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European validation of a real-time PCR-based method for detection of *Listeria monocytogenes* in soft cheese

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ABSTRACT

The classical microbiological method for detection of *Listeria monocytogenes* requires around 7 days for final confirmation, and due to perishable nature of RTE food products, there is a clear need for an alternative methodology for detection of this pathogen. This study presents an international (at European level) ISO 16140-based validation trial of a non-proprietary real-time PCR-based methodology that can generate final results in the following day of the analysis. This methodology is based on an ISO compatible enrichment coupled to a bacterial DNA extraction and a consolidated real-time PCR assay. Twelve laboratories from six European countries participated in this trial, and soft cheese was selected as food model since it can represent a difficult matrix for the bacterial DNA extraction and real-time PCR amplification. The limit of detection observed was down to 10 CFU per 25 of sample, showing excellent concordance and accordance values between samples and laboratories (>75%). In addition, excellent values were obtained for relative accuracy, specificity and sensitivity (82.75%, 96.70% and 97.62%, respectively) when the results obtained for the real-time PCR-based methods were compared to those of the ISO 11290-1 standard method. An interesting observation was that the *L. monocytogenes* detection by the real-time PCR method was less affected in the presence of *Listeria innocua* in the contaminated samples, proving therefore to be more reliable than the reference method. The results of this international trial demonstrate that the evaluated real-time PCR-based method represents an excellent alterative to the ISO standard since it shows a higher performance as well as reduce the extent of the analytical process, and can be easily implemented routinely by the competent authorities and food industry laboratories.

1. Introduction

Listeria monocytogenes is a ubiquitous, Gram-positive, intracellular pathogen that is the causative agent of human listeriosis. It affects mainly fetuses, newborn infants, the elderly and those with weakened immune systems. Symptoms vary, ranging from mild flu-like symptom and diarrhea to life-threatening infections characterized by septicaemia and meningoencephalitis. However, it is often severe with high hospitalization and mortality rates being the leading cause of mortality in food-borne infections in industrialized countries (Slutsker and Schuchat, 1999; Ryser, 1999). Human listeriosis outbreaks are most

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often associated with ready-to-eat food products that are consumed without prior cooking. A wide range of raw and processed foods can be contaminated with *L. monocytogenes* during and after processing; the highest proportions of contaminated food at point of retail were observed in ready-to-eat (RTE) fishery products, cheeses and fermented sausages (Anonymous, 2013). As a result, the presence of *L. monocytogenes* in food products is a safety problem that warrants attention and improvements in detection and tracking, and to err on the side of caution, food safety regulations have tended to adopt a zero-tolerance attitude for *L. monocytogenes* in these products (Gallagher et al., 2003; Anonymous, 2005b).

The traditional microbiological techniques for detecting *L. monocytogenes* require more than 6 days for a final confirmation (Donnelly, 1999), and consequently are not sufficiently rapid to assure the safety of food products at consumption, as the risk of delaying withdrawal of not compliant food from the market. Despite these challenges, classical techniques still remain the official method used although many alternative molecular methods already exist. PCR is one of the most promising alternative techniques for the rapid detection of microorganisms in food and provides an excellent analytical sensitivity for detection improving the likelihood of detecting bacterial pathogens (Rodríguez-Lázaro et al., 2007). In recent years, real-time PCR has replaced conventional PCR as it reduces the time for final results and the carry-over contamination due to its closed-tube format.

Due to length of the standard method for detection of *L. monocytogenes* and the short shelf life of some RTE food products, there is a clear need for availability of an alternative non-proprietary real-time PCR method that can generate final results in, at least, the following day of the analysis. This will reduce the extent of the analytical process, and the method can be easily implemented routinely by the competent authorities and food industry laboratories. Therefore, the aim of this study was to validate a simple detection strategy based on an ISO compatible enrichment coupled to a bacterial DNA extraction and a consolidated real-time PCR assay for the rapid and specific detection of *L. monocytogenes* in food. We selected soft cheese as food model due to its relevance in *Listeria* transmission and to that it is a difficult matrix for real-time PCR-based detection of foodborne pathogens. The validation approach was based on a collaborative and inter-laboratory study in accordance with the ISO 16140:2003 (Anonymous, 2003), and the use of the ISO 11290-1:1996+Amd.1:2004 (Anonymous, 2004), as reference method. This is the first study, to our knowledge, of a validation of a non-proprietary real-time PCR method for the detection of *L. monocytogenes*, involving more than 10 laboratories from different countries.

