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

3

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30 Abstract:

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

43

44 Keywords: interlaboratory validation; *Listeria monocytogenes*; qPCR; Ready-to-eat; fish
45 products; alternative methods

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46 1. Introduction

47 *Listeria monocytogenes* is a well-known human pathogen. It is a ubiquitous, Gram-positive, rod-
48 shaped, non-spore forming bacterium, and it is highly resistant to harsh environments being able to
49 persist, and even grow, in a wide range of pH, temperatures and water activity (a_w) (Leong et al., 2016;
50 Zilelidou & Skandamis, 2018). All these features make it a particularly problematic pathogen
51 associated with ready-to-eat (RTE) foods (Abdollahzadeh et al., 2016; Kramarenko et al., 2016; Ziegler
52 et al., 2019). Susceptible individuals such as immunocompromised people, elderly and/ or pregnant
53 women, may be infected through the consumption of contaminated foods, and they may develop
54 listeriosis (Warriner & Namvar, 2009). The disease is relatively rare but potentially serious reaching
55 mortality rates above 24 %. There are two major forms of the disease, the non-invasive, which
56 manifests as a febrile gastroenteritis, and the invasive form which causes septicemia or
57 meningoencephalitis. The bacteria may be passed to a fetus via the placenta of the infected mother,
58 leading to abortion, and meningitis in the neonate, among other manifestations (Allerberger &
59 Wagner, 2010; Lepe, 2020).

60 In 2019, the SEAFOOD-AGE project started with the aim of tackling a challenge in the Atlantic area
61 region, the aging of the population (https://seafoodage.eu/). For a healthy aging, among other
62 preventive measures, a healthy diet is important, and seafood products can provide essential
63 nutrients not always accessible to older adults. Thus, in the framework of the project a novel RTE
64 seafood product was developed taking advantage of natural resources (fish discards and seaweeds
65 among others), and by-products (shells and fish protein hydrolysates) from this region (Alter et al.,
66 2022; Henriques et al., 2021). This new product supports the growth of *L. monocytogenes* thus it is
67 classified in the food category 1.2 of "Chapter 1. Food safety criteria" of the European Regulation
68 2073/2005, more specifically "Ready-to-eat foods able to support the growth of *L. monocytogenes*,
69 other than those intended for infants and for special medical purposes". The legal criterion indicated
70 by the mentioned Regulation indicates "not detected/ 25 g" (Commission Regulation (EC) No

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71 2073/2005, 2005). Additionally, considering that the target group of age of the novel product is > 65
72 years old, who is a risk group of listeriosis, it was of particular importance to develop a rapid method
73 for the detection of this pathogen. Furthermore, its presentation as an open formula, ready-to-use kit
74 (freely available oligonucleotide sequences and reagents) was consider of interest for any potential
75 final user, testing laboratories as well as food producers. In the mentioned Regulation, the reference
76 method indicated is the ISO standard 11290-1 which is culture-based (ISO, 2017), and like most
77 standard microbiological methods, has been reported as lengthy and tedious to perform (Rohde et al.,
78 2017; Villamizar-Rodríguez et al., 2015). In this context, molecular methods, particularly those based
79 on Polymerase Chain Reaction (PCR) and real-time PCR (qPCR), have been reported during the last
80 decades, as a suitable alternative to overcome the limitations of culture-based approaches (Bavisetty
81 et al., 2018; Dalmasso et al., 2014) in addition of being capable of detecting Viable But Non-Culturable
82 (VBNC) bacteria, stage in which the microorganisms may enter under stress conditions such as the
83 presence of disinfectants used to clean food industries (Brauge et al., 2020). One of the typical claims
84 against the extended use of qPCR-based methods relies on its incapacity to discriminate among DNA
85 coming from live or dead cells. However, in recent years solutions to overcome this limitation have
86 been reported such as the detection of mRNA, or the implementation of Ethidium/ Propidium
87 Monoazide (EMA/ PMA) (Garcia et al., 2015; González-Escalona et al., 2009). It is noteworthy that,
88 from a risk assessment point of view, one must not oversee the interest of these so-called “false
89 positive” results which do not represent a direct risk as the bacteria are dead, but highlights a clear
90 hygiene issue as the pathogen detected was viable at some point in the food product under analysis.

