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Rapid Same-Day Detection of *Listeria monocytogenes*, *Salmonella* spp., and *Escherichia coli* O157 by Colorimetric LAMP in Dairy Products

Sarah Azinheiro[1,2] · Foteini Roumani[1,2] · Marta Prado[1] · Alejandro Garrido-Maestu[1]

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Abstract
Foodborne illnesses are being reported everyday; thus, there is an obvious need for faster and sensitive methodologies to detect foodborne pathogens in order to assure the safeness of foods. In the present study, the detection of *L. monocytogenes*, *Salmonella* spp., and *E. coli* O157 was performed combining a multiplex short enrichment of 7 h in Tryptic Soy Broth, with a colorimetric LAMP-based naked-eye detection. The LAMP reaction obtained a similar sensitivity to that of real-time PCR. The methodology was evaluated in UHT, fresh and raw milk were tested, achieving a LoD95 of 1.6 CFU/25 mL for *Salmonella* spp. and *E. coli* O157 respectively without matrix interference, and for *L. monocytogenes*, the LoD95 was calculated to be 79.0 CFU/25 mL, showing some interference when a higher natural microflora was present in the sample. The methodology can be applied in the food industry with reliability, as the evaluation obtained a *k* index of 1.0 for *L. monocytogenes* and *Salmonella* spp. and 0.94 for *E. coli* O157.

Keywords Rapid methods · Loop-mediated isothermal amplification · Naked-eye detection · Colorimetric · Pathogens · Dairy

Introduction

With the globalization in the food industry, the production systems have been intensified and the products reach the consumer even faster. This also led to less time to ensure the safety of the food and easier spread of the contamination between countries. With 5175 foodborne outbreaks reported by the EFSA and ECDC in 2019, the food supply chain continues to need improvements and more rapid analysis in order to keep up with the intense production existing nowadays (EFSA & ECDC 2021). *Salmonella* spp. and Shiga toxin-producing *Escherichia coli* (STEC) are among the top three reported causes of hospitalization associated with food, and these numbers have not decrease over the last 5 years for the first, and even showed an increase for the second. On the other hand, *Listeria monocytogenes* presents a lower incidence, but has the highest mortality rate from all zoonoses monitored.

The standard methodologies are based on culture techniques which need from 48 h to 1 week to obtain a final result depending on the pathogen to be detected. Despite the effort to improve the analysis, these ones have been focused mostly in the detection part, where DNA-based analysis such as PCR/real-time PCR (qPCR) was developed and validated for their application in food analysis (ISO 2006, 2011). Isothermal amplification techniques appear as a promising alternative, as they allow to reduce the cost in terms of equipment needed, and result simpler due to naked-eye result interpretation. Loop-mediated isothermal amplification (LAMP) is the most popular isothermal technique, and assays have been developed for different applications, from clinical to food authenticity and safety (Niessen et al. 2013; Lakshmi and Kim 2021; Moehling et al. 2021). In addition to this, nowadays, several commercial master mixes are available which allow to visualize the results by the presence of turbidity or a color change (Azinheiro et al. 2018; Pang et al. 2019; Shi et al. 2021).

Before the detection, a crucial step must to be perform, the enrichment, in order to have enough bacteria to be

✉ Alejandro Garrido-Maestu
alejandro.garrido@inl.int

[1] Food Quality and Safety Research Group, International Iberian Nanotechnology Laboratory, Av. Mestre José Veiga s/n, 4715-330 Braga, Portugal

[2] Department of Analytical Chemistry, Faculty of Veterinary Science, University of Santiago de Compostela, 27002 Lugo, Spain

detected. Most of the optimization strategies implemented in the ISO standard are focused in different general and selective media, to allow a better recovery of the targeted microorganism (Rohde et al. 2017); however, no significant changes have been able to significantly reduce the time of analysis. In addition to this, the enrichment step tends to be specific for the targeted pathogen and it is not conceived for the growth of different types of pathogens simultaneously. Alternative methodologies have been studied to overcome this step, and a short enrichment combined with a treatment of the sample, to eliminate potential interfering compounds, seemed like a promising approach. Fachmann et al. described such an approach targeting *Salmonella* spp. in meat products (Fachmann et al. 2017), and later, Garrido-Maestu et al. adapted the method for the detection of *E. coli* O157 in simplex and even in multiplex to also detect *Salmonella* spp. (Garrido-Maestu et al. 2020b, a). The detection Gram-positive bacteria was likewise described targeting *L. monocytogenes* in a simplex format (Azinheiro et al. 2022) demonstrating its applicability of the methodology for the detection of different types of microorganisms.

The present study aimed to develop a new methodology shortening the enrichment step for the growth of three foodborne pathogens, namely *Salmonella* spp., *E. coli* O157:H7, and *L. monocytogenes*, in multiplex, along with a naked-eye colorimetric LAMP in order to simplify the result visualization.

Materials and Methods

Bacterial Culture Preparation

Reference strains of *Listeria monocytogenes* (WDCM 00,021), *Salmonella* Typhimurium (WDCM 00,031), and *Escherichia coli* O157:H7 (WDCM 00,014) were obtained from the Spanish Type Culture Collection (CECT) to evaluate the novel, rapid method and to be used in spiking experiments. In addition to these, a panel of target and non-targeted bacteria was selected to evaluate the inclusivity and exclusivity of the LAMP assay (see Table 1). For these experiments, as well as for the spiking ones, fresh cultures were prepared by resuspending a colony in 4 mL of Nutrient Broth (NB, Biokar diagnostics S.A., France) and incubated overnight (ON) at 37 °C.

Enrichment

In order to obtain a sensitive detection of the three pathogens targeted, their growth in a same enrichment medium was optimized, evaluating the performance of different non-selective and selective broths for this purpose, as well as the most appropriate time of incubation. *L. monocytogenes* presents a slower growth rate compared to the other bacteria selected, and for this reason, the enrichment media were chosen according to its ability to increase the concentration of this bacterium.

