The entire procedure of sample preparation took about 60 min to complete and no significant outgrowth of the pathogen was noticed during this period (results not shown). DNA extracted from artificially contaminated untreated or autoclaved cheese homogenates was subjected to PCR. From Table 2 it can be seen that this protocol enables detection of 1–10 CFU ml^{-1} or 0·04–0·4 CFU g^{-1} for mozzarella, 10^2 CFU ml^{-1} or 4 CFU g^{-1} for crescenza and 10^3 CFU ml^{-1} or 40 CFU g^{-1} for ricotta. No significant differences were noticed between untreated or autoclaved samples. Recovery of *L. monocytogenes* was confirmed by plating the contaminated homogenates on Oxford medium and by confirmation of suspected colonies following the ISO 11620-2 procedure (Anon. 1996). Figure 2 shows a typical gel electrophoresis of the PCR DNA amplicons generated from untreated mozzarella cheese homogenates contaminated with serial dilutions of *L. monocytogenes*.

To investigate the influence of the storage on the sensitivity of the above described sample preparation protocol for the recovery of sub-lethally injured bacteria, cheeses were also directly inoculated with serial dilutions of *L. monocytogenes* and incubated for 7 days at 4°C. Then, artificially contaminated samples were homogenized and immediately submitted to DNA extraction procedure. In parallel, after homogenization, duplicate samples were further incubated in enrichment broth for 18 h at 30°C to allow the growth of eventually injured bacteria. The results demonstrated that incubation of stored mozzarella and ricotta samples in enrichment broth was a fundamental step, as the PCR assay enabled detection of recovery values of bacteria equal or higher than the initial inoculum level only after incubation of samples in this medium (results not shown). In contrast, PCR was unable to recover *L. monocytogenes* from crescenza, maintained for 7 days at 4°C, even after incubation in enrichment broth. Plating of the contaminated enriched homogenates confirmed these results.

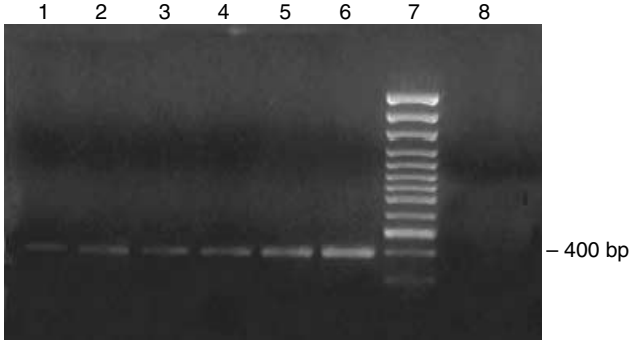

Fig. 2 Agarose gel electrophoresis of the amplification products of the *actA* gene of *Listeria monocytogenes* generated from serial dilutions of artificially contaminated mozzarella cheese homogenates. Lanes: 1, 1 CFU ml^{-1}; 2, 10 CFU ml^{-1}; 3, 10^2 CFU ml^{-1}; 4, 10^3 CFU ml^{-1}; 5, 10^4 CFU ml^{-1}; 6, 10^5 CFU ml^{-1}; 7, molecular weight markers 100 pb ladder; 8, negative control

DISCUSSION

In the last decades several surveys have dealt with the incidence of *L. monocytogenes* in cheese and particular attention has been addressed to Italian soft cheeses (Ryser 1999b). Among these, crescenza, ricotta and a pasta filata-type soft cheese, i.e. mozzarella, although prepared with pasteurized milk, can be contaminated by low numbers of *L. monocytogenes* cells that may survive in an acidic environment and heat treatment during the manufacture, or occur in a postprocessing step (Buazzi *et al.* 1992a). The survival over a wide range of temperatures (Cole *et al.* 1990) and the ability to mount an acid tolerance response (Phan-Thanh *et al.* 2000) correlate in *L. monocytogenes* with an increased virulence (O'Driscoll *et al.* 1996; Gahan and Hill 1999; Conte *et al.* 2000; Saklani-Jusforgues *et al.* 2000). Exposure of bacteria to acidic pH can occur either during soft cheese production or during cheese storage, such as for instance in mozzarella cheese, which is conserved in a conditioning liquid (pH 3·8–4·2) until delivered to consumers (Gahan *et al.* 1996; Villani *et al.* 1996). As a matter of fact soft cheeses may represent a serious risk for the public health, also taking into account that experiments of postprocessing contamination and storage of mozzarella increased up to 10 000-fold the number of listeriae (Stecchini *et al.* 1995). Recovery of *L. monocytogenes* from soft cheese can be complicated because low numbers of this