2. Materials and methods

2.1. Participating laboratories

The Istituto Superiore di Sanità (Italy) was the organizing laboratory and led the international exercise. Twelve laboratories from six different European countries participated in the trial. They compromised the Centro Nacional de Tecnologia y Seguridad Alimentaria (Spain); Instituto Tecnológico Agrario de Castilla y León (Spain); the Istituto Zooprofilattico Sperimentale dell'Abruzzo e Molise (Italy); the Istituto Zooprofilattico Sperimentale del Lazio e Toscana (Italy); the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (Italy); the Istituto Zooprofilattico Sperimentale delle Venezie (Italy); the National Food Chain Safety Office (Hungary); the Norwegian Veterinary Institute (Norway); University of Bologna (Italy); the University of Copenhagen (Denmark); and the University of Zagreb (Croatia). Nine out of twelve laboratories are accredited according to EN ISO/IEC17025 (Anonymous, 2005a,), and two laboratories are also National Reference Laboratory for *L. monocytogenes* in food; the National Food Chain Safety Office in Hungary, and the IZS dell'Abruzzo e Molise in Italy. Each participant was provided with a standard operating procedure (SOP) for performance of this trial.

2.2. Trial materials

Soft cheese was selected as food model since it can support *Listeria* growth due to its chemical–physical characteristics (A_w and pH), and since it can represent a difficult matrix for the bacterial DNA extraction and real-time PCR amplification due to its high fat and protein content. Food samples were prepared at the ISS by specific personnel not to be involved in the actual trial, and sized and weighted for preparing individual samples of 25 g. Prior to use, six samples were analyzed by culture method to check the absence of *L. monocytogenes*, and all samples were found to be negative. Total aerobic counts at 30 °C and lactic acid bacteria were found by standard methods to be an average of 16.000 CFU/g; and 5.000 CFU/g, respectively.

Lyophilized bacteria were prepared by IZS Venezie and submitted to the organizing lab. Lyophilized bacteria were prepared to include 3 contamination levels: a medium level (M) that included 100 CFU and 8 CFU of *L. monocytogenes* ATCC 19111 and *Listeria innocua* ATCC 33090, respectively; a low level (L) containing 10 CFU and 2 CFU of *L. monocytogenes* ATCC 19111 and *L. innocua* ATCC 33090, respectively; and a non-*Listeria* contamination level (B) that included 100 CFU of *Escherichia coli* ATCC 25922 and *Salmonella* Agbeni CNRS 463/S03 (National Centre of Reference for *Salmonella* spp.).

The organizing laboratory provided laboratories with the foodstuffs and the lyophilized strains in ready to use, coded containers, as well as all reagents to perform the real-time PCR assay by a courier service. This means that the laboratories performing the analysis were blind to the actual content of *L. monocytogenes* in each sample.

2.3. Artificial contamination and pre-enrichment in Half Fraser Broth

The laboratories performed the artificial contamination of 24 soft cheese samples using 8 blind coded lyophilized bacterial containers of each level of contamination (M, L, and B). One sample of soft cheese not artificially contaminated and one sample of Half Fraser Broth without inoculated food were used as negative controls. After the inoculation of the bacteria to samples, a ten-fold dilution of each sample in Half Fraser Broth was performed. Samples were homogenized for 90 s, and incubated at 30 °C ± 1 °C for 24 h + 2 h. Samples were subsequently analyzed following the two methodological alternatives: traditional culture method (ISO 11290-1:1996+Amd.1:2004) and alternative method (real-time PCR-based method).

