Brazilian Journal of Microbiology
https://doi.org/10.1007/s42770-024-01353-7

FOOD SAFETY AND QUALITY - RESEARCH PAPER

Comparison of real-time PCR and nested PCR based on the *HlyA* gene for the detection of *Listeria monocytogenes*. Application on cheese samples

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Received: 2 January 2024 / Accepted: 22 April 2024

Abstract

The aim of the present study was to compare the performance of a nested polymerase chain reaction (nPCR) and a real-time PCR based on the amplification of the *HlyA* gene from *Listeria monocytogenes* using a plasmid DNA standard. Nested PCR was developed with an internal amplification control (IAC). Both techniques were validated in soft cheese samples by comparing their results with the results of the microbiological reference method ISO 11290–1:2017. Cheese samples artificially contaminated with 3.5 to 3,500 UFC/25 g were processed by ISO 11290–1:2017 and, at several times of culture, DNA samples were extracted. All cheeses contaminated with *L. monocytogenes* were positive for the microbiological method 96 h post contamination and for nPCR and real-time PCR 48 h post contamination. At this time, the *HlyA* gene was amplified in all contaminated samples. Both molecular techniques showed the same sensitivity, 30 copies/reaction or 3.5 UFC/25 g, when plasmid DNA standard or artificially contaminated cheese samples were used. Finally, eighty soft cheese samples obtained from local retail stores and tested by three methods were negative, indicating a 100% concordance in results. The development of an nPCR with IAC reinforces the reliability of the negative results without increasing the costs of the reaction. Besides, nPCR showed less sensitivity to the presence of inhibitory substances in the reaction. The use of one of these molecular techniques could be easily coupled to the microbiological method, serving as a screening method in the food industry for hygiene monitoring and early identification of contaminated foods.

Keywords Nested PCR · Real-time PCR · *Listeria monocytogenes* · Soft cheese · Food

Introduction

Listeriosis is a serious infection caused by *Listeria monocytogenes*. The main route of transmission to humans is through the consumption of contaminated food [1]. These bacteria cause food-borne disease because they have the ability to grow across a broad range of temperatures (0–45 °C) and pH (4.7–9.2), water activity above 0.92, and salt concentrations up to 14% [2]. Several outbreaks of listeriosis have been associated with the contamination of dairy products [3, 4]. The ISO 11290–1:2017 reference method for the detection of *L. monocytogenes* requires more than 6 days for final confirmation [5]. Thus, molecular techniques came to satisfy the demand for a result in a short period of time [6]. Several molecular assays that amplify virulence genes specific for *L. monocytogenes*, such as the *HlyA* gene that codifies for listeriolisin O, were developed [7, 8]. A limitation for PCR application to food-contaminating microorganisms is the presence of inhibitory substances generated from food

Responsible Editor: Luis Augusto Nero

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Published online: 30 April 2024

matrices that can lead to amplification failure [9]. Thus, the choice of a proper DNA extraction method and the use of internal amplification controls (IAC) are critical for a successful result. Commercial DNA extraction kits are time-consuming, expensive and differ in extraction efficiency, DNA purity, and DNA suitability for amplification [9, 10]. The boiling method produces a greater amount but lower purity of DNA than commercial kits [10].

The U.S. Food and Drug Administration and the Ministry of Food have established zero tolerance for *L. monocytogenes* in cooked, ready-to-eat food. Therefore, in order to ensure the detection of low numbers of viable *L. monocytogenes*, pre-enrichment procedures are necessary [11]. The combination of prior enrichment and molecular detection increased the sensitivity of the determinations [12, 13]. Real-time PCR and nested PCR (nPCR) enhance the sensitivity of the conventional PCR. Some authors have found that real-time PCR is more sensitive than nPCR [14, 15], while others differ [16, 17].

The aim of this study was to compare the performance of an nPCR and a real-time PCR based on the amplification of the *L. monocytogenes HlyA* gene by using the plasmid DNA standard, and to validate their use with artificially contaminated soft cheese samples. We combined culture enrichment with PCR and evaluated, by means of an IAC, which was the best sample to be processed by nPCR and real-time PCR. Afterwards, these methods were used for *L. monocytogenes* detection in samples of soft cheese obtained in local retail stores.

Materials and methods

Bacterial strains

Listeria monocytogenes ATCC 19115 was used as positive control. *Listeria innocua*, *Streptococcus thermophilus,* and *Lactobacillus acidophilus* were used as negative controls. *Listeria* strains were maintained on Tryptose agar, and all other strains were maintained on Nutrient Agar (Oxoid).