pathogen may be present injured or in association with high numbers of other micro-organisms of natural flora (Ryser 1999b). The European Community directive on milk and milk-based products specifies zero tolerance for soft cheese (Anon. 1992). Thus, rapid and reliable procedures to verify in real time the putative presence of *L. monocytogenes* in these dairy products are required.

We present here a highly specific and sensitive DNA-based method (PCR) for detecting *L. monocytogenes* in pure cultures and in Italian soft cheeses that are among the most economically important. The use of a pair of primers annealing to the coding region of the *actA* gene proved positive with the DNA from different *L. monocytogenes* strains of clinical and food origin, while with the DNA from other *Listeria* spp. strains tested it was proved negative. Moreover, the choice of the *actA* gene is not only appropriate because *actA* is regarded as a gene critically involved in virulence (Tilney and Portnoy 1989; Vazquez-Boland *et al.* 2001) and unique to the species *L. monocytogenes*, but also relevant for epidemiological approaches as the primers can discriminate between two different alleles, naturally occurring in *L. monocytogenes* and probably involved in cell-to-cell spread (Wiedmann *et al.* 1997).

The DNA extraction procedure developed by us and the primers chosen to perform the DNA amplification for the recovery of *L. monocytogenes* in freshly manufactured soft cheeses are suitable and render this technique advantageous, compared with other conventional detection methods. The assay has low complexity and low cost, and is faster than other molecular identification methods, allowing a rapid detection of low levels of *L. monocytogenes* in mozzarella and crescenza fresh cheeses in the presence of competitive flora (1–10 CFU ml^{-1} or 0·04–0·4 CFU g^{-1} for mozzarella and 10^2 CFU ml^{-1} or 4 CFU g^{-1} for crescenza). The sensitivity achieved was comparable to that obtained by the procedure proposed by Uyttendaele *et al.* (2000). The detection limit for bacteria was 100-fold higher in ricotta (10^3 CFU ml^{-1} or 40 CFU g^{-1}) than in mozzarella, suggesting a possible interference with PCR reaction inhibitory factors present in this cheese, as already described for other soft cheeses (Fluit *et al.* 1993). Moreover, no striking differences were noticed between untreated or autoclaved samples, providing evidence that in these experimental conditions the contaminating bacterial flora did not hinder the detection of *L. monocytogenes*.

DNA-based methods often include a lengthy enrichment procedure prior to *L. monocytogenes* identification (Donnelly 1999) that must take into account the recovery of both healthy and heat-injured bacterial cells. Our results indicate that a brief enrichment step (6 h) is required for the recovery of *L. monocytogenes* after storage of mozzarella and ricotta for 7 days at 4°C. When incubation in enrichment broth was prolonged, the contaminating flora frequently found in these cheeses, especially in mozzarella, grows faster than the inoculated *L. monocytogenes*, thus enhancing the difficulty in recovering this pathogen by the molecular procedure (unpublished results). The inability to detect the contamination caused by *L. monocytogenes* in crescenza, even after enrichment step, indicates that after 7-day incubation at 4°C the viability of bacteria was affected, and also suggests the presence of inhibiting factors developed in this cheese during storage, as already postulated for other dairy products (Ryser 1999b).

Taking into account that Italian-style soft cheeses are consumed generally within 48 h after manufacture, this DNA-based detection procedure can be appropriate for the detection in real-time of low *L. monocytogenes* levels, to avoid the risk of food-borne infection in immunocompromised hosts.

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