2.4. Real-time PCR-based method protocol

After the pre-enrichment step, 2 ml of the sample was taken and transferred into a new clean microcentrifuge tube, and centrifuged for 5 min at 10,000 ×*g* at 4 °C. The supernatant was carefully removed and the pellet was washed with 1 ml of PBS, and centrifuged for 5 min at 10,000 ×*g* at 4 °C. Afterwards, the pellet was re-suspended in 180 μl of enzymatic lysis buffer containing 20 mg/ml lysozyme; 20 mM Tris·HCl, pH 8.0, 2 mM EDTA, pH 8.0; and 1.2% Triton X-100, and bacterial DNA was extracted using the QIAamp DNA Mini kit (Qiagen, Hilden Germany) following the manufacturer's instructions (Rodríguez-Lázaro et al., submitted for publication).

Three microliters of DNA extract was used as template for the *L. monocytogenes*-specific real-time PCR detection assay (Rodríguez-Lázaro et al., 2004, 2005). The ring trial was performed using 96-well plates. PCR mix for 96 reactions was prepared using 1200 μl of Master mix (QuantiTec Multiplex PCR No Rox Master Mix — Qiagen, Hilden, Germany), 7.20 μl of 100 μM of each forward and reverse primers and 2.40 μl of 100 μM *hly* and IAC probes (*hly* and IAC probes were labeled with FAM and HEX, respectively). Primer sequences internal probe and IAC sequences were as previously reported (Rodriguez-Lazaro

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Table 1
Interlaboratory results of detection of *Listeria monocytogenes* by culture and qPCR method in soft cheese samples artificially contaminated with 10 CFU *L. monocytogenes* (LOW level).

Laboratory	Sample A		Sample B		Sample C		Sample D		Sample E		Sample F		Sample G		Sample H	
	ISO	qPCR	ISO	qPCR	ISO	qPCR	ISO	qPCR	ISO	qPCR	ISO	qPCR	ISO	qPCR	ISO	qPCR
1	−	−	+	+	−	−	−	+	−	+	−	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	−	+	−	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+
7	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+
8	+	+	−	−	−	−	−	+	+	+	+	+	+	+	+	+
9	+	+	−	+	−	−	+	+	−	+	+	+	+	+	+	+
10	+	+	+	+	+	+	−	+	−	+	+	+	+	+	−	+
11	+	+	+	+	−	−	−	−	+	+	+	+	+	+	+	+

et al., 2005). Two negative and positive PCR controls were prepared, adding 4.0 μl of water for molecular biology or 3.0 μl of DNA standard and 1.0 μl of water for molecular biology to 21 μl of master mix, respectively. Ninety-two microliters of the IAC (about 100 copies per reaction) were added to the remaining master mix tube. The real-time PCR mix was mixed and centrifuged briefly and 22.0 μl were aliquoted in the PCR plate. For the internal amplification control test reactions, 3.0 μl of water for molecular biology was added to each well. For the negative medium control (NMC) 3.0 μl of extract of HFB in the absence of a test sample was added to each well. For the positive PCR control/internal amplification control (IAC) 3 μl of DNA standard was added to each well. PCR 96-well-plate was spun-down, transferred into the real-time PCR platform cycler. Five different real time PCR platforms were used in the study: Applied BioSystems 7500 fast – Applied BioSystems, Foster City, USA – (two labs); Applied BioSystems 7900HT (one lab); Bio-Rad CFX96 – Bio-Rad, Hercules, USA – (three labs); Roche Light Cycler 96 – Roche Diagnostics, Mannheim, Germany – (one lab) and Stratagene Mx3005P – Agilent technologies, Santa Clara, USA – (six labs). The amplification was performed using an initial hot-start step at 95 °C for 15 min, followed by 40 cycles of a denaturation step at 95 °C for 15 s and an annealing/extension step at 63 °C for 60 s. The fluorescence was recorded only at the end of annealing/extension step. Three PCR replicates were used for each sample.

