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Interlaboratory validation of a multiplex qPCR method for the detection of *Listeria monocytogenes* in a ready-to-eat seafood product

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A B S T R A C T

Listeria monocytogenes is a major foodborne pathogen which mainly infects susceptible individuals through the consumption of contaminated foods. To this end, ready-to-eat (RTE) food products are of particular concern as this microorganism is widely distributed, can survive, and even grow, under adverse conditions, and thus must be carefully controlled. In the present study, an interlaboratory ring trial was organized to evaluate an open formula qPCR-based method for the detection of *L. monocytogenes*. The molecular method was evaluated on a novel RTE seafood product, developed in the framework of a European project, the SEAFOODAGE (EAPA_758/2018). Six laboratories located in Spain and Portugal participated in the study, and the results obtained indicated that this new method presented high diagnostic sensitivity (100%) reaching a low limit of detection (<10 CFU/25 g) with an overall agreement with the reference method, attending to the Cohen's k, of 0.97 that is interpreted as "almost complete agreement".

1. Introduction

Listeria monocytogenes is a well-known human pathogen. It is a ubiquitous, Gram-positive, rod-shaped, non-spore forming bacterium, and it is highly resistant to harsh environments being able to persist, and even grow, in a wide range of pH, temperatures and water activity (a_w) (Leong et al., 2016; Zilelidou & Skandamis, 2018). All these features make it a particularly problematic pathogen associated with ready-to-eat (RTE) foods (Abdollahzadeh et al., 2016; Kramarenko et al., 2016; Ziegler et al., 2019). Susceptible individuals such as immunocompromised people, elderly and/or pregnant women, may be infected through the consumption of contaminated foods, and they may develop listeriosis (Warriner & Namvar, 2009). The disease is relatively rare but potentially serious reaching mortality rates above 24%. There are two major forms of the disease, the non-invasive, which manifests as a febrile gastroenteritis, and the invasive form which causes septicemia or meningoencephalitis. The bacteria may be passed to a fetus via the placenta of the infected mother, leading to abortion, and meningitis in

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the neonate, among other manifestations (Allerberger & Wagner, 2010; Lepe, 2020).

In 2019, the SEAFOOD-AGE project started with the aim of tackling a challenge in the Atlantic area region, the aging of the population (https://seafoodage.eu/). For a healthy aging, among other preventive measures, a healthy diet is important, and seafood products can provide essential nutrients not always accessible to older adults. Thus, in the framework of the project a novel RTE seafood product was developed taking advantage of natural resources (fish discards and seaweeds among others), and by-products (shells and fish protein hydrolysates) from this region (Alter et al., 2022, pp. 0–19; Henriques et al., 2021). This new product supports the growth of *L. monocytogenes* thus it is classified in the food category 1.2 of "Chapter 1. Food safety criteria" of the European Regulation 2073/2005, more specifically "Ready-to-eat foods able to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes". The legal criterion indicated by the mentioned Regulation indicates "not detected/25 g" (Commission Regulation (EC) No 2073/2005, 2005). Additionally, considering that the target group of age of the novel product is > 65 years old, who is a risk group of listeriosis, it was of particular importance to develop a rapid method for the detection of this pathogen. Furthermore, its presentation as an open formula, ready-to-use kit (freely available oligonucleotide sequences and reagents) was consider of interest for any potential final user, testing laboratories as well as food producers. In the mentioned Regulation, the reference method indicated is the ISO standard 11290-1 which is culture-based (ISO, 2017), and like most standard microbiological methods, has been reported as lengthy and tedious to perform (Rohde et al., 2017; Villamizar-Rodríguez et al., 2015). In this context, molecular methods, particularly those based on Polymerase Chain Reaction (PCR) and real-time PCR (qPCR), have been reported during the last decades, as a suitable alternative to overcome the limitations of culture-based approaches (Bavisetty et al., 2018; Dalmasso et al., 2014) in addition of being capable of detecting Viable But Non-Culturable (VBNC) bacteria, stage in which the microorganisms may enter under stress conditions such as the presence of disinfectants used to clean food industries (Brauge et al., 2020). One of the typical claims against the extended use of qPCR-based methods relies on its incapacity to discriminate among DNA coming from live or dead cells. However, in recent years solutions to overcome this limitation have been reported such as the detection of mRNA, or the implementation of Ethidium/Propidium Monoazide (EMA/PMA) (Garcia et al., 2015; González-Escalona et al., 2009). It is noteworthy that, from a risk assessment point of view, one must not oversee the interest of these so-called "false positive" results which do not represent a direct risk as the bacteria are dead, but highlights a clear hygiene issue as the pathogen detected was viable at some point in the food product under analysis.