Media Optimization

Tryptic Soy Broth (TSB, Biokar diagnostics S.A., France) was the general medium chosen, as *L. monocytogenes* demonstrated to grow faster in this broth, allowing a detection in 5 h (Azinheiro et al. 2022). Different conditions were tested in this study in order to improve the sensitivity of the methodology. Two times of enrichment, 6 h or 7 h, in TSB were tested, and in an attempt to overcome the influence of other bacteria growths during the enrichment, the combination with a selective enrichment was analyzed. For this, after a pre-enrichment of 3 h in TSB, an aliquot of 100 µL, or 1 mL, was transferred to Full Fraser (FF, Biokar Diagnostics S.A., France) for a selective enrichment of 3 h, and a mixture of these two media was analyzed. The

Table 1 Specificity of colorimetric LAMP for the different targets

Bacterium	Source	*N*	*plcA**	*rfbE**	*invA**
L. monocytogenes	WDCM 00,021, mollusk, chestnut, chicken	17	+	–	–
L. seeligeri	CECT 917	1	–	–	–
L. innocua	WDCM 00,017, CECT 5376, 4030; CUP 1141, 1325, 2110	6	–	–	–
C. coli	UM	1	–	–	–
S. aureus	WDCM 00,033, WDCM 00,034	2	–	–	–
Y. enterocolitica	WDCM 00,038	1	–	–	–
E. coli	WDCM 00,013, AMC 73, 75,81, 171, 178, 278	7	–	–	–
E. coli O157:H7	WDCM 00,014, AMC 76	2	–	+	–
Salmonella spp.	(AMC 28, 60, 82, 84, 90, 96, 198. 200, 238, 253, 255, 260, 261, UB, WDCM 00,031)	15	–	–	+

N, number of strains; *WDCM*, World Data Centre for Microorganisms reference; *CUP*, Catholic University of Porto; *UM*, University of Minho; *UB*, University of Bristol. *The *plcA* was selected for the detection of *L. monocytogenes*, the *rfbE* for *E. coli* O157, and the *invA* for *Salmonella* spp

enrichment of 7 h also combining these two media was tested, analyzing only the FF portion to reduce the concentration of other bacteria.

Short Enrichment Protocol

For the enrichment of the three bacteria in multiplex, the protocol described by Azinheiro et al. was followed (Azinheiro et al. 2022). To this end, 25 mL of sample was weighted in a stomacher bag with filter (< 250 µm) and mixed with 25 mL of pre-enrichment broth, TSB, pre-warmed at 37 °C. The matrix was homogenized for 30 s in a Stomacher 400 Circulator (Seward Limited, UK) and incubated at 37 °C for 7 h with a constant agitation at 200 rpm. After incubation, the whole liquid was recovered and transferred to a conical 50-mL tube. The tube was centrifuged at 8960 × g for 10 min, the supernatant was discarded, and the pellet was resuspended in 45 mL of protease buffer (1/100 dilution in PBS of Alcalase and Neutrase (Sigma-Aldrich, USA), and incubated horizontally at 37 °C for 10 min at 200 rpm. After digestion, the samples were centrifuged again under the same conditions. Once more, the supernatant was discarded, and the pellet was resuspended in PBS with 0.35% of a surfactant mixture with a hydrophilic/lipophilic balance equivalent to Lutensol AO-07 (surfactant previously applied by Maryl et al. (Mayrl et al. 2009)), followed by a new centrifugation step. Finally, the new pellet was resuspended in 1.5 mL of washing buffer, transferred to a clean tube, and centrifuged for 5 min at 11,000 × g. An additional washing step with 1 mL of PBS was performed, and the resulting pellet with the clean bacteria was used for downstream DNA extraction.

DNA Extraction

Pure Cultures

To obtain the DNA from each microorganism to be used for the inclusivity and exclusivity of the LAMP reaction, a simple thermal lysis protocol was applied. Briefly, 1 mL of an ON pure culture was centrifuged for 5 min at 16,000 × g, and the pellet was washed with 1 mL of TE 1X (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) and centrifuged again under the same conditions. The supernatant was discarded and the pellet resuspended in 300 µL of TE 1X. The aliquots were incubated at 99 °C for 15 min with constant agitation at 1400 rpm, in a dry bath (Thermomixer comfort, Eppendorf AG, Germany). A final centrifugation was performed at 16,000 × g for 5 min at 4 °C to separate the DNA from cellular debris.

Food Samples

The DNA extraction from food samples spiked with the different microorganisms was performed based on the Lysis-GuSCN method described by Kawasaki et al. and modified by Garrido et al. (Garrido et al. 2013). The bacterial pellets were resuspended in 200 µL of an enzymatic solution containing 1 mg/mL of achromopeptidase and 20 mg/mL of lysozyme (Sigma-Aldrich, St. Louis, USA) in TE 2X with 1.2% of Triton X-100. The enzymatic lysis was performed for 30 min at 37 °C, with a constant agitation of 1000 rpm. After the incubation, 300 µL of a solution containing 4 M of guanidine isothiocyanate and 1% of Tween 20 were added, and 400 µL of this solution was transferred to 400 µL of 100% isopropanol, and the mixture was centrifuged for 10 min at 16,000 × g. The pellet was rinsed with 1 mL of 75% isopropanol, resuspended in 160 µL of sterile Milli-Q water, and incubated at 70 °C, for 3 min. Finally, a 5-min centrifugation at 16,000 × g and 4 °C was performed to separate the DNA from any remaining debris prior to the analysis. All reagents were purchased from Sigma-Aldrich (USA).

Colorimetric LAMP

The LAMP reaction was first optimized to amplify three different genetic targets, *rfbE*, *invA*, and *plcA*, in order to detect, in parallel reactions, *E. coli* O157, *Salmonella* spp., and *L. monocytogenes*, respectively. The *plcA* and *invA* primers were previously designed and evaluated for real-time fluorescence, and turbidity, and were now incorporated in this new approach. For *E. coli* O157, a new set of primers were specifically designed targeting the *rfbE* gene using PrimerExplorer V5 (https://primerexplorer.jp/e/index.html). A detailed list of the LAMP primers selected is provided in Table 2. The LAMP assay was performed in a final reaction volume of 25 µL, composed of 15 µL of WarmStart® Colorimetric LAMP 2X Master Mix (New England BioLabs, NEB), 40 mM of guanidine hydrochloride (GuHCl, Sigma-Aldrich USA), and 3 µL of template DNA for the reaction targeting *rfbE* and *invA* and 6 µL for *plcA*. The standard primer concentration recommended by the supplier was used, corresponding to 1600 nM FIB/BIP, 200 nM F3/B3, and 400 nM loop primers for *rfbE* and *invA*. For the amplification of the *plcA* gene, the loop primer concentration was increased to 600 nM. The amplification was performed at 65 °C, over 30 min for *E. coli* O157 and *Salmonella* spp. and 1 h for *L. monocytogenes.*