Soft cheese samples

Artificially contaminated cheese

A cheese sample, which tested negative for *L. monocytogenes* according to ISO 11290–1:2017, was used for artificial contamination. Fifteen aliquots of 25 g of this cheese sample were transferred to sterile plastic bags with 225 mL of half-Fraser broth (Oxoid) and inoculated in triplicate with 1 mL of serial dilutions obtained from overnight cultures of *L. monocytogenes* ATCC 19115 in phosphate-buffered saline (PBS) pH 7,2 to obtain inoculum levels of 3.5, 35, 350 and 3,500 CFU/mL. The negative control consisted of three aliquots of cheese inoculated with 1 mL of sterile PBS. The mixtures were homogenized for 2 min in a Stomacher 400 (Seward Ltd, United Kingdom) and incubated at 30ºC for 24 h. An aliquot of 0.1 mL was then transferred to 10 mL of Fraser broth (Oxoid) and incubated at 30ºC for 24 h. The cultures obtained were streaked on chromogenic *Listeria* agar (Oxoid) and incubated at 35ºC for 24 and 48 h. Samples of 1 mL of half-Fraser broth homogenate at 0 and 24 h of incubation, 1 mL of Fraser broth homogenate at 24 h of culture, and three colonies grown from chromogenic *Listeria* agar were processed for analysis by nPCR and real-time PCR.

Commercial cheese samples

Eighty soft cheese samples obtained from local retail stores were analyzed for the presence of *L. monocytogenes* according to ISO 11290–1:2017. Samples of 1 mL of Fraser broth homogenate at 24 h of culture were processed for analysis by nPCR and real-time PCR.

DNA isolation

Pure bacterial cultures

DNA extraction from *L. innocua* and *L. monocytogenes* control strains was performed by boiling 1 mL of overnight cultures at 100 °C for 12 min in the presence of PBS-0.05% Tween 20 (PBST). Cell debris was removed by centrifugation at 12,000 × g, and 1 µL of the supernatant was used in the nPCR and real-time PCR reaction. DNA extraction from *S. thermophilus* and *L. acidophilus* was performed using Wizard® Genomic DNA Purification Kit, following the manufacturer's instructions. DNA samples were stored at -20 °C.

Cheese samples

DNA extraction was carried out following the protocol previously described by Longhi et al. (2003) [18].

Molecular biology

Development of an internal amplification control (IAC) for nested PCR

An IAC was designed to evaluate the presence of PCR inhibitors. A double-stranded DNA sequence of 85 bp (tgaagttcaatgacccgggttcaaaagcttagacagatggaacggccaatgttgatcactctagaggctacgtcgctcaattcaa) was synthesized (Macrogen, Seoul). This sequence has 9-bp at the 5´-terminal region complementary to the HlyA-FI primer, and 10-bp complementary to the 3'-terminal region of the HlyA-RI primer

(Table 1). Amplification of the synthesized DNA sequence with HlyA-FI and HlyA-RI primers yields a 106 bp product. This PCR product was cloned into pGEM®-T easy vector (Promega), thus producing the pGEMT-IAC plasmids. These were transformed into One Shot TOP10F´ *Escherichia coli* competent cells (Invitrogen). Recombinant plasmids from white colonies were purified using Wizard® Plus SV Minipreps DNA Purification System (Promega) and sequenced using the SP6 and T7promoter vector primers (Macrogen). The concentration of pGEMT-IAC was determined using the Nanodrop system (Thermo Fisher Scientific Inc, USA). The copy number (copies/µL) of plasmid per milliliter was calculated using Eq. 1:

$$copy\frac{number}{\mu L} = \frac{6 \times 10^{23}\left(\frac{copies}{mol}\right) \times concentration\left(\frac{g}{\mu L}\right)}{DNA\ length(bp) \times 660\left(\frac{g}{molpb}\right)} \quad (1)$$

where DNA length is 3121 bp.

Generation of a plasmid DNA standard to compare the performance of molecular techniques

The *HlyA* gene of *L. monocytogenes* ATCC 19115 was amplified using HlyA-EF and HlyA-ER primers (Tabla 2) and cloned as described above for IAC, thus yielding plasmid DNA standard. The plasmid linearized with *Nco* I endonuclease and purified by Wizard® SV Gel and PCR Clean-Up System (Promega) was used as DNA standard. The copy number (copies/µL) was calculated using Eq. 1, where the plasmid DNA length was 3560 bp.

