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Comparative evaluation of impedanciometry combined with chromogenic agars or RNA hybridization and real-time PCR methods for the detection of *L. monocytogenes* in dry-cured ham

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## 1 Title

2 Comparative evaluation of impedanciometry combined with chromogenic agars  
3 or RNA hybridization and real-time PCR methods for the detection of *L.*  
4 *monocytogenes* in dry-cured ham

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## 26 Abstract

27 *Listeria monocytogenes* is an important foodborne pathogen of particular  
28 relevance in “Ready To Eat” products. Food producers require rapid methods to  
29 detect *L. monocytogenes*, since the reference method (ISO 11290-1) is  
30 laborious, lengthy and costly. The aim of this study was to evaluate three  
31 alternative methods to detect *L. monocytogenes* in dry-cured ham following the  
32 ISO 16140-2:2016 standard: (A) impedance measurement followed by plating  
33 onto chromogenic agars; (B) impedance measurement followed by RNA  
34 hybridisation, and (C) real-time PCR.

35 Inclusivity and exclusivity were evaluated. The limits of detection 50 (LOD<sub>50</sub>)  
36 and the relative limits of detection (RLOD) were obtained by analysing dry-  
37 cured ham samples inoculated with *L. monocytogenes* at three different levels  
38 of contamination. The sensitivity study of alternative methods, as well as the  
39 relative specificity (SP), sensitivity (SE), and Kappa Cohen’s index were  
40 calculated analysing 93 samples of sliced dry-cured ham. The inclusivity and  
41 exclusivity tests of three methods showed no interference in pathogen  
42 detection. LOD<sub>50</sub> were very low for the three methods evaluated (<1 cfu / 25 g  
43 dry-cured ham). The RLOD values of the three alternative methods were below  
44 the acceptability limit established by ISO 16140. For methods A and C, good  
45 results were obtained in the sensitivity study, as well as in the SP and SE.  
46 However, method B showed poorer results in the