91 Even though a plethora of qPCR-based methods have been reported in the scientific literature, most
92 of them lack proper assay validation, being this a key point to assure optimal performance, and to
93 encourage its use by the food industry as a reliable self-monitoring tool. Even though many PCR/ qPCR
94 methods have been reported for the detection of *L. monocytogenes*, as well as for many other
95 pathogens, very few have undergone a proper interlaboratory evaluation to determine their
96 performance. Covering this gap was the aim of a European project granted in 2000 where PCR-based

97 methods were developed and evaluated for the specific detection of *Salmonella* spp. (Malorny et al.,
98 2003), *L. monocytogenes* (D'Agostino et al., 2004), *Escherichia coli* O157 (Abdulmawjood et al., 2004)
99 and thermotolerant *Campylobacter* spp. (Lübeck et al., 2003). In addition to the mentioned studies,
100 very few others have been reported in the literature being this a true limitation for the wider adoption
101 of this type of methodologies.

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

107

108 2. Materials & Methods

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

111 2.1. Strains, culture media and inoculation procedure

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

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

132

133 2.2. DNA extraction

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

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146 ## 2.3. Multiplex qPCR

147 Primers from Roumani et al. targeting the *hly* gene, along with a competitive internal amplification
148 control (IAC) were selected (Roumani et al., 2021). The primers and probes were provided as a 10X
149 mixture, sequences provided in Table 2. The qPCR was performed in a final reaction volume of 20 µL,
150 containing 10 µL of NZYSupreme qPCR Probe Master Mix (NZYTech, Lisbon, Portugal), 2 µL of the 10X
151 primer mix, 3 µL of template DNA and 5 µL of sterile, DNase, RNase free water.

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

155

156 ## 2.4. Results reporting and interpretation

157 Along with the different materials detailed in supporting information, each laboratory also received
158 an Excel spreadsheet to report the results and the Standard Operations Procedure (SOP) detailing all
159 the steps for performing the method, as well as a guide for the interpretation of the results. In this
160 regard, a sample was considered as positive whenever a positive result was obtained for *hly*, with/
161 without positive IAC; it was considered as negative when *hly* was negative with a positive IAC
162 (expected Cq value ~30); and inconclusive with a negative result for *hly* and IAC (in this case the sample
163 should be re-analyzed along with a 1/2-1/10 dilution of the original DNA extract). As indicated in M&M
164 2.1, all the samples were plated on ALOA, or similar media, for confirmation.

165

166 ## 2.5. Evaluation of the method

167 The samples were classified as Positive or Negative Agreement (PA/ NA) if the result obtained by the
168 alternative method, the multiplex qPCR under evaluation, matched the expected ones (positive for
169 samples inoculated with *L. monocytogenes* and negative for the samples inoculated with *L. innocua*).

170 Likewise, the samples were classified as Positive or Negative Deviations (PD/ ND) if the results did not
171 match. The culture-based method described in M&M 2.1 was used for the confirmation of the results.
172 The samples deviating from the expected results were re-classified after results confirmation
173 (presence of typical colonies by the culture-based method), in this sense, the ND was classified as False
174 Negative (FN) if typical colonies were observed, and the PD were classified as True Positives (TP) or
175 False Positives (FP) if the typical colonies were obtained or not, respectively. In Table S1 a summary of
176 the results interpretation, and classification, is provided. These parameters were used to determine
177 the diagnostic sensitivity, specificity and accuracy (SE, SP and AC, respectively) along with the Cohen's
178 kappa (k). The definition of the different parameters, and the formulae for their calculation, were
179 obtained from the NordVal regulation (NordVal, 2017).

180

181 3. Results

182 3.1. Results from each laboratory

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

190 In Table 3 the results of all the laboratories, per inoculation level, are summarized. At the lowest
191 inoculation level (9.3 CFU/ 25 g) 14 out of the 15 samples analyzed considering all 5 laboratories were
192 positive by the qPCR method and also presented typical colonies on the chromogenic media. One
193 sample was negative; however no typical colonies were obtained on the selective media thus this was
194 not considered as a deviation. Regarding the intermediate inoculation level (1.4 x 10^2 CFU/ 25 g), all

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195 15 samples were positive and presented typical colonies. Finally, regarding the high inoculation level
196 (4.9 x 10^2 CFU/ 25 g), just like with the low inoculation level, 14 out of 15 samples were positive and
197 once more all the positive samples presented typical colonies, while the sample which was negative
198 by qPCR did not present any typical colonies on chromogenic media. The average Cq values obtained
199 for the low inoculation level was ~30 while for the medium and high levels it was ~25, these results
200 are graphically depicted in Figure 1.