Listeria monocytogenes nPCR

Nested PCR for *L. monocytogenes* was based on the amplification of the conserved *HlyA* gene (GenBank accession number AF253320.1). The external primers were HlyA-EF (forward primer, nucleotide position 868–888) and HlyA-ER (reverse primer, complement of 1392–1412). The internal primers were HlyA-IF (forward primer, nucleotide position 1052–1071) and HlyA-IR (reverse primer, complement of 1285–1306) (Table 1). Two rounds of amplification were performed, the PCR1 in a final reaction volume of 50 µL and the PCR2 in 25 µL. The PCR1 amplification mix contained 10 µL of DNA sample, 100,000 IAC copies, 0.3 µM of each external primer, 1X DreamTaq reaction buffer containing 2 mM Cl_2Mg, 0.2 mM of each deoxynucleotide triphosphate, and 0.6 U of DreamTaq™ DNA polymerase (Thermo Fisher Scientific Inc). The cycling protocol comprised: preheating at 94ºC for 5 min; 20 cycles of 94ºC for 30 s, 55ºC for 30 s, and 72ºC for 35 s; with a final extension at 72ºC for 5 min. The PCR2 used 2 µL from the PCR1 as a DNA template, 0.3 µM of each internal primer, 1X DreamTaq reaction buffer containing 2 mM Cl_2Mg, 0.2 mM of each deoxynucleotide triphosphate, and 1 U of DreamTaq™ DNA polymerase. The cycling protocol comprised: preheating at 94ºC for 5 min; 35 cycles of 95ºC for 30 s, 55ºC for 30 s, and 72ºC for 20 s; with a final extension at 72ºC for 5 min. Ten microliters of the reaction products were analyzed by electrophoresis in 2% agarose gels stained with 0.015% ethidium bromide.

Table 1 Oligonucleotide used for the detection of *Listeria monocytogenes*

Target gene	Name	Oligonucleotide sequence (5'–3')	Amplicon size (bp)
Listeriolysin O (*HlyA*)	HlyA-EF	CCTGCATATATCTCAAGT GTG	545
	HlyA-ER	GGCAAATAGATGGACGAT GTG	
	HlyA-IF	CCGCAAAAGATGAAG TTCAA	255
	HlyA-IR	CCCAAGAGATGTTGAATT GAG	

Listeria monocytogenes real-time PCR

Real-time PCR tests, based on the amplification of the *HlyA* gene, were carried out in 12 µL reaction. Briefly, reaction mixtures comprised 6 µL 2X Master Mix (Biodynamic, Argentina), 1 µL of each primer (HlyA-IF and HlyA-IR, 10 µM), 1 µL of DNA sample and 3 µL of DNasa-free water. The amplification was performed with Rotor Gene Q (Qiagen, Hilden, Germany), and the analysis was performed with version 1.7 of the Rotor-Gene Q Series Software. The amplification program steps were pre-incubation at 95 °C for 3 min followed by 40 cycles: denaturalization at 95ºC for 15 s, annealing at 60ºC for 20 s, and extension at 72ºC for 20 s. Fluorescence acquisition was obtained at the end of the extension stage of each cycle. Lastly, a final extension at 72ºC for 2 min and a high-resolution melting curve, with a ramp from 72 to 90 °C at a transition rate of 0.2 ºC/s with continual monitoring of fluorescence, were performed. A no-template negative control was included in each run. The standard curve was constructed from the amplification of serial tenfold dilutions of the plasmid DNA standard ranging from 3×10^0 to 3×10^7 copies/mL. The reaction efficiency (E) was determined according to Eq. 2 [19].

$$E(\%) = \left(\left(10^{-1/slope}\right) - 1 \times 100\right) \quad (2)$$

Evaluation of cheese matrix effect on PCR performance

The inhibitor effect on nPCR and real-time PCR of the substances present in the DNA solution extracted from cheese samples was evaluated replacing 10 µL or 1 µL of water of the PCR master mix for the same volume of MES (matrix effect sample), respectively. The MES was the sample of DNA extracted from culture for 24 h in Fraser broth of the *L. monocytogenes* negative cheese.