2.5. Reporting and interpretation of data

Raw data were reported by each participant to the organizing laboratory (ISS), who translated the codes and analyzed the data in collaboration with ITACyL. When an assay showed a quantification cycle (Cq) value ≤37 independently of the IAC Cq value, the result was interpreted as positive. When an assay showed a Cq value ≥37 with the IAC Cq value ≤37, the result was interpreted as negative. When an assay showed both the target and its corresponding IAC Cq values ≥37, the reaction was considered to have failed. When a participant reported that at least one of the three PCR replicate was positive, they were considered to have identified the sample as being *L. monocytogenes* contaminated. When a participant reported that the three PCR replicates were negative, but at least one replicate IAC assay was positive, they were considered to have identified the sample as being *L. monocytogenes* uncontaminated. When a participant reported that both *L. monocytogenes* and IAC assays had failed, they were considered to have reported that the analysis of that sample had failed.

2.6. Statistical analysis

The raw data sent by each laboratory were statistically analyzed according to the recommendations of Scotter et al. (2001) and by the methods of Langton et al. (2002). Accordance (repeatability of qualitative data), concordance (reproducibility of qualitative data) and the concordance odds ratio (COR) values were calculated. The first two values take into account different levels of replication in different laboratories by weighting results appropriately, and the latter evaluates (Langton et al., 2002). Confidence intervals for accordance, concordance and COR were calculated by the method of Davison and Hinckley (1997); each laboratory was considered representative of all laboratories in the "population" of laboratories, not just those participating in this analysis. In addition, other important parameters included in the ISO 16140:2033 were calculated: relative accuracy, diagnostic sensitivity, diagnostic specificity, positive and negative predictive values, false negative results and the relative specificity and sensitivity.

3. Results

3.1. Results of the laboratories in the collaborative trial

One of the laboratories was excluded from the results as it reported serious problems during the preparation of the trial materials leading to negative results from both ISO and alternative methods. Table 1 shows

Table 2
Interlaboratory results of detection of *L. monocytogenes* by culture and qPCR method in soft cheese samples artificially contaminated with 100 CFU *L. monocytogenes* (MEDIUM level).

Laboratory	Sample A		Sample B		Sample C		Sample D		Sample E		Sample F		Sample G		Sample H	
	ISO	qPCR	ISO	qPCR	ISO	qPCR	ISO	qPCR	ISO	qPCR	ISO	qPCR	ISO	qPCR	ISO	qPCR
1	−	+	−	+	+	+	−	+	−	+	−	+	−	+	−	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+
8	−	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+
9	−	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+
10	+	+	−	+	−	+	+	+	−	+	−	+	+	+	+	+
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

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Table 3
Interlaboratory results of detection of *L. monocytogenes* by culture and qPCR method in soft cheese samples artificially contaminated with 100 CFU *Escherichia coli* ATCC 25922 and *Salmonella* Agbeni CNRS 463/S03 (BLANK).

Laboratory	Sample A		Sample B		Sample C		Sample D		Sample E		Sample F		Sample G		Sample H	
	ISO	qPCR	ISO	qPCR	ISO	qPCR	ISO	qPCR	ISO	qPCR	ISO	qPCR	ISO	qPCR	ISO	qPCR
1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

the results of each participant from the analysis of soft cheese samples contaminated with a low level load of *L. monocytogenes* (10 CFU per 25 g of sample) and concomitant contamination of *L. innocua* (3 CFU per sample). At this level of contamination, 70.45% (62 out 88) of the samples were considered as positive using the reference method (ISO 11290-1:1996+Amd.1:2004) (Anonymous, 2004), whereas 87.50% (77 out of 88) were considered as positive using the real-time PCR-based method. As a result, the real-time PCR-based method detected significantly more samples positive than the reference method (22.22% more samples). Interestingly, while only two samples (2.27%) were positive by the reference method and negative by the real-time PCR-based method, 17 (19.32%) were positive by the real-time PCR-based method and negative by the reference one. All laboratories reported 6 or more samples (≥75% samples) as positive using the real-time PCR-based method, and 4 (36.36%) reported all samples as positive, whereas only 7 laboratories (63.64%) reported 6 or more samples (≥75%) as positive using the reference method and only three (27.27%) reported all as positive. Five laboratories (45.45%) reported the same results using both methods, whereas one participant (9.09%) reported all the results different.