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

205

206 3.2. Evaluation of the method

207 Laboratories 3, 4 and 5 did not report any deviation from the expected results. In addition to this, the
208 culture-based method perfectly matched the results obtained by qPCR, thus they obtained values of
209 100 % for the SE, SP and AC along with a k of 1.00.

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

214 Finally, Laboratory 2 reported 1 ND which corresponded to the sample inoculated with *L. innocua* that
215 was reported to obtain a positive result by qPCR (positive for *hly* and the IAC). This sample was
216 classified as a FP after results confirmation due to the fact that no typical colonies were observed on
217 the chromogenic media. This FP generated the following results for the performance parameters: SE
218 of 100 %, an SP of 0 %, an AC of 90 % and a k of 0.88. For this particular laboratory, it was observed

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219 that the Cq values reported for all the samples were lower than those of all the others, which may
220 indicate that the misidentified sample was the result of an incorrect assignment of the threshold, see
221 Figure 1.

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

225

226 4. Discussion

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

238 In the present manuscript, an open formula qPCR method for the detection of *L. monocytogenes* was
239 evaluated in an interlaboratory ring trial to determine its capacity to detect this microorganism in a
240 novel RTE seafood product, which was experimentally determined to support the growth of *L.*
241 *monocytogenes* (data not shown). The method includes an enrichment in a selective medium, thus
242 ONE Broth *Listeria* was selected as according to Azinheiro et al. a good, and faster recovery, of *L.*

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243 *monocytogenes* could be obtained in one single step compared to the two-step enrichment indicated
244 in the ISO method (Azinheiro et al., 2020). For the DNA extraction a simple and economic thermal lysis
245 was as well evaluated to avoid expensive chemicals; this approach was previously reported as suitable
246 for its combination with qPCR assays (David Rodríguez-Lázaro et al., 2004; David Rodríguez-Lázaro et
247 al., 2014). Two commercial reagents were tested, namely PrepMan Ultra and Chelex, from Applied
248 Biosystems and Bio-Rad respectively, and considering the results, cost and complexity of the
249 protocols, the Chelex extraction was selected (see supporting information Table S2 and Figure SF1).
250 Finally, the assay described by Roumani et al., which consisted in a multiplex qPCR targeting *hly* along
251 with a competitive IAC, was selected (Roumani et al., 2021). For ease-of-use the primers, probes and
252 IAC DNA were pre-mixed concentrated 10X, and the mixture was stored in the fridge for up to 70 days
253 since the amplification efficiency was evaluated on regular intervals without significant changes (see
254 supporting information Figure SF2).

255 Laboratories 3, 4 and 5 correctly identified all the samples provided and did not report any problem
256 following the SOP provided. However, Laboratory 1 indicated that they experienced some problems
257 when preparing the initial bacterial suspension in the 50 mL tubes provided, even though the process
258 was supposed to be simple, by just adding 20 mL of sterile water, the bacterial tablet was not easily
259 dissolvable, so it was hypothesized that this may have caused issues in the uniformity of suspension
260 thus leading to the deviations observed. It is worth to note that no typical colonies were obtained by
261 this Laboratory in the 2 samples where the ND were identified thus these were classified as NA after
262 the confirmation, and so the SE and SP values obtained by this laboratory were 100 %.

263 Another discrepant result was obtained in the interlaboratory study, and this was from Laboratory 2
264 who reported a positive result by qPCR in sample 10, which was the one inoculated with *L. innocua*,
265 while it was negative by the culture-based approach. Considering that only one negative control was
266 included among the 10 samples, this generated that the SP of the method for this laboratory was 0 %,
267 while the SE 100 %. In order to understand the reported result, the data of the qPCR run was requested

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268 to the laboratory, and after a detailed analysis, it was observed that the reported result for this
269 particular sample was most likely associated to an incorrect setting of the threshold. The amplification
270 plots of all the samples are included in the supporting information Figure SF3, where it can be
271 observed that there was no actual amplification. Furthermore, this particular laboratory reported
272 significantly lower Cq values compared to all the other participants, that agrees with an incorrect
273 threshold setting, by placing it excessively low the positive samples reported very low Cq values, and
274 due to this the background noise of the negative sample was interpreted as a positive signal by the
275 software. As no specific parameters were provided in the SOP, in order to leave it open to any
276 thermocycler and software, the results provided by this laboratory were included just as reported,
277 meaning that sample 10 was considered a FP.