Even though a plethora of qPCR-based methods have been reported in the scientific literature, most of them lack proper assay validation, being this a key point to assure optimal performance, and to encourage its use by the food industry as a reliable self-monitoring tool. Even though many PCR/qPCR methods have been reported for the detection of *L. monocytogenes*, as well as for many other pathogens, very few have undergone a proper interlaboratory evaluation to determine their performance. Covering this gap was the aim of a European project granted in 2000 where PCR-based methods were developed and evaluated for the specific detection of *Salmonella* spp. (Malorny et al., 2003), *L. monocytogenes* (D'Agostino et al., 2004), *Escherichia coli* O157 (Abdulmawjood et al., 2004) and thermotolerant *Campylobacter* spp. (Lübeck et al., 2003). In addition to the mentioned studies, very few others have been reported in the literature being this a true limitation for the wider adoption of this type of methodologies.

The present manuscript reports the results obtained in an interlaboratory validation ring trial where an open formula, ready-to-use kit for the detection of *L. monocytogenes* developed in the framework of the SEAFOOD-AGE project, was evaluated. In this study, the RTE fish-based dish, which was developed in the framework of the SEAFOOD-AGE project, was used as the commodity of choice, and a total of six independent laboratories, from Spain and Portugal, were involved.

2. Materials & methods

A detailed list of all the materials provided to each one of the participants can be found in the supporting information.

2.1. Strains, culture media and inoculation procedure

The strain WDCM 00021 of *L. monocytogenes*, purchased from the Spanish Type Culture Collection (CECT 935) was used as positive control. For the spiking of the samples, certified and quantified reference materials were purchased from ielab (Alicante, Spain) and distributed to the participants freeze-dried. Three different concentrations of *L. monocytogenes* WDCM 00021 were assayed (low, medium, and high), along with one of *Listeria innocua* (WDCM 00017) which served as negative control. Thus each participant received four 50 mL tubes with one freeze-dried tablet to be reconstituted following the manufacturer's instructions. Additional details on the reference materials are provided in Table 1. Three samples were inoculated at each concentration level, along with the negative control thus making a total of 10 samples per laboratory.

The samples were processed as follows. Twenty five grams of the RTE fish product were weighted in a stomacher bag, 3 mL of the reconstituted bacteria detailed above were added and then 225 mL of ONE Broth *Listeria* (ONE, OXOID, Hampshire, UK) were added. The matrixes were mixed in a laboratory homogenizer (Stomacher, or similar device) and then were incubated for 24 h at 30 °C. Once completed the incubation, the enriched samples were used for DNA extraction as detailed below, and also they were plated on ALOA, the medium indicated by the ISO standard 11290 (ISO, 2017) or any other commercial chromogenic medium with a similar formulation, for confirmation purposes and to serve as reference. The plates were incubated at 37 °C for 24–48 h and screened for typical colonies (blue – turquoise surrounded by a halo). This culture-based approach was used for the confirmation of the qPCR method, and was based on the protocol with AFNOR validation from OXOID (http://www.oxoid.com/pdf/uk/27363_Listeria_Precis.pdf).