Confirmation Methodologies

The results obtained by LAMP were compared with reference methodologies, in order to evaluate the performance of the developed method. A culture-based analysis was applied

Table 2 Primers used in LAMP reactions

Target microorganism	Target gene	Primer	Sequence 5′-3′	References
L. monocytogenes	*plcA*	plcA-FIP	GCA GCG CTC TCT ATA CCA GGT ACA *ttt*AAT GTC CAT GTT ATG TCT CCG TTA	Garrido-Maestu et al. (2018a)
		plcA-BIP	AGG TTT GTT GTG TCA GGT AGA GCG *ttt*CGC TTA ATA ACT GGA ATA AGC CAA	
		plcA-F3	TGT GTT TGA GCT AGT GGT TTG G	
		plcA-B3	CCC ATT AGG CGG AAA AGC ATA T	
		plcA-LB	CAT CCA TTG TTT TGT AGT TAC AGA G	
Salmonella spp.	*invA*	HK-FIP	GAC GAC TGG TAC TGA TCG ATA GTT TTT CAA CGT TTC CTG CGG	Hara-Kudo et al. (2005)
		HK-BIP	CCG GTG AAA TTA TCG CCA CAC AAA ACC CAC CGC CAG G	
		HK-F3	GGC GAT ATT GGT GTT TAT GGG G	
		HK-B3	AAC GAT AAA CTG GAC CAC GG	
		HK-LF	GAC GAA AGA GCG TGG TAA TTA AC	
		HK-LB	GGG CAA TTC GTT ATT GGC GAT AG	
E. coli O157	*rfbE*	rfbE-FIP	TGC CAA TAT TGC CTA TGT ACA GCT A *tttt* GAC AAA ACA CTT TAT GAC CGT TG	This study
		rfbE-BIP	GGA TGA CAA ATA TCT GCG CTG CTA T *ttt*TCA GCA ATT TCA CGT TTT CGT	
		rfbE-F3	GGT GGA ATG GTT GTC ACG AA	
		rfbE-B3	GTG GAC TTG TAC AAG ACT GTT GAT	
		rfbE-LB	AGG ATT AGC CCA GTT AGA ACA AGC	

After optimization, for the detection of *Salmonella* spp. the HK-LB was excluded from the reaction due to non-specific amplification. All the other primers were included in the final methodology. "*tttt*" is a linker between F2 and F1c and B2 and B1c which form the FIP and BIP primers respectively

after enrichment, as well the analysis of the DNA extracts by qPCR, the gold-standard amplification technique.

Culture-Based Methodology

To confirm the presence of the three pathogens after enrichment, a loopful, approximately 10 µL, was plated on COMPASS *Listeria* (Biokar diagnostics S.A., France), CHROMagar™ *E. coli* O157, and CHROMagar™ *Salmonella* Plus (CHROMagar, France) for the differentiation of *L. monocytogenes*, *E. coli* O157, and *Salmonella* spp., respectively. The plates were then incubated at 37 °C ON and screened for typical colonies.

qPCR

Alternative to the amplification with the colorimetric LAMP, a multiplex qPCR was performed targeting *rfbE*, *ttr*, and *hly* for the detection of *E. coli* O157, *Salmonella* spp., and *L. monocytogenes*, being the primers already described and evaluated in previous studies (Garrido-Maestu et al. 2018b; Azinheiro et al. 2020b, a). A non-competitive internal amplification control (NC-IAC) was also included in the reaction as previously described to confirm the absence of amplification inhibition (Garrido-Maestu et al. 2019; Azinheiro et al. 2020a). In a final volume of 20 µL, the sets of primers at the final concentrations detailed in Table 3 were mixed with 10 µL TaqMan™ Fast Advanced Master Mix (Applied Biosystems™, Foster City, CA, USA). The thermal profile consisted on an UDG treatment at 50 °C for 2 min, followed by a hot-start activation at 95 °C for 2 min, and 40 cycles of 95 °C for 1 s and 63 °C for 20 s. The qPCR reaction was performed in a QuantStudio 5 Real-Time PCR System (Applied Biosystems™, USA) and results were analyzed using respective software QuantStudio™ Design & Analysis version 1.5.1.

Evaluation

Colorimetric LAMP Evaluation

The colorimetric LAMP was evaluated in terms of its dynamic range and its inclusivity/exclusivity. For the dynamic range, the lowest DNA concentration detectable was determined for the three targets performing ten-fold serial dilutions of a pure DNA extract obtained as described in the "Pure Cultures" section. The DNA concentration was quantified using Qubit™ 1X dsDNA Assay Kits, high sensitivity (HS) and respective equipment (Invitrogen™, USA).

To evaluate the inclusivity and exclusivity of the colorimetric LAMP, the different bacterial species and strains

Table 3 Primer and probes used in qPCR reactions

Target microorganism	Primer	Sequence 5′-3′	Concentration
L. monocytogenes	*hly* F	GCA ACA AAC TGA AGC AAA GGA T	200 nM
	hly R	CGA TTG GCG TCT TAG GAC TTG C	
	hly P	FAM-CAT GGC ACC-ZEN-ACC AGC ATC TCC G-IABkFQ	150 nM
Salmonella spp.	*ttr* F	GGC TAA TTT AAC CCG TCG TCA G	200 nM
	ttr R	GTT TCG CCA CAT CAC GGT AGC	
	ttr P	NED-AAG TCG GTC TCG CCG TCG GTG-MGBNFQ	150 nM
E. coli O157	*rfbE* F	TCA ACA GTC TTG TAC AAG TCC AC	200 nM
	rfbE R	ACT GGC CTT GTT TCG ATG AG	
	rfbE P	FAM-ACT AGG ACC-ZEN-GCA GAG GAA AGA GAG GAA-IABkFQ	150 nM
NC-IAC	NC-IAC F	TTA AGA CTT GCT TTG CCA GAG AC	100 nM
	NC-IAC R	GGT GGT GGA AAT TCG AAT GAG C	
	IAC P	YY-AGT GGC GGT -ZEN- GAC ACT GTT GAC CT- IABkFQ	

NC-IAC, non-competitive internal amplification control

detailed in Table 1 were tested. Both analyses were also performed by qPCR in order to compare the results obtained.