Specificity, sensitivity, and limit of detection of nPCR and real-time PCR

Primers specificity was evaluated in silico using Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). The 545-bp and 255-bp DNA fragments (positions 868 to 1412 and 1052 to 1306 in GenBank under accession no. AF253320.1) were subjected to a homology search in FastA (https://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html). The specificity of nPCR and real-time PCR was evaluated using *L. innocua*, *S. thermophillus,* and *L. acidophilus* as negative controls.

The sensitivity of these primers to detect several strains and isolates of *L. monocytogenes* was assessed by sequence annealing using the Basic Local Alignment Search Tool (Blast, http:// blast.ncbi.nlm.nih.gov/). Analytical sensitivity was assessed by testing serial tenfold dilutions of the plasmid DNA standard (3×10^0 to 3×10^7 copies/µL) in triplicate. Serial dilutions of the plasmid DNA standard (3×10^0 to 3×10^3 copies/µL) were tested in 10 repetitions to determine the limit of detection (LOD), where the detection rate of 10 repetitions was 100%.

Repeatability and reproducibility of nPCR and real-time PCR

For intra-assay repeatability, each dilution of the plasmid DNA standard (3×10^0 to 3×10^7 copies/µL) was tested three times in one run. For inter-assay reproducibility, each dilution was tested in triplicate in three independent experiments on three different days. Moreover, the assay reproducibility was measured by amplification of gDNA from the control *L. monocytogenes* strain. Three tenfold dilutions containing 1.7×10^{-10}, 1.7×10^{-11}, and 1.7×10^{-12} g/µL of gDNA were used. The copy number of the *HlyA* gene was calculated using Eq. 1 by using the *L. monocytogenes* genome length of 2.94 Mb (geneBanck AL591824.1). One microliter of plasmid DNA standard or gDNA control or sample was used for the reaction. The coefficients of variation (CV) of the threshold cycle (Ct) and the percentage of positive replicas (%P) for intra- and inter-assay were the parameters measured for real-time PCR and nPCR, respectively. Additionally, analytical sensitivity, intra-assay repeatability, and inter-assay reproducibility were evaluated by adding MES into the mix reaction to determine the matrix cheese effect.

Results

Optimization of nPCR with IAC

Several IAC copies/reaction were evaluated during the nPCR set-up. The use of 100,000 copies for reaction allowed the amplification of plasmid DNA standards in all ranges tested (3×10^0 to 3×10^7) (Fig. 1). Of the 100,000 copies of IAC added, only 4,000 remain in the PCR2 where the specific primers are found. In this condition, 30 copies of plasmid DNA were detected, indicating that such competition is not problematic for detection. The amplified band of 106-pb of the IAC was observed in all negative controls, in the no-template control, and in every sample where there was no amplification of the *HlyA* gene.

DNA calibration curve for real-time PCR

Two standard curves of the *L. monocytogenes HlyA* gene were constructed by real-time PCR ranging from 3×10^1 to 3×10^7 copies of plasmid DNA standard/reaction since the results obtained at a concentration of 3×10^0 copies/reaction were not reproducible. Dilutions containing 5.25×10^6, 5.25×10^5, and 5.25×10^4 copies of *L. monocytogenes* gDNA/µL were evaluated. The control standard curve showed a high linearity, with a correlation coefficient (r^2) of 0.9963. The curve had a slope of -3.076 and an intercept of 33.67. The amplification efficiency (E) was 111%. The

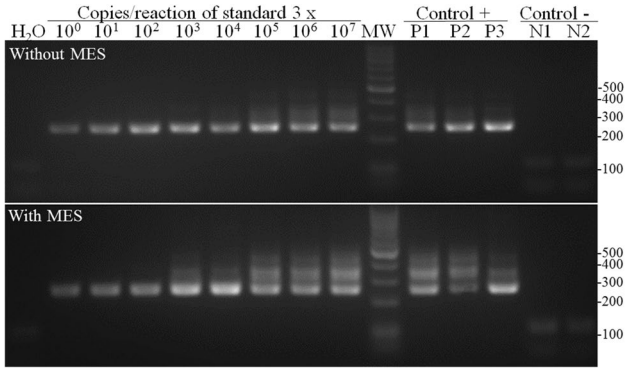

Fig. 1 Detection of *Listeria monocytogenes* DNA using serial dilutions of lineal plasmid DNA standard by nested polymerase chain reaction (nPCR) with and without the addition of matrix effect sample (MES). Lanes: H_2O; copies/reaction of plasmid DNA standard (tenfold serial dilutions from 3×10^7 to 3×10^0 copies/reaction of lineal pGEMT-*HlyA)*; MW: 100-bp DNA ladder (PB-L Productos Bio-Logicos®, Argentina); Control + (P1 to P3, tenfold serial dilution from *L. monocytogenes* ATCC 19115 genomic DNA); Control – (N1: *Listeria innocua* and N2: *Lactobacillus acidophilus*)