2.2. DNA extraction

One milliliter was taken from the enriched samples, centrifuged at $900\times g$ for 1 min and the supernatant was transferred to a clean tube and centrifuged at $16000\times g$ for 2 min, the supernatant was discarded, the pellet was resuspended in 1 mL of PBS (pH 7.4 ± 0.2) and centrifuged again under the same conditions. The supernatant was further discarded and the clean bacterial pellet was resuspended in 300 μL of Chelex 6% (Bio-Rad Laboratories, Inc., USA). The samples were then heated at 56 °C for 15 min under constant agitation (1000 rpm) and afterwards, the bacteria were thermally lysed at 99 °C for 10 min under constant agitation (1400 rpm). Whenever available the heating steps were performed in a dry bath such as a Thermomixer comfort (Eppendorf AG, Germany) or similar devices, if not available the tubes were mixed by hand. The lysates were finally centrifuged at $16000\times g$ for 2 min at 4 °C. The DNA extracts were stored at 4 °C (for longer term storage the samples were kept at −20 °C).

2.3. Multiplex qPCR

Primers from Roumani et al. targeting the *hly* gene, along with a competitive internal amplification control (IAC) were selected (Roumani et al., 2021). The primers and probes were provided as a 10X mixture, sequences provided in Table 2. The qPCR was performed in a final reaction volume of 20 μL, containing 10 μL of NZYSupreme qPCR Probe Master Mix (NZYTech, Lisbon, Portugal), 2 μL of the 10X primer

Table 1
Bacterial species used for sample spiking.

Species	WDCM[a]	Amount per tablet (CFU)	95% Confidence	Concentration (CFU/mL)[b]	Final concentration (CFU/25 g)[c]
L. monocytogenes	00021	6.2×10^1	3.5×100–1.1×10^1	3.1×100	9.3×100
	00021	9.1×10^2	5.0×10^2–1.7×10^3	4.6×10^1	1.4×10^2
	00021	5.0×10^3	2.0×10^3–5.3×10^3	1.6×10^2	4.9×10^2
L. innocua	00017	4.4×10^3	1.3×10^2–1.5×10^3	2.2×10^2	6.6×10^2

[a] WDCM: World Data Center for Microorganisms.
[b] Calculated considering that each tablet was resuspended in 20 mL of sterile water as indicated by the manufacturer.
[c] Final concentration indicated in CFU/25 g after the addition of 3 mL of the reconstituted tablets.

Table 2
Multiplex qPCR primers and probes for the detection of *L. monocytogenes*.

Primer	Sequence 5' → 3'	Concentration (nM)	Modifications	Reference
hly-P3F	CGC AAC AAA CTG AAG CAA AGG A	200	–	Roumani et al. (2021)
hly-P3R	CGA TTG GCG TCT TAG GAC TTG C	200	–	
hly-P3P	CAT GGC ACC// ACC AGC ATC TCC G	150	FAM/ZEN/ IABkFQ	
IAC-P	AGT GGC GGT// GAC ACT GTT GAC CT	100	YY/ZEN/ IABkFQ	
IAC-DNA	GGA TTA CCC TAG AGT GGC GGT GAC ACT GTT GAC CTT CTA TTA CCT C	10^3[a]	b	

[a] Copies of IAC DNA added per reaction.
[b] Sequence flanked at 5′ and 3′ ends by hly-P3F and hly-P3R primers to construct the qPCR competitive IAC. YY (Yakima Yellow), IABkFQ (Iowa Black®FQ) and ZEN (secondary, internal quencher) are trademarks from IDT.

mix, 3 μL of template DNA and 5 μL of sterile, DNase, RNase free water.

The thermal profile consisted on a hot-start step of 5 min at 95 °C followed by 40 cycles of Denaturation at 95 °C for 15 s and Annealing-Extension at 63 °C for 60 s. Each participating laboratory used the real-time thermocycler available at their premises.