Table 2 shows the results from the analysis of soft cheese samples artificially contaminated with a medium level load of *L. monocytogenes* (100 CFU per 25 g of sample) and concomitant contamination of *L. innocua* (8 CFU per sample). All the samples except two (97.73%; 86 out 88) were correctly reported as contaminated when the real-time PCR based method was used, whereas only 72.73% (64 out 88) of the samples were considered as positive using the ISO 11290 method. It represents 33.90% more samples correctly identified as contaminated using the real-time PCR method. While only one sample (1.14%) was positive by the reference method and negative by the real-time PCR-based method, 23 samples (26.14%) were positive by the real-time PCR-based method and negative for the ISO method. All laboratories reported 7 or more samples (≥87.5% samples) as positive using the real-time PCR-based method, and 9 (81.82%) reported all samples as positive, whereas only 7 laboratories (63.64%) reported 6 or more samples (≥75%) as positive using the reference method and only six (54.55%) reported all as positive. As for low level of contamination, five laboratories (45.45%) reported the same results using both methods, whereas one participant (1.14%) reported all the results different.

Table 3 shows the results from the analysis of the soft cheese samples not contaminated with *L. monocytogenes*. All samples were correctly reported as uncontaminated by both the reference and the alternative methods.

3.2. Statistical analysis

Table 4 shows the diagnostic specificity, diagnostic sensitivity, positive and negative predictive values, accordance and concordance values and the concordance odds ratio for the collaborative trial of both analytical methods for the detection of *L. monocytogenes* on soft cheese. Both methods showed 100% diagnostic specificity and consequently 100% accordance and concordance for samples that did not contain *L. monocytogenes*. However, some differences were observed when *L. monocytogenes* containing samples were analyzed. Although high values for diagnostic sensitivity, positive predictive values (i.e. values above 70%) were obtained, significantly better results were obtained when the real-time PCR-based method was used for the detection of *L. monocytogenes*: an overall diagnostic specificity (LOW + MEDIUM) of 92.61% vs 71.59%, or a diagnostic specificity at low level of 87.50% vs 70.45%. Similarly, better results were observed for the accordance and concordance values for the real-time PCR-based method and the

Table 4
Statistical analysis of the data obtained in the collaborative trial. Values in parentheses are the lower and upper 95% confidence intervals.

	Contamination level	Diagnostic Specificity	Diagnostic Sensitivty	Positive predictive value	Negative predictive value	Accordance (%)	Concordance (%)	Concordance odd ratio (COR)
ISO	LOW	–	70.45	–	–	72.11 (58.42, 86.02)	56.60 (47.63, 76.02)	1.98 (1.0, 4.78)
	MEDIUM	–	72.73	–	–	83.79 (69.53, 96.09)	57.80 (46.79, 84.81)	3.77 (1.3, 13.8)
	LOW + MEDIUM	–	71.59	100.00	–	78.52 (65.98, 90.7)	57.32 (47.4, 79.9)	2.72 (1.17, 6.87)
	NONE	100.0	–	–	87.12	100.00	100.00	1.00
qPCR	LOW	–	87.50	–	–	76.6 (64.62, 87.72)	76.10 (65.78, 87.18)	1.03 (0–90, 1.16)
	MEDIUM	–	97.73	–	–	96.10 (88.3, 100.00)	95.50 (87.03, 100.00)	1.20 (1.04, 1.17)
	LOW + MEDIUM	–	92.61	100.00	–	86.45 (79.19, 93.63)	86.24 (79.74, 93.44)	1.02 (0.98, 1.07)
	NONE	100.0	–	–	64.72	100.00	100.00	1.00

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Table 5
ISO 16140 evaluation parameters obtained for the qPCR-based method from the trial data. Values in parentheses are the lower and upper 95% confidence intervals.