standard curve with added MES showed a high linearity too, with a r^2 of 0.9929, a slope of -3.147, an intercept of 34.067, and an E of 108%. The melting temperature (Tm) for the plasmid DNA standard ranged from 79.15–79.5 ºC while it was 79.25 ºC for the *L. monocytogenes* gDNA. When MES was added to the mix reaction, the Tm for the plasmid DNA standard ranged from 79.50–79.75 ºC while it was 79.50 ºC for the *L. monocytogenes* gDNA. Thus, the addition of MES to the real-time PCR did not alter the amplification of the plasmid DNA standard (Fig. 2, Table 2) or gDNA.

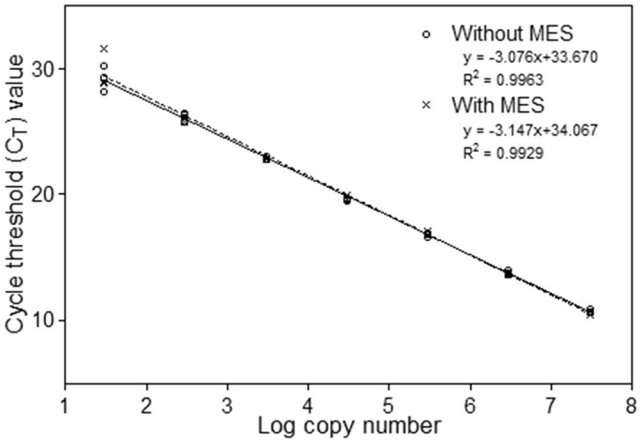

Fig. 2 Standard curves of amplification of the *Listeria monocytogenes HlyA* gene by real-time PCR with and without matrix effect sample (MES) added to the mix reaction. Ten-fold dilutions of the plasmid DNA standard were tested in triplicate

Specificity, sensitivity, and limit of detection of nPCR and real-time PCR

Specific PCR primers directed against the *HlyA* gene were designed for *L. monocytogenes* (Table 1). Only fragments of 545 bp or 255 bp from several strains and isolates of *L. monocytogenes* were amplified in silico using external (HlyA-EF and HlyA-ER) or internal primers (HlyA-IF and HlyA-IR), respectively. The search for sequence similarity using the FastA tool revealed no other identical sequences than those reported for the *HlyA* gene from *L. monocytogenes*. In addition, amplification products were not obtained using DNA from *L. innocua*, *S. thermophillus,* and *L. acidophilus*.

The search for a similitude of sequences in the database of the National Center for Biotechnology Information showed that at least the first 250 results correspond to the sequence for *L. monocytogenes* strains and isolates. The *HlyA* gene sequence identity was > 99.8% and the conservation in the region of primers annealing was 100%. The preliminary assessment for analytical sensitivity of *L. monocytogenes* real-time PCR and nPCR assay was performed with tenfold

Table 2 Repeatability (A) and reproducibility (B) of the *Listeria monocytogenes* real-time PCR and nPCR assay. The mean ± standard deviation (M ± SD) threshold cycle (Ct) value and coefficient of variation (CV) of intra- (A) and inter-assay (B)