2.4. Results reporting and interpretation

Along with the different materials detailed in supporting information, each laboratory also received an Excel spreadsheet to report the results and the Standard Operations Procedure (SOP) detailing all the steps for performing the method, as well as a guide for the interpretation of the results. In this regard, a sample was considered as positive whenever a positive result was obtained for *hly*, with/without positive IAC; it was considered as negative when *hly* was negative with a positive IAC (expected Cq value ~30); and inconclusive with a negative result for *hly* and IAC (in this case the sample should be re-analyzed along with a 1/2-1/10 dilution of the original DNA extract). As indicated in M&M 2.1, all the samples were plated on ALOA, or similar media, for confirmation.

2.5. Evaluation of the method

The samples were classified as Positive or Negative Agreement (PA/NA) if the result obtained by the alternative method, the multiplex qPCR under evaluation, matched the expected ones (positive for samples inoculated with *L. monocytogenes* and negative for the samples inoculated with *L. innocua*). Likewise, the samples were classified as Positive or Negative Deviations (PD/ND) if the results did not match. The culture-based method described in M&M 2.1 was used for the confirmation of the results. The samples deviating from the expected results were re-classified after results confirmation (presence of typical colonies by the culture-based method), in this sense, the ND was classified as False Negative (FN) if typical colonies were observed, and the PD were classified as True Positives (TP) or False Positives (FP) if the typical colonies were obtained or not, respectively. In Table S1 a summary of the results interpretation, and classification, is provided. These parameters were used to determine the diagnostic sensitivity, specificity and accuracy (SE, SP and AC, respectively) along with the Cohen's kappa (k). The definition of the different parameters, and the formulae for their calculation, were obtained from the NordVal regulation (NordVal, 2017).

3. Results

3.1. Results from each laboratory

One of the laboratories was excluded from the final evaluation due to the fact that they did not report the results in the provided, standard Excel sheet, and inconsistencies in the spiking procedure were identified. Considering this, the interlaboratory trial included 5 independent laboratories located in Spain and Portugal. In terms of equipment, it was reported that 2 of the participating laboratories used Bio-Rad CFX96 (Bio-Rad Laboratories, Inc., USA), another 2 made the analysis on a 7500 Fast Real Time PCR System Thermal Cycler, and the fifth laboratory used a QuantStudio™ 12 K Flex (7500 and QuantStudio™ are machines from Applied Biosystems. Foster City, CA, USA).

In Table 3 the results of all the laboratories, per inoculation level, are summarized. At the lowest inoculation level (9.3 CFU/25 g) 14 out of the 15 samples analyzed considering all 5 laboratories were positive by the qPCR method and also presented typical colonies on the chromogenic media. One sample was negative; however no typical colonies were obtained on the selective media thus this was not considered as a deviation. Regarding the intermediate inoculation level (1.4×10^2 CFU/25 g), all 15 samples were positive and presented typical colonies. Finally, regarding the high inoculation level (4.9×10^2 CFU/25 g), just like with the low inoculation level, 14 out of 15 samples were positive and once more all the positive samples presented typical colonies, while the sample which was negative by qPCR did not present any typical colonies on chromogenic media. The average Cq values obtained for the low inoculation level was ~30 while for the medium and high levels it was ~25, these results are graphically depicted in Fig. 1.

Table 3
Summary of the results obtained by all the participating laboratories at the different inoculation levels.

Inoculation level	N	Before Confirmation				After Confirmation		
		PA	NA	PD	ND	FP	FN	TP
L0	5	0	4	1	0	1	0	0
L1	15	14	1	0	0	0	0	0
L2	15	15	0	0	0	0	0	0
L3	15	14	1	0	0	0	0	0

N: number of samples. PA: Positive Agreement. NA: Negative Agreement. PD: Positive Deviation. ND: Negative Deviation. FP: False Positive. FN: False Negative. TP: True Positive. L0: negative control inoculated with *L. innocua*, 6.6×10^2 CFU/25 g. L1: low inoculation level, 9.3 CFU/25 g. L2: medium inoculation level, 1.4×10^2 CFU/25 g. L3: high inoculation level, 4.9×10^2 CFU/25 g.