Full Methodology Evaluation

The methodology developed was evaluated in different types of milk, including UHT, fresh, and raw milk. The evaluation process consisted on the determination of the limit of detection (LoD) and then the analysis of the fitness for purpose.

LoD To determine the LoD in each type of milk, samples were inoculated with decreasing concentrations until reaching negative results. The LoD_{50} and LoD_{95} were calculated using PODLOD calculation program version 9, developed by Wilrich and Wilrich (Wilrich and Wilrich 2009), which also allowed to predict the probability of detection (POD). Extra, non-inoculated samples were also analyzed to assure absence of the pathogens in the original matrix.

Fit for purpose To assess if the developed protocol was suitable to be used in the food industry, the following parameters were calculated having into account the samples above the LoD_{95} and following the NordVal International Protocol for the validation of alternative methods (NordVal 2017). The samples analyzed were classified as positive and negative agreement (PA/NA) and positive and negative deviation (PD/ND) comparing the result obtained after analysis, against the reference qPCR methodology. Samples are considered PA and NA when both methodologies obtained the same result. The PD reports a negative result by the alternative methodology when the reference obtained a positive result, while the ND state for the opposite, a positive result by the alternative and negative by the reference In addition to this, the results were confirmed by plating the TSB enrichment on selective media, being the ND considered false negative (FN) when the reference method was confirmed as positive, and PD becomes true positive (TP) when the alternative method is confirmed positive, and false positive (FP) when the confirmation was negative. The interpretation of the results is summarized in Table S1. Using these data, the relative sensitivity, specificity, accuracy (SE/SP/AC), and the Cohen's kappa (k) were calculated following the formulae detailed in the NordVal protocol.

Results

Colorimetric LAMP Optimization

For the detection of *L. monocytogenes* and *E. coli* O157, only one loop primer, LB, was designed and used in the reaction not affecting the specificity of the assay. Regarding the detection of *Salmonella* spp., non-specific amplification was observed using both loop primers previously described, presenting false-positive results in negative non-spiked milk samples. For this reason, the loop primers to be use in the colorimetric LAMP reaction were optimized, testing them separately. Figure 1A shows the results obtained after different incubation times, from what can be observed that the LB primer is the one causing the false positives after 30 min of incubation. It was also observed that the LF primer also generated unspecific amplification after 40 min of incubation. Attending to these results, it was decided to only use the LF primer. The time of amplification was also evaluated for the different targets. The detection of both *E. coli* O157 and *Salmonella* spp. was possible within 30 min of incubation without presenting unspecific amplification (Fig. 1B and C). However for *L. monocytogenes*, a longer amplification time was needed. Figure 2 depicts the optimized reactions.

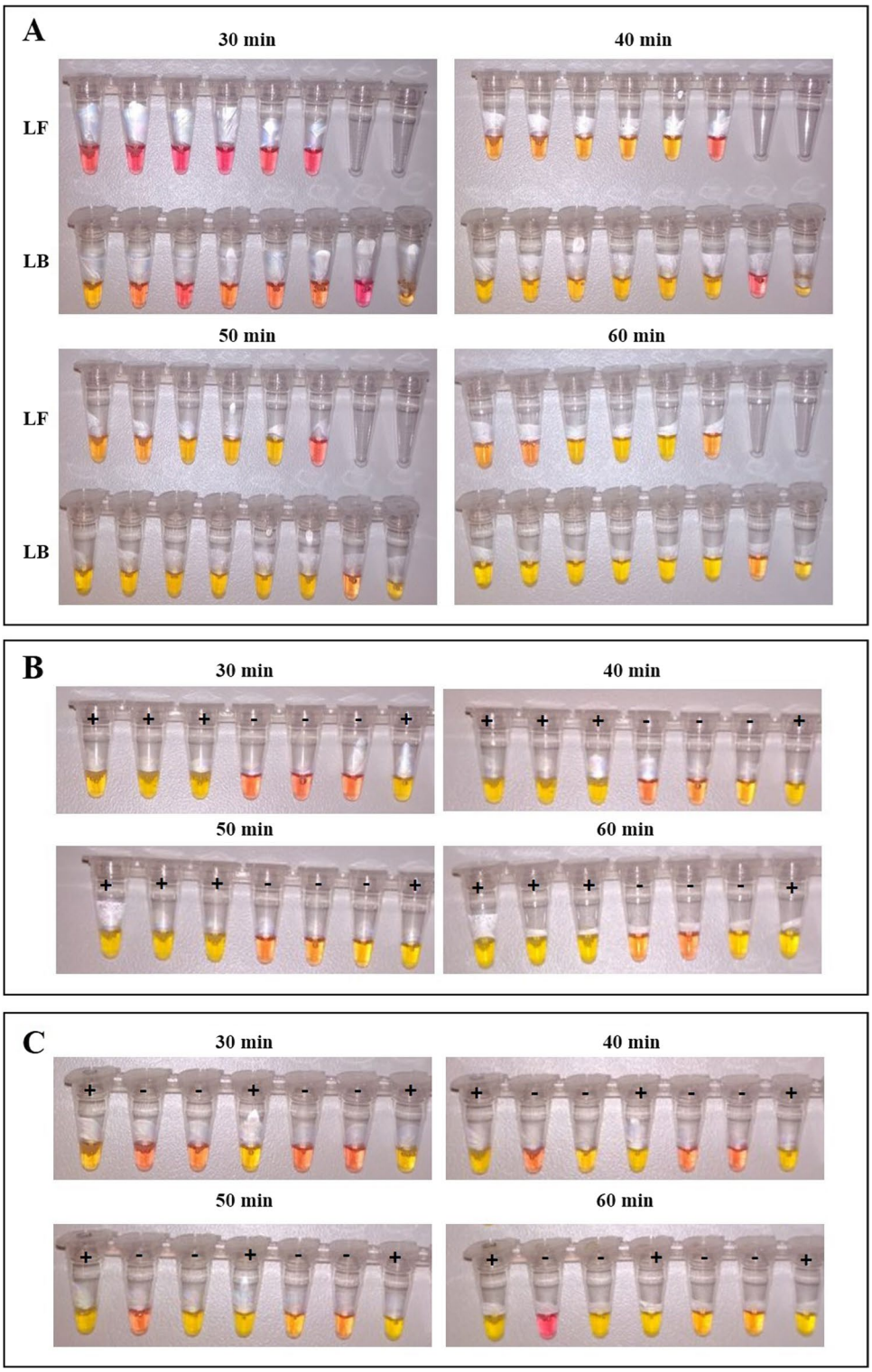

Fig. 1 Optimization of the colorimetric LAMP for the detection of *Salmonella* spp. and *E. coli* O157. **A** Comparison between the use of LB or LF loop primers of *invA* gene when negative samples were tested. **B**, **C** Optimization of the time of amplification targeting *invA* using LF primer and *rfbE*, respectively, with both negative (−) and positive samples (+). The positive results are yellow and the negatives pink

Colorimetric LAMP Evaluation

With the colorimetric LAMP optimized, the assay was evaluated in order to evaluate the dynamic range, and verify the inclusivity and exclusivity.