Without MES	A) Repeatability			B) Reproducibility		
	Real-time PCR		nPCR	Real-time PCR		nPCR
Concentration (copies/µL)	M ± SD Ct value	CV (%)	%P	M ± SD Ct value	CV (%)	%P
3×10^7	10.77 ± 0.16	1.54	100% (3/3)	11.34 ± 0.92	8.02	100% (9/9)
3×10^6	13.81 ± 0.15	1.12	100% (3/3)	14.45 ± 1.12	7.76	100% (9/9)
3×10^5	16.83 ± 0.13	0.80	100% (3/3)	17.64 ± 0.99	5.60	100% (9/9)
3×10^4	19.61 ± 0.13	0.69	100% (3/3)	20.35 ± 0.78	3.83	100% (9/9)
3×10^3	22.86 ± 0.11	0.50	100% (3/3)	23.45 ± 0.71	3.02	100% (9/9)
3×10^2	26.19 ± 0.32	1.22	100% (3/3)	26.87 ± 0.91	3.40	100% (9/9)
3×10^1	29.21 ± 1.03	3.53	100% (3/3)	30.16 ± 1.29	4.27	100% (9/9)
3×10^0	ND		66% (2/3)	ND		56% (5/9)
With MES	A) Repeatability			B) Reproducibility		
	Real-time PCR		nPCR	Real-time PCR		nPCR
Concentration (copies/µL)	M ± SD Ct value	CV (%)	%P	M ± SD Ct value	CV (%)	%P
3×10^7	10.63 ± 0.16	1.5	100% (3/3)	11.51 ± 1.16	10.08	100% (9/9)
3×10^6	13.73 ± 0.06	0.44	100% (3/3)	14.62 ± 1.27	8.72	100% (9/9)
3×10^5	16.93 ± 0.09	0.56	100% (3/3)	17.74 ± 1.23	6.93	100% (9/9)
3×10^4	19.83 ± 0.20	1.03	100% (3/3)	20.71 ± 1.14	5.50	100% (9/9)
3×10^3	22.87 ± 0.03	0.15	100% (3/3)	23.83 ± 1.08	4.55	100% (9/9)
3×10^2	26.02 ± 0.22	0.84	100% (3/3)	27.02 ± 1.15	4.26	100% (9/9)
3×10^1	29.82 ± 1.53	5.14	100% (3/3)	30.45 ± 1.25	4.11	100% (9/9)
3×10^0	ND		66% (2/3)	ND		56% (5/9)

nPCR, nested polymerase chain reaction; *MES*, matrix effect sample; *%P*, percentage of positive replicas

dilutions of the plasmid DNA standard. Amplification was observed in all three replicates when the concentration of the plasmid DNA standard was ≥ 30 copies/µL. Next, we tested 10 replicates of the 3, 30, and 300 copies/µL by the both methods, and the LOD for real-time PCR and nPCR was 30 copies/µL, which was the concentration where the 10 replicates were positive. Only two or five samples were positive by real-time PCR or nPCR when 3 copies/µL were used (Fig. S1).

Repeatability and reproducibility of nPCR and real-time PCR

The repeatability and reproducibility of the real-time PCR evaluated through the determination of the CV intra- and inter-assay ranged from 0.5 to 3.53% and 3.02 to 8.02%, respectively. When MES was added to the mix reaction, the CV intra- and inter-assay ranged from 0.15 to 5.14% and 4.11 to 10.08%, respectively (Table 2). The CV inter-assay for three concentrations of genomic DNA with and without added MES ranged from 5.59 to 18.57% and from 9.06 to 12.50%, respectively.

The repeatability and reproducibility of the nPCR assay was 100% when the number of plasmid DNA standard by reaction was ≥ 30 copies, with and without the added MES (Table 2).

Evaluation of the performance of the nPCR and real-time PCR using artificially contaminated cheese samples

The analysis of the amplification of the *HlyA* gene in cheese samples artificially contaminated with 3.5 to 3,500 UFC/25 g, obtained at different times during the ISO 11290–1:2017 protocol, allowed us to establish that the sample obtained from the culture at 24 h in Fraser broth was the best sample to be processed by nPCR and real-time PCR. At this time, the *HlyA* gene was amplified in all *L. monocytogenes*-contaminated cheese samples by real-time PCR and nPCR. On the other hand, in DNA samples obtained at 0 h from half-Fraser broth, both techniques failed to amplify the *HlyA* gene. The absence of amplification of the IAC by nPCR revealed the presence of PCR inhibitors (Fig. 3). In DNA samples taken from half-Fraser broth after 24 h of incubation, the *HlyA* gene was not amplified by real-time PCR either, but the *HlyA* gene amplification was observed in all samples with the exception of one of the samples contaminated with 3.5 UFC by nPCR. The IAC was amplified in the sample negative for the *HlyA* gene, which indicated that the lack of amplification was not due to PCR inhibition. The *HlyA* gene was amplified by both techniques in all colonies grown from artificially contaminated samples in chromogenic *Listeria* agar (Fig. 3).