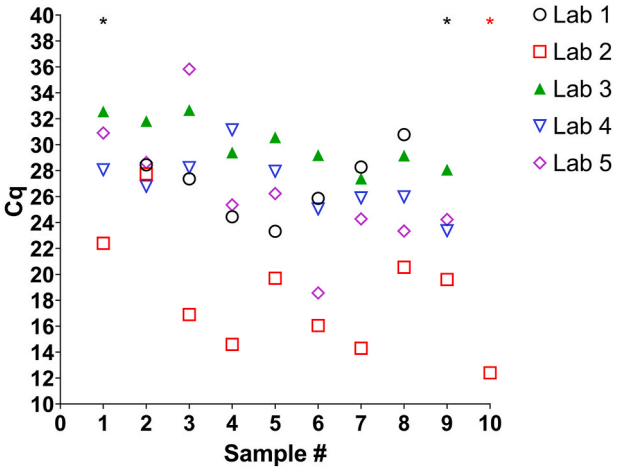

Fig. 1. Average Cq values obtained by each participating laboratory for every sample. *Indicates samples which obtained a negative qPCR result, but did not present typical colonies on chromogenic media. *Sample expected to be negative which was reported to have a positive qPCR result but no typical colonies on chromogenic media.

One participating laboratory reported a positive result in the sample inoculated with *L. innocua*, which served as negative controls. There were no typical colonies of *L. monocytogenes* on the chromogenic media. The other 4 laboratories reported a negative qPCR result along with absence of typical colonies on selective media.

3.2. Evaluation of the method

Laboratories 3, 4 and 5 did not report any deviation from the expected results. In addition to this, the culture-based method perfectly matched the results obtained by qPCR, thus they obtained values of 100% for the SE, SP and AC along with a k of 1.00.

Regarding the Laboratory number 1, they missed to detect one sample at the lower inoculation level, and another at the highest one, however these samples did not present typical colonies after confirmation thus were classified as NA, and so did not affect the SE value obtained. Thus, the SE, SP and AC values were 100% and the k was 1.00.

Finally, Laboratory 2 reported 1 ND which corresponded to the sample inoculated with *L. innocua* that was reported to obtain a positive result by qPCR (positive for *hly* and the IAC). This sample was classified as a FP after results confirmation due to the fact that no typical colonies were observed on the chromogenic media. This FP generated the following results for the performance parameters: SE of 100%, an SP of 0%, an AC of 90% and a k of 0.88. For this particular laboratory, it was observed that the Cq values reported for all the samples were lower than those of all the others, which may indicate that the misidentified sample was the result of an incorrect assignment of the threshold, see Fig. 1.

Jointly analyzing all the results provided from the 50 samples analyzed by the 5 independent participants, the values obtained for the current method were a SE of 100%, SP of 85.7%, AC of 98% and a k of 0.97. All these results are summarized in Table 4.

4. Discussion

Fish is known to be a healthy food product due to its high content in vitamins, minerals and high quality proteins among other factors (Belton et al., 2018). Its presentation as RTE food product can increase its consumption due to the convenience of the format. This is of particular relevance for the elderly, who could benefit from this nutritious food in a simple manner thanks to this format. However, this might pose specific challenges from a food safety point of view due to the lack of any post-processing treatment that could eliminate potential microbial pathogens (Gambarin et al., 2012). *L. monocytogenes* represents a particular threat, being explicitly regulated in RTE foods in most countries. In order to cope with the intensive production systems, and many times the short shelf-life of certain food products, including RTE, rapid microbiological methods are needed and, even though many have already been described, very few have been validated in interlaboratory trials to evaluate their fitness-for-purpose and robustness.