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

283 In the current study the lowest inoculation level tested was 9.3 CFU/ 25 g. This concentration was
284 detected by all the laboratories in all the samples spiked at this concentration, thus it was
285 demonstrated that the method can detect a very low concentration of *L. monocytogenes* (<10 CFU/
286 25 g), being this a similar value to the one reported in previous open formula validation studies
287 targeting *L. monocytogenes* like the one of D'Agostino et al. who reached a LOD of 20 CFU/ 25 mL of
288 milk combining a two-step enrichment and PCR (D'Agostino et al., 2004). Similarly, Oravcová et al.
289 managed to detect 1 CFU/ 25 g of *L. monocytogenes* in various food matrixes including smoked
290 salmon, implementing a two-step enrichment protocol (Oravcová et al., 2007). In a later study from
291 Gattuso et al., using meat as a model, they reached levels of 1-10 CFU/ 25 g implementing a single
292 enrichment step in Half Fraser Broth (Gattuso et al., 2014), and following a similar approach

293 Gianfranceschi et al., obtained similar results in fresh cheese (Gianfranceschi et al., 2014). More
294 recently, Vizzini et al. published a study where they reported been capable of detecting 10 CFU/ g of
295 *L. monocytogenes* in smoked salmon after an enrichment step in One Broth Listeria (Vizzini et al.,
296 2020), which is ten times higher than the value reported by Amagliani et al. in a similar matrix, salmon,
297 however they could have benefitted from an immunomagnetic separation step to concentrate the
298 bacteria of interest (Amagliani et al., 2010), however no proper evaluation of the LOD was performed
299 in either study and none of them were tested by independent laboratories. In terms of time of analysis,
300 the assay under evaluation in the present study reported results in line with previous studies which
301 implemented a single enrichment step, ~27 h, including the culture, DNA extraction and qPCR analysis.

302

303 5. Conclusions

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

311

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321 Tables:

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324 Figures:

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

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Table 1. Bacterial species used for sample spiking

Species	WDCM*	Amount per tablet (CFU)	95 % Confidence	Concentration (CFU/ mL)**	Final concentration (CFU/ 25 g)***
	00021	6.2×10^{1}	$3.5 \times 10^{0} – 1.1 \times 10^{1}$	3.1×10^{0}	9.3×10^{0}
L. monocytogenes	00021	9.1×10^{2}	$5.0 \times 10^{2} – 1.7 \times 10^{3}$	4.6×10^{1}	1.4×10^{2}
	00021	5.0×10^{3}	$2.0 \times 10^{3} – 5.3 \times 10^{3}$	1.6×10^{2}	4.9×10^{2}
L. innocua	00017	4.4×10^{3}	$1.3 \times 10^{2} – 1.5 \times 10^{3}$	2.2×10^{2}	6.6×10^{2}

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

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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*}	**	

*Copies of IAC DNA added per reaction. **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.

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

	Before Confirmation					After Confirmation		
Inoculation level	N	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 x 10^2 CFU/ 25 g. L1: low inoculation level, 9.3 CFU/ 25 g. L2: medium inoculation level, 1.4 x 10^2 CFU/ 25 g. L3: high inoculation level, 4.9 x 10^2 CFU/ 25 g.

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Table 4. Method evaluation summary

	Before Confirmation					After Confirmation						
Participant	N	PA	NA	PD	ND	FP	FN	TP	SE	SP	AC	k
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 to 0.8) and "almost complete concordance" (0.81 to 1.00) according to previous references (Anderson et al., 2011; DG, 1991).