Dynamic Range

The lowest concentration of DNA from each target was assessed by analyzing ten-fold serial dilutions of DNA extracts from the obtained pure cultures (see Fig. 3). Similar results

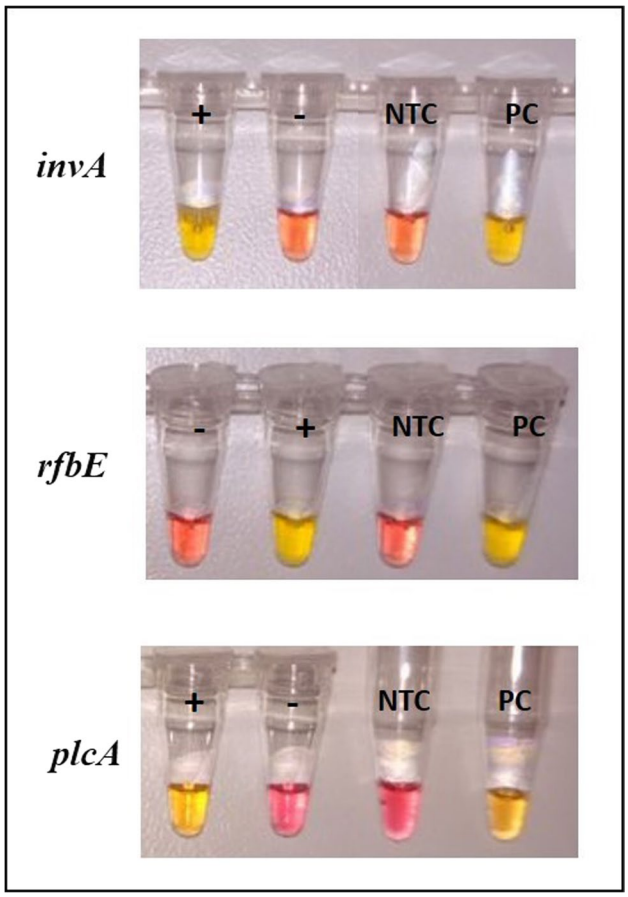

Fig. 2 Optimized colorimetric LAMP reaction targeting *invA*, *rfbE*, and *plcA* for the detection of *Salmonella* spp., *E. coli* O157, and *L. monocytogenes.* NTC, non-template control (water); PC, positive control

were obtained for *E. coli* O157 and *Salmonella* spp., with a dynamic range covering 5 orders of magnitude dilutions reaching 14.8 pg/µL and 19.3 pg/µL, respectively. Regarding *L. monocytogenes*, the dynamic range covered 4 serial dilutions, considering that the initial DNA concentration was 10 times lower than for the other two bacteria; a similar sensitivity was reached, 22.0 pg/µL. The same concentrations were also tested by qPCR, and the results are presented in Fig. 4, showing comparable results to those of colorimetric LAMP.

Inclusivity and Exclusivity

To evaluate the inclusivity/exclusivity of the colorimetric LAMP with the different set of primers, 52 bacteria which included different species and strains were tested. This panel of bacteria included 15 *Salmonella* spp., 17 *L. monocytogenes*, and 2 *E. coli* O157:H7; and to evaluate the exclusivity, another 14 related strains and species were tested, such as non-O157 *E. coli* from different sources (clinical, mussels, and water) and other *Listeria* spp. (*L. ivanovii* and *L. innocua*), as well as 4 non-related bacteria (*S. aureus*, *Y. enterocolitica*, and *C. coli*). A positive result with a change of color to yellow was originated with the target strains, while no color change occurred with the non-target bacteria; the results are presented in Table 1.

Methodology Evaluation

To evaluate this new method, a total of 109 milk samples (51 UHT, 32 fresh, and 26 raw) were spiked with different concentrations of the targeted microorganisms, and the results obtained are summarized in Table 4.

These results were used to calculate the LoD and the fit for purpose. For *E. coli* O157 and *Salmonella* spp., a lower LoD_{95}, compared to that of *L. monocytogenes*, was achieved. For both Gram-negative pathogens, the LoD_{95} was calculated to be 1.6 CFU/25 mL (Tables 5 and 6), while for *L. monocytogenes*, the value obtained was 79.0 CFU/25 mL (Table 7).

The LoD was calculated for each type of milk presentation in order to understand the influence of the matrix in the detection of the pathogens. For the Gram-negative bacteria, no significant difference in the probability of detection was observed among the types of samples tested (Tables 5 and 6). However for *L. monocytogenes*, an increase in the LoD was observed from UHT, to fresh and then raw milk; more specifically, the values were 52, 82, and 130 CFU/25 mL respectively (Table 7).

Regarding the fit for purpose, it was also calculated based on the results from the previously mentioned samples, considering those above the LoD_{95} for each type of matrix (see Table 8). Due to the high bacterial background present in the raw milk samples, the confirmation on selective solid medium was not possible, making difficult the isolation and identification of typical colonies. The evaluation revealed no deviation between the LAMP and reference methodologies for *L. monocytogenes* and *Salmonella* spp., reaching a *k* 1.0, as well as an AC, SE, and SP of 100%. Regarding the detection of *E. coli* O157, 1 deviation was observed in raw milk samples spiked with a concentration of 2 CFU/25 mL. These samples returned a positive result by colorimetric LAMP, while negative by qPCR, being consider FP. Attending to these results, the AC, SE, and SP for *E. coli* O157 detection obtained a value of 99, 100, and 90%, respectively, and a lower *k* index of 0.94.