In the present manuscript, an open formula qPCR method for the detection of *L. monocytogenes* was evaluated in an interlaboratory ring trial to determine its capacity to detect this microorganism in a novel RTE seafood product, which was experimentally determined to support the growth of *L. monocytogenes* (data not shown). The method includes an enrichment in a selective medium, thus ONE Broth *Listeria* was selected as according to Azinheiro et al. a good, and faster recovery, of *L. monocytogenes* could be obtained in one single step compared to the two-step enrichment indicated in the ISO method (Azinheiro et al., 2020). For the DNA extraction a simple and economic thermal lysis was as well evaluated to avoid expensive chemicals; this approach was previously reported as suitable for its combination with qPCR assays (David Rodríguez-Lázaro et al., 2004; David Rodríguez-Lázaro et al., 2014). Two commercial reagents were tested, namely PrepMan Ultra and Chelex, from Applied Biosystems and Bio-Rad respectively, and considering the results, cost and complexity of the protocols, the Chelex extraction was selected (see supporting information Table S2 and Figure SF1). Finally, the assay described by Roumani et al. which consisted in a multiplex qPCR targeting *hly* along with a competitive IAC, was selected (Roumani et al., 2021). For ease-of-use the primers, probes and IAC DNA were pre-mixed concentrated 10X, and the mixture was stored in the fridge for up to 70 days since the amplification efficiency was evaluated on regular intervals without significant changes (see supporting information Figure SF2).

Laboratories 3, 4 and 5 correctly identified all the samples provided and did not report any problem following the SOP provided. However, Laboratory 1 indicated that they experienced some problems when preparing the initial bacterial suspension in the 50 mL tubes provided, even though the process was supposed to be simple, by just adding 20 mL of sterile water, the bacterial tablet was not easily dissolvable, so it

Table 4
Method evaluation summary.

Participant	N	Before Confirmation				After Confirmation			SE	SP	AC	k
		PA	NA	PD	ND	FP	FN	TP				
Lab 1	10	7	3	0	0	0	0	0	100	100	100	1.00
Lab 2	10	9	0	1	0	1	0	0	100	0	90	0.88
Lab 3	10	9	1	0	0	0	0	0	100	100	100	1.00
Lab 4	10	9	1	0	0	0	0	0	100	100	100	1.00
Lab 5	10	9	1	0	0	0	0	0	100	100	100	1.00
Total	50	43	6	0	0	1	0	0	100	85.7	98.0	0.97

N: number of samples. PA: Positive Agreement. NA: Negative Agreement. PD: Positive Deviation. ND: Negative Deviation. FP: False Positive. FN: False Negative. TP: True Positive. SE: relative sensitivity. SP: relative specificity. AC: relative accuracy. k: Cohen's kappa, interpreted as "substantial agreement" (0.61–0.8) and "almost complete concordance" (0.81–1.00) according to previous references (Anderson et al., 2011; DG, 1991).

was hypothesized that this may have caused issues in the uniformity of suspension thus leading to the deviations observed. It is worth to note that no typical colonies were obtained by this Laboratory in the 2 samples where the ND were identified thus these were classified as NA after the confirmation, and so the SE and SP values obtained by this laboratory were 100%.

Another discrepant result was obtained in the interlaboratory study, and this was from Laboratory 2 who reported a positive result by qPCR in sample 10, which was the one inoculated with *L. innocua*, while it was negative by the culture-based approach. Considering that only one negative control was included among the 10 samples, this generated that the SP of the method for this laboratory was 0%, while the SE 100%. In order to understand the reported result, the data of the qPCR run was requested to the laboratory, and after a detailed analysis, it was observed that the reported result for this particular sample was most likely associated to an incorrect setting of the threshold. The amplification plots of all the samples are included in the supporting information Figure SF3, where it can be observed that there was no actual amplification. Furthermore, this particular laboratory reported significantly lower Cq values compared to all the other participants, that agrees with an incorrect threshold setting, by placing it excessively low the positive samples reported very low Cq values, and due to this the background noise of the negative sample was interpreted as a positive signal by the software. As no specific parameters were provided in the SOP, in order to leave it open to any thermocycler and software, the results provided by this laboratory were included just as reported, meaning that sample 10 was considered a FP.