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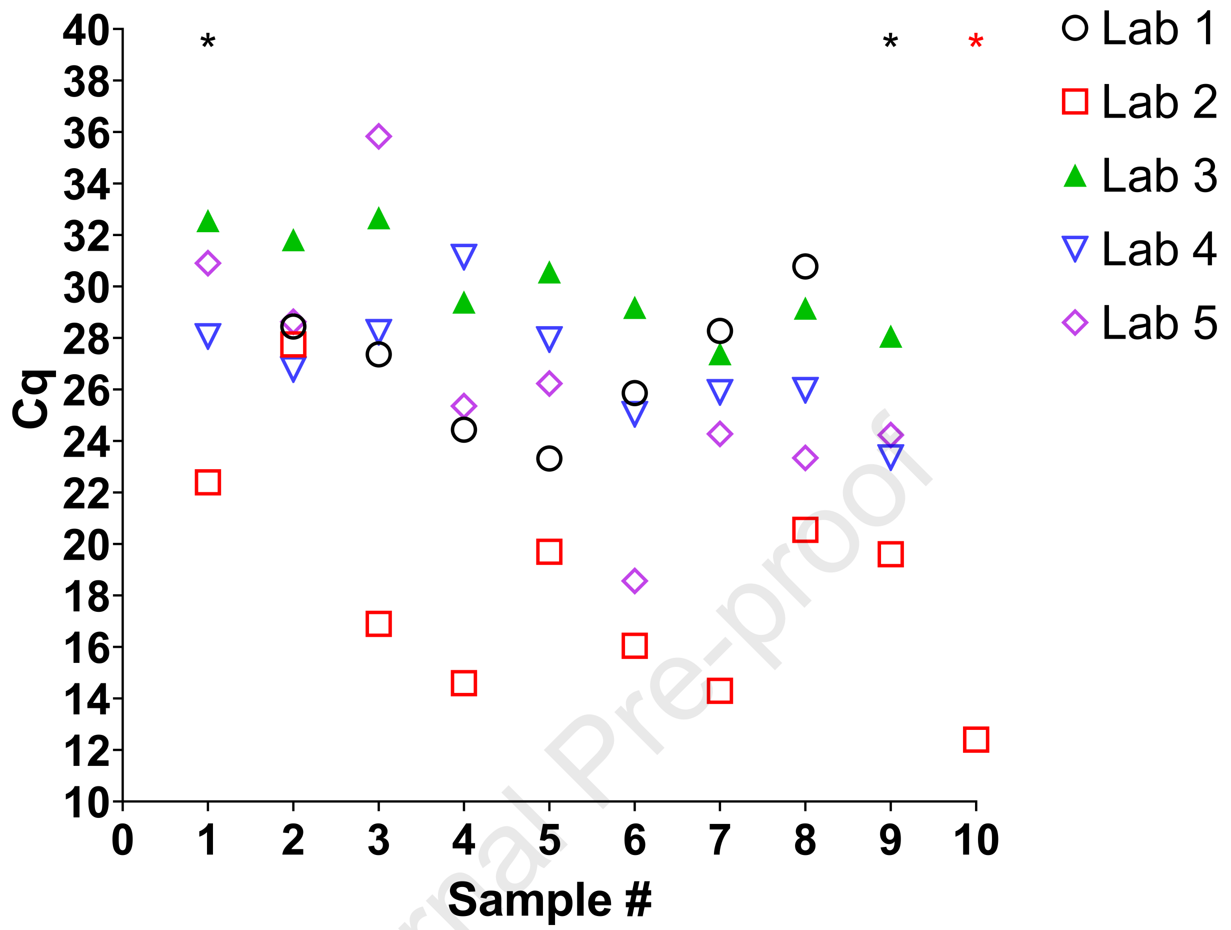

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Highlights

- An open formula qPCR assay to control *L. monocytogenes* in a novel ready-to-eat seafood product was evaluated.
- The evaluation was performed in an international interlaboratory ring trial.
- The assay targets the *hly* gene and includes a competitive internal amplification control.
- It was possible to detect < 10 CFU/ 25 g by all the participating laboratories.
- The Cohen's k obtained was 0.97, interpreted as "almost complete agreement".

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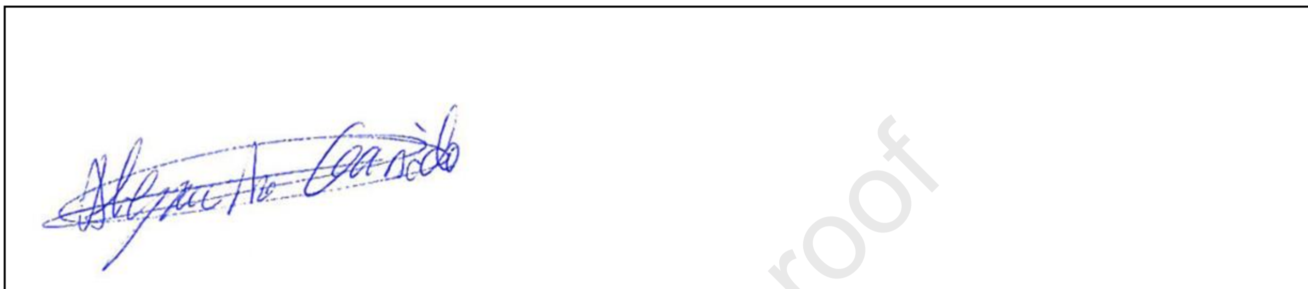
Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



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