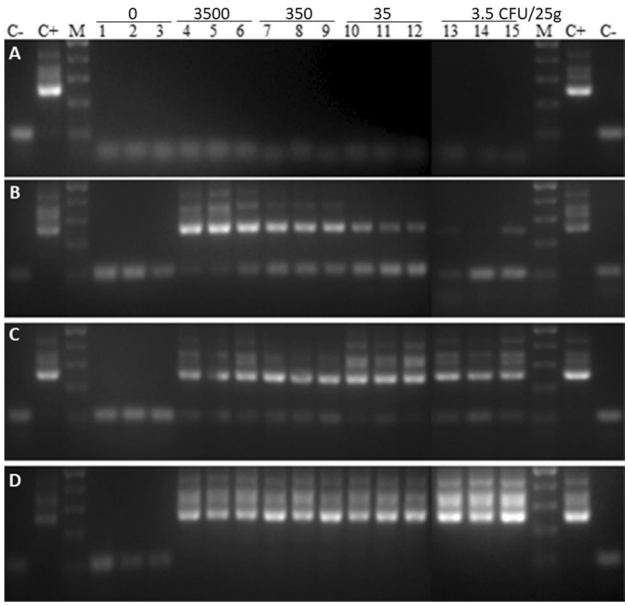

Fig. 3 Detection of the *Listeria monocytoges HlyA* gene in artificially contaminated cheese by nPCR. DNA samples from 1 mL of half-Fraser broth at 0 (**A**) and 24 h of incubation (**B**), 1 mL of Fraser broth at 24 h of culture (**C**), and three colonies grown from chromogenic *Listeria* agar (**D**) were processed. Lanes 1–3: DNA extracted from cheese samples without *L. monocytogenes* contamination; 4–6, 7–9, 10–12 and 13–15: DNA extracted from artificially contaminated cheese samples with 3,500, 350, 35, and 3.5 CFU, respectively; lane C-, *L. innocua* negative control; lane C+, *L. monocytogenes* ATCC 19115 positive control; line M: 100-bp DNA ladder (PB-L Productos Bio-Logicos®, Argentina)

Detection of *Listeria monocytogenes* in commercial soft cheese samples

Eighty soft cheese samples obtained from local retail stores were negative for *L. monocytogenes* by microbiological and molecular methods. Amplification of IAC was observed in all samples by nPCR.

Discussion

The aim of this study was to develop an nPCR and a real-time PCR for *L. monocytogenes* detection, compare their performance, and validate their application in soft cheese samples. Real-time PCR and nPCR are two highly sensitive molecular techniques and are used as a complement for microbiological methods for the detection of food-borne pathogens [20]. While many research works used a real-time PCR for the detection of *L. monocytogenes* in food [10, 21, 22], a few papers described the use of nPCR [8, 23]. It is clear that the main advantages of real-time PCR over nPCR lie in the fact that it is quantitative and the results are obtained in less time, while the main disadvantage consists

of the cost of reagents and equipment. Regarding the sensitivity of these techniques, some works recognize real-time PCR as the most sensitive technique [14, 15] and others the nPCR [16, 17]. Multiple factors, such as the selection of the gene to be amplified, the primers used, the nature of the sample, and the DNA extraction method, influence the results of molecular techniques [24]. In this work, nPCR and real-time PCR developed using the same primers (internal primers of nPCR were used for real-time PCR) had the same sensitivity (LOD, 30 plasmid DNA standard copies/reaction).

As mentioned above, the extraction and purification of DNA from complex food matrices is another key point to take into account. Montoya et al. (2010) show that the agreement between nPCR and real-time PCR depended on the extraction methods and that the real-time PCR results were more dependent on the extraction method than the nPCR results. In this work, DNA extraction was carried out by boiling method because, despite the purity of the obtained DNA being lower than that obtained with a commercial kit [10], it is the simplest and most economical method and it is available to all laboratories. In this condition, real-time PCR was also more sensitive to the composition of the sample than nPCR since there was no amplification by real-time PCR, but there was amplification by nPCR in the samples obtained 24 h after contamination. Therefore, the nPCR would be able to detect positive samples in less time than real-time PCR. This could be attributed to the two rounds of PCR that allow the dilution of PCR inhibitory substances. For this reason, the use of nPCR could be more versatile to be applied to samples with complex matrices, such as cheeses, than real-time PCR. In this work, the sample of choice for the comparison of the techniques was the one obtained after 48 h of culture, a sample in which the *HlyA* gene is amplified by both techniques. We did not evaluate the use of commercial extraction kits, but they could allow obtaining results in less time.