Discussion

Despite all the research performed to improve food analysis focused on the detection of pathogenic microorganism, the sample treatment continues to be the major bottleneck, as it

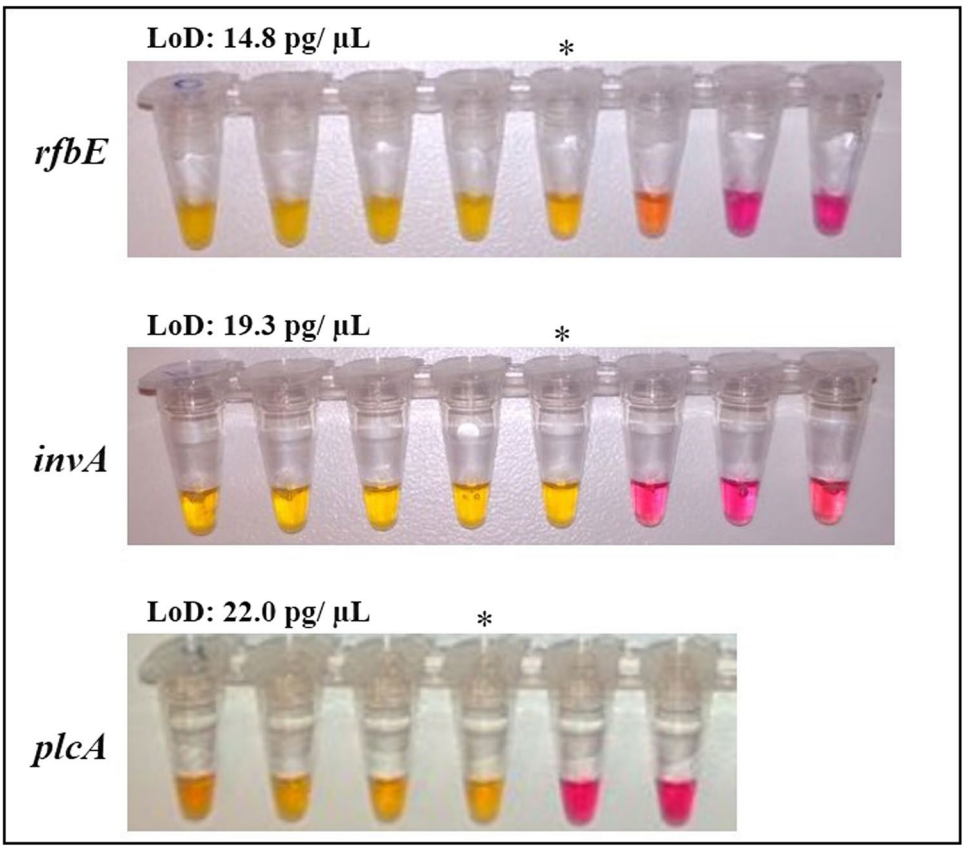

Fig. 3 Dynamic range of the colorimetric LAMP for the different targets. Determination of the lowest DNA concentration detectable, using a ten-fold diluted DNA extract from a pure culture of each pathogen. "*" represents the last dilution to be considered positive

still relies on prolonged enrichments which delay the results. The "short pre-enrichment" approach was already evaluated, and showed promising results for the detection of *Salmonella* spp., *E. coli* O157, and *L. monocytogenes* in simplex (Fachmann et al. 2017; Garrido-Maestu et al. 2020a; Azinheiro et al. 2022), as well as in multiplex for the detection of *E. coli* O157 and *Salmonella* spp. (Garrido-Maestu et al. 2020b). The simultaneous detection of these three bacteria can be challenging due to growth competition, among them, or even with other microorganisms present in the sample. In this regard, *L. monocytogenes* was reported to be the most challenging, and for this reason, the enrichment conditions were optimized for its recovery.

Different alternatives, including the combination of a non-selective pre-enrichment along with a selective enrichment, were tested, in an attempt to improve the detection of *L. monocytogenes*. The inoculation with different volumes, 100 µL and 1 mL, of the primary pre-enrichment in TSB after 3 h, to a selective broth, FF, for the same incubation time allowed to decrease the Cq obtained by qPCR, indicating that transferring 1 mL increased the concentration of *L. monocytogenes*. However, this selective medium seemed to interfere with the colorimetric LAMP, producing an initial color shift when the sample was loaded, and at the end of the incubation, no further variation was observed (see supporting information). This could also explain why the mixture of TSB and FF (6 h), which obtained a similar Cq than direct pre-enrichment in TSB for 7 h, did develop a yellow color. The change of color before amplification also suggests the presence of compounds which may interfere with the master mix. FF broth was already reported to inhibit the qPCR (Rossen et al. 1992; D'Agostino et al. 2004).

The colorimetric LAMP master mix selected relies on a pH-sensitive dye (phenol red) which generates a color change due to the release of protons caused by the nucleotide incorporation, leading to a drop of the pH in positive reactions (Zhang et al. 2020a). Due to this, it is recommended by the manufacturer to elute the DNA extracts in water as other buffers may interfere with the pH when added in higher volume than 10%, which emphasizes the possible interference of different compounds which may remain in the DNA extract, as it was observed with the FF broth. Despite the dilution and purification of the sample which allow to solve this issue, no change of color was observed after amplification. This could be due to the reaming of inhibitory compounds or the decrease of the DNA concentration after treatment. For this reason, the final methodology consisted on the direct pre-enrichment in TSB for 7 h.

Another effected observed during the culture optimization was the difference in the Cq obtained for *L. monocytogenes* when only one other target was simultaneously spiked. These