Relative accuracy (%)	Relative specificity (%)	Relative sensitivity (%)	False negative ratio (%)	False positive ratio (%)
82.75 (78.01, 87.48)	96.70 (93.00, 100.00)	97.62 (96.00, 100.00)	1.18	0.0

concordance odd ratio value was close to 1.00 in all the levels (1.03, 1.2 and 1.02 for low, medium levels and overall, respectively) (Table 4).

Table 5 shows the values obtained in the collaborative trial for relevant parameters defined in ISO 16140 for validation of alternative methods in food microbiology: relative accuracy, relative specificity, relative sensitivity, and false negative and positive ratios. All the parameters show an excellent performance with a reduced percentage of false negative and positive results (1.18 and 0%, respectively).

4. Discussion

Recent foodborne crises have demonstrated the importance of monitoring food safety (Schoder et al., 2012). In terms of microbiological criteria, food safety requires the reliable detection of pathogens such as *L. monocytogenes* along the entire food chain by appropriate analytical methods. This study presents an international effort to validate an alternative method based on an ISO-compatible pre-enrichment coupled to bacterial DNA extraction and real-time PCR detection. The real-time PCR assay used is based on the co-amplification of a specific region of the *L. monocytogenes hly* gene and an internal amplification control (IAC); the simultaneous use in a single reaction of two differently labeled fluorescent probes makes it possible to detect the target and if negative results are obtained for the target PCR, the positive IAC signal can confirm that the negative result is not due of an inhibition during the amplification (Hoorfar et al., 2004). Importantly, the real-time PCR-based method has previously been shown to detect *L. monocytogenes* robustly down to 1 CFU of *L. monocytogenes* in 25 g of cheese samples and has also been successfully tested in naturally contaminated cheese samples (Rodríguez-Lázaro et al., submitted for publication). The results obtained in this international trial corroborate those findings, and demonstrate that the internationally validated real-time PCR method can be used for solid verification that cheese samples fulfill the microbiological criteria for *L. monocytogenes*.

In this interlaboratory trial food samples were artificially contaminated with *L. monocytogenes* and co-contaminated with *L. innocua* to mimic as much as possible the real scenario found in food samples (Ryser, 1999; Gravani, 1999). Previous studies have highlighted the possibility of an overgrowth of *L. monocytogenes* by *L. innocua*, during selective enrichment, leading to high rates of false-negative results (Keys et al., 2013; Zitz et al., 2011; Besse et al., 2010; Oravcová et al., 2008). A significant decrease in the detectability of *L. monocytogenes* could be quantified at ratios of 2:1 at very low concentration representative of natural contamination levels often found in foods and environments (Zitz et al., 2011; Besse et al., 2010; Oravcová et al., 2008). An important observation from the results of this inter-laboratory study was that the reference method was not completely reliable for the detection of *L. monocytogenes* in the presence of *L. innocua*. From a practical perspective, it implies the possibility of false negative results with serious consequences on public health since the non-compliant food lots will not be withdrawn from the market. In addition, false negative results can complicate the ability of public health investigators to trace-back the source of contamination, allowing the spread of contamination. As a result, there is a need for improving the reference method for *L. monocytogenes* detection or alternatively shift to more sensitive methods, like the molecular method used in the current study. Interestingly, the *L. monocytogenes* detection by the real-time PCR method was affected to a lower extent in the same situation, which in the current study resulted in a more reliable detection of positive samples, than the reference method. On top of that, the real-time PCR method is cost effective (3 € vs 15 €) and time saving (27 h vs 7 days) and it provided a satisfactory reproducibility when carried out by different laboratories with different real-time PCR platforms. However, false negative results were also produced by this method, and the complexity of the DNA extraction procedure could have contributed to produce these results, especially in laboratories with less experience in applying molecular methodology.

Due to the high cost of an inter-laboratory study, this trial was performed only for one food category (dairy product) among those listed in the ISO 16140. Other validation studies must be conducted for other food categories (e.g. meat and seafood products) to corroborate the satisfactory results obtained in this interlaboratory exercise in soft cheese.

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