Heo et al. (2022) found differences in the LOD of the real-time PCR for *L. monocytogenes* detection using several commercial DNA extraction kits and, in addition, differences using the same kit with different types of artificially contaminated food samples were found, thus evidencing the complexity of food matrices. In this study, the main parameters of the nPCR and the real-time PCR evaluated using plasmid DNA standard were not modified when MES was added to the master mix PCR to validate their use on cheese samples obtained 48 h post contamination (Figs. 1 and 2, Table 2). The CV of intra- and inter-assay were satisfactorily low with (less than 6% and 11%, respectively) and without the MES addition (less than 5% and 10%, respectively) (Table 2).

Additionally, nPCR was developed with the use of an IAC. The presence of an IAC signal in all negative results of artificially contaminated cheese demonstrates that each of these negative results is a true negative and not the result of PCR inhibition, reinforcing the value of the diagnostics (Hoorfar et al., 2003). In addition, the incorporation of an IAC does not make the nPCR reaction more expensive, which does occur in the real-time PCR since reagents (probes) and equipment are necessarily more expensive.

The contaminated cheese samples were analyzed following ISO 11290–1:2017. This requires more than 96 h to make an accurate identification of *L. monocytogenes*. In this work, *L. monocytogenes* could be detected after 24 h or 48 h of microbiological culture in specific media using nPCR or real-time PCR, respectively. When the *L. monocytogenes* detection was analyzed in DNA samples from 48 h culture, the sensitivity of nPCR and real-time PCR was the same (3.5 UFC/25 g). Similar results had been reported by other authors using different incubation times of prior enrichment and DNA extraction methods in dairy products [23, 25, 26]. Ready-to-eat foods containing less than 10^2 CFU/g are estimated to pose little risk, whereas foods linked to outbreaks have been found to contain 10^3 CFU/g [27]. The detection limit of 3.5 UFC/25 g detected in this study is below the minimal infectious dose [28].

Finally, we processed 80 soft cheese samples by ISO 11290–1:2017 method and molecular techniques. The concordance between the microbiological method and the two molecular methods was 100% due to all samples being negatives. These results reinforce the specificity of nPCR and real-time PCR, since no false positive results were obtained. In addition, molecular methodologies could complement the results of the microbiological method, thus providing results in less time [29]. Furthermore, another advantage shown by molecular methodologies is the identification of DNA from viable but non-culturable (VNC) *L. monocytogenes* [30], hence reducing the possibility of false negatives in ISO standard methodology.

Although one of the advantages attributed to real-time PCR over nPCR is the greater specificity [29], in our work no loss of specificity was evident with the use of nPCR. The no detection of contaminated cheeses with *L. monocytogenes* is in agreement with previous reports made by other authors from South America [31–33].

In conclusion, we developed and compared the nPCR and real-time PCR for the detection of the *HlyA* gene from *L. monocytogenes*. Moreover, we validated them for their use in soft cheese samples. Both techniques showed the same performance (LOD: 30 plasmid DNA copies/reaction and 3.5 UFC/25 g of cheese), but nPCR was less sensitive to the inhibitors present in the sample, being able to reduce the times needed to obtain results. Likewise, nPCR is cheaper than real-time PCR and only requires a conventional thermalcycler, which makes it an easier technique to apply in food microbiology laboratories. The incorporation of the IAC in the nPCR does not make the test more expensive and has a very important role in the reliability of the negative

result. The combination of prior enrichment and one of these molecular techniques could serve as a screening method that provides support for the microbiological method. Besides, the procedure employs the same enrichment medium as that used for ISO 11290–1, hence facilitating its integration into routine laboratory diagnostics.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42770-024-01353-7.

Author Contributions MVZ participated in conceptualization, investigation, formal analysis, methodology, validation, visualization, writing – review & editing. NM and CC participated in conceptualization, methodology, writing – review & editing. MLS participated in conceptualization, writing – review & editing. MEP participated in conceptualization, project administration, supervision, investigation, formal analysis, methodology, validation, visualization, writing – original draft, writing – review & editing.

Funding This project has received funding from National University of Rafaela (UNRAf), INTA, and Asociación Cooperadora INTA Rafaela (TCP #426100).

Availability of data and material The data and material are available.

Code availability Not applicable.

Declarations

Competing Interests The authors have no conflicts of interest associated with this research work.

Ethics statement This work does not contain studies with human participants or live animals, or hazardous or sensitive material.

Consent to participate Not applicable.

Consent for publication Not applicable.

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