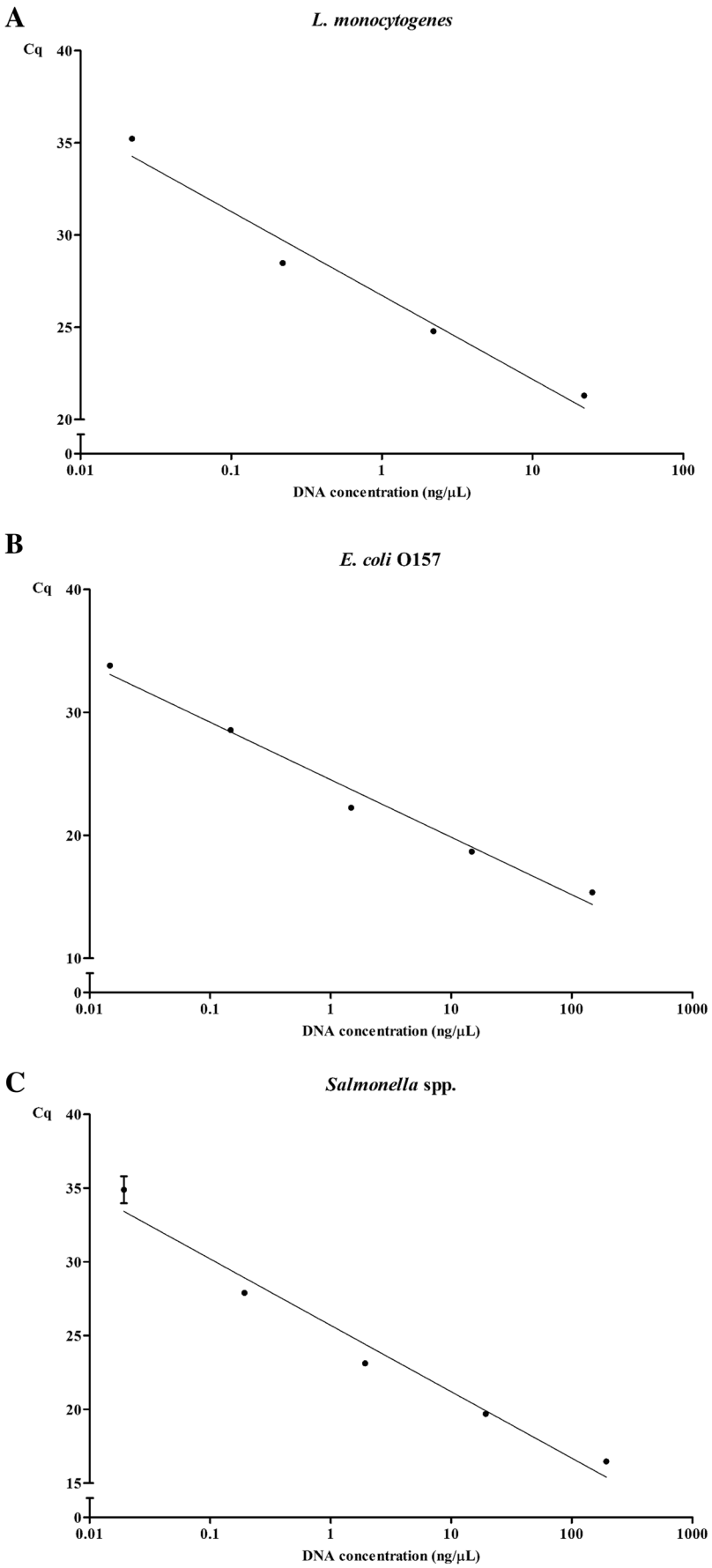

Fig. 4 Dynamic range obtained by multiplex qPCR for the different targets *hly* (**A**), *rfbE* (**B**), and *invA* (**C**). Ten-fold dilutions of the DNA extracted from pure cultures of each pathogen were tested in duplicate

Table 4 Results obtained by colorimetric LAMP, by qPCR and by plating in spiked milk samples

Sample	Contamination level				Results								
	Lm	ST	Ec O157	*N*	*L. monocytogenes*			*Salmonella* spp.			*E. coli* O157		
Milk					LAMP	qPCR	Plate	LAMP	qPCR	Plate	LAMP	qPCR	Plate
UHT	89	124	30	6	+[a]	+[a]	+[a]	+[a]	+[a]	+[a]	+[a]	+[a]	+[a]
	38	48	24	6	+	+	+	+[a]	+[a]	+[a]	+[a]	+[a]	+[a]
	23	49	16	6	+	+	+	+[a]	+[a]	+[a]	+[a]	+[a]	+[a]
	34	6	5	6	+(2) − (4)	+(2) − (4)	+	+[a]	+[a]	+[a]	+[a]	+[a]	+[a]
	11	18	3	6	−	−	+*	+[a]	+[a]	+[a]	+[a]	+[a]	+[a]
	7	17	4	6	−	−	+(3)* − (3)	+[a]	+[a]	+[a]	+[a]	+[a]	+[a]
	39	4	2	6	+	+	+	+[a]	+[a]	+[a]	+[a]	+[a]	+[a]
	37	1	1	6	+	+	+	+(3) –(3)	+(2) − (4)	+(2) − (4)	+	+	+
	30	1	1	6	+	+	+	+(3) − (3)	+(5) − (1)	+(5) − (1)	+(5) − (1)	+(5) − (1)	+
	67	-	4	1	+[a]	+[a]	+[a]	−[a]	−[a]	−[a]	+[a]	+[a]	+[a]
	-	-	-	2	−[a]	−[a]	−[a]	−[a]	−[a]	−[a]	−[a]	−[a]	−[a]
Fresh	81	88	12	3	+[a]	+[a]	+[a]	+[a]	+[a]	+[a]	+[a]	+[a]	+[a]
	61	66	9	3	+	+	+	+[a]	+[a]	+[a]	+[a]	+[a]	+[a]
	40	57	11	6	+(5) − (1)	+(5) − (1)	+	+[a]	+[a]	+[a]	+[a]	+[a]	+[a]
	20	28	13	6	+(5) − (1)	+(5) − (1)	+	+[a]	+[a]	+[a]	+[a]	+[a]	+[a]
	36	2	1	5	+(2) − (3)	+(4) − (1)	+	+[a]	+[a]	+[a]	+(4) − (1) [a]	+(4) − (1) [a]	+(4) − (1) [a]
	20	1	1	6	+(3) –(3)	+	+	+(3) − (3)	+(4) − (2)	+(4) − (2)	+	+	+
	-	-	-	3	−[a]	−[a]	−[a]	−[a]	−[a]	−[a]	−[a]	−[a]	−[a]
Raw	-	636	195	1	−[a]	−[a]		+[a]	+[a]		+[a]	+[a]	
	407	636	-	1	+[a]	+[a]		+[a]	+[a]		−[a]	−[a]	
	407	-	195	1	+[a]	+[a]		−[a]	−[a]		+[a]	+[a]	
	122	191	59	3	+	+		+[a]	+[a]		+[a]	+[a]	
	96	1	2	5	+	+		+(4) − (1)	+(4) − (1)		+[a]	+(4) − (1) [a]	
	61	1	1	6	+(3) –(3)	+		+	+		+(3) − (3)	−	
	36	3	1	6	+(4) –(2)	+		+[a]	+[a]		+(3) − (3)	−	
	-	-	-	3	−[a]	−[a]		−[a]	−[a]		−[a]	−[a]	