Taken together all the results reported by the different participants, a very high diagnostic sensitivity was reached (100%) as well as diagnostic accuracy (98%). Only the diagnostic specificity was slightly lower than expected (85.7%) due to one single FP result reported. These good results were translated into a very high Cohen's k value (0.97) that is interpreted as in "Almost complete concordance" with the reference method (DG, 1991).

In the current study the lowest inoculation level tested was 9.3 CFU/25 g. This concentration was detected by all the laboratories in all the samples spiked at this concentration, thus it was demonstrated that the method can detect a very low concentration of *L. monocytogenes* (<10 CFU/25 g), being this a similar value to the one reported in previous open formula validation studies targeting *L. monocytogenes* like the one of D'Agostino et al. who reached a LOD of 20 CFU/25 mL of milk combining a two-step enrichment and PCR (D'Agostino et al., 2004). Similarly, Oravcová et al. managed to detect 1 CFU/25 g of *L. monocytogenes* in various food matrixes including smoked salmon, implementing a two-step enrichment protocol (Oravcová et al., 2007). In a later study from Gattuso et al. using meat as a model, they reached levels of 1–10 CFU/25 g implementing a single enrichment step in Half Fraser Broth (Gattuso et al., 2014), and following a similar approach Gianfranceschi et al. obtained similar results in fresh cheese (Gianfranceschi et al., 2014). More recently, Vizzini et al. published a study where they reported been capable of detecting 10 CFU/g of *L. monocytogenes* in smoked salmon after an enrichment step in One Broth Listeria (Vizzini et al., 2020), which is ten times higher than the value reported by Amagliani et al. in a similar matrix, salmon, however they could have benefitted from an immunomagnetic separation step to concentrate the bacteria of interest (Amagliani et al., 2010), however no proper evaluation of the LOD was performed in either study and none of them were tested by independent laboratories. In terms of time of analysis, the assay under evaluation in the present study reported results in line with previous studies which implemented a single enrichment step, ~27 h, including the culture, DNA extraction and qPCR analysis.

5. Conclusions

A ready-to-use method for the detection of *L. monocytogenes* in RTE fish-based foods was successfully developed, and validated in an international interlaboratory ring trial. The method was capable of detecting <10 CFU/25 g of sample after a single-step enrichment, followed by an economic and simple DNA extraction protocol based on thermal lysis, and a multiplex qPCR implementing a competitive IAC to assure absence of reaction inhibition. The overall evaluation indicated that this molecular method reached "Almost complete concordance" with the reference method selected (culture-based) attending to the Cohen's k value that was reached (0.97).

CRediT authorship contribution statement

Sarah Azinheiro: Investigation, Writing – review & editing. **Pedro Rodríguez-López:** Investigation, Writing – review & editing. **Antonio Lozano-León:** Formal analysis, Writing – review & editing. **Hugo Guedes:** Investigation, Writing – review & editing. **Patricia Regal:** Investigation, Writing – review & editing. **Carlos M. Franco:** Investigation, Writing – review & editing. **Alberto Cepeda:** Investigation, Writing – review & editing. **Pilar Teixeira:** Investigation, Writing – review & editing. **Luís D.R. Melo:** Investigation, Writing – review & editing. **Daniela Silva:** Investigation, Writing – review & editing. **Ana Fernández:** Investigation, Writing – review & editing. **Márcia Faria:** Investigation, Writing – review & editing. **Foteini Roumani:** Investigation, Writing – review & editing. **Juan Herrera:** Investigation, Writing – review & editing. **Marta Prado:** Funding acquisition, Supervision, Writing – review & editing. **Marta López-Cabo:** Investigation, Writing – review & editing. **Alejandro Garrido-Maestu:** Conceptualization, Methodology, Formal analysis, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.foodcont.2023.109769.

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