The contamination level is expressed in cfu/25 g of sample. Lm, ST, and Ec O157 stand for *L. monocytogenes*, *S.* Typhimurium, and *E. coli* O157. *N* indicates the number of samples. "*" only observed between 1 and 4 colonies. [a]Samples considered for the evaluation of the fit for purpose, which were above the LoD_{95}

Table 5 Limit of detection (LoD) calculated for *E. coli* O157

Sample type	LoD_{50}*			LoD_{95}*		
	LoD	Lower conf. Limit	Upper conf. Limit	LoD	Lower conf. Limit	Upper conf. Limit
UHT	0.2	0.1	0.5	1.1	0.5	2.3
Fresh	0.3	0.1	0.6	1.1	0.5	2.5
Raw	0.6	0.3	1.2	2.6	1.3	5.0
Combined	0.4	0.3	0.6	1.6	1.1	2.4

"*" cfu/25 mL

Table 6 Limit of detection (LoD) calculated for *Salmonella* spp

Sample type	LoD_{50}*			LoD_{95}*		
	LoD	Lower conf. Limit	Upper conf. Limit	LoD	Lower conf. Limit	Upper conf. Limit
UHT	0.5	0.2	1.2	2.1	0.9	5.0
Fresh	0.5	0.2	1.2	2.1	0.9	5.2
Raw	0.2	0.1	0.6	1.1	0.5	2.4
Combined	0.4	0.2	0.6	1.6	1.0	2.7

"*" cfu/25 mL

Table 7 Limit of detection (LoD) calculated for *L. monocytogenes*

Sample type	LoD_{50}*			LoD_{95}*		
	LoD	Lower conf. Limit	Upper conf. Limit	LoD	Lower conf. Limit	Upper conf. Limit
UHT	12.1	7.5	19.5	52.3	32.4	84.2
Fresh	18.9	11.5	31.0	81.5	49.6	133.8
Raw	30.1	16.7	54.2	130.0	72.2	234.1
Combined	18.3	13.6	24.6	79.0	58.7	106.4

"*" cfu/25 mL

Table 8 Results of the fit for purpose for each pathogen detected

Target	*N*	PA	NA	FN	TP	FP	AC	SE	SP	p0	pe	*k*
L. monocytogenes	21	12	9	0	0	0	100	100	100	1.00	0.49	1.00
Salmonella spp.	87	77	10	0	0	0	100	100	100	1.00	0.20	1.00
E. coli O157	87	74	9	0	0	1	99	100	90	0.95	0.20	0.94

N, number of samples; *PA*, positive agreement; *NA*, negative agreement; *FN*, false negative; *TP*, true positive; *FP*, false positive; *AC*, relative accuracy (%); *SE*, relative sensitivity (%); *SP*, relative specificity (%); *p0*, proportion of agreement; *pe*, expected frequency of agreement; *k*, Cohen's kappa. Samples above the LoD_{95} were considered for these analyses

results highlight the increased interference for the detection of *L. monocytogenes* when all were grown simultaneously.

The colorimetric LAMP was developed to target *rfbE*, *invA*, and *plcA* for the detection of *E. coli* O157, *S.* Typhimurium, and *L. monocytogenes*, respectively. The last two have been already used for the detection in real-time fluorescence. A typo was detected in the information provided by Garrido-Maestu et al., for the detection of *L. monocytogenes*. It was observed that the sequences provided targeted the *plcA* gene, and not the *hly* as indicated by the authors. However, this error did not have any relevance in regard to the performance of the assay (Garrido-Maestu et al. 2018a). All the reactions were supplemented with GuHCl due to its capacity to enhance LAMP reactions, as already reported by other studies (Lalli et al.

2020; Zhang et al. 2020b), which was crucial for the detection of *L. monocytogenes*, combined with a higher DNA template volume, 6 µL, and a longer amplification time, 1 h.

In terms of LoD_{95}, a very low concentration was reached, 1.6 CFU/25 mL, for *Salmonella* spp. and *E. coli* O157, without significant influence of the matrix type. These results are in line with those previously reported by Brewster and Paul who reached 4 CFU/mL of *Salmonella* spp. By qPCR in only 3 h (Brewster and Paul 2016), similarly, Paul et al. detected O157 in spiked raw milk samples in only 3 h and reached a limit of detection of 1 CFU/mL (Paul et al. 2013). In regard to *L. monocytogenes*, a higher concentration was determined, 79 CFU/25 mL, which was roughly 1 log higher than that of the Gram-negative pathogens tested; this observation somewhat agrees with the observation Paul et al. who, even though reported an LoD of 1 CFU/mL for *L. monocytogenes* by qPCR in only 3 h, also indicated issues in the quantification below 10 CFU/mL (Paul et al. 2015). It is worth to note that the studies previously mentioned were performed in 10 mL of milk, which would make them unsuited for legal requirements in many countries as the regulations tend to indicate a minimum sample size of 25 g or mL (European Commision 2019).

The detection of *L. monocytogenes* seemed to be influenced by the type of matrix. An increase of the LoD was observed from UHT, fresh, and raw milk. The increase in other interfering bacteria present in the sample could be involved in this observation. To confirm this hypothesis, the mesophilic bacteria present were counted in triplicate for each type of milk, following ISO 4833–1:2013 (ISO 2013). The analysis showed a concentration of < 10 CFU/mL for UHT and 2 replicates of fresh milk. The third replicate of fresh milk presented a bacterial concentration of 10 CFU/g, while the raw milk samples had an average of 5.9 ± 0.2 log CFU/mL, explaining the higher LoD obtained in this type of sample. The results obtained are in agreement with Banik et al., who reported similar microbial content in these samples (Banik et al. 2015).

The methodology developed had a turnaround time of 9 h, including the pre-enrichment, DNA extraction, and amplification/detection, demonstrating promising for its application in the food industry.

Conclusions

After the optimization of the methodology to enhance the detection of three different foodborne pathogens in milk samples, an economic and fast analysis of 9 h was obtained, showing a promising possibility for its implementation in the food industry.

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Foteini Roumani: investigation, revision, and edition.

Marta Prado: funding acquisition, supervision, revision, and editing.

Alejandro Garrido-Maestu: analyzed the data, supervision, revision, and editing.

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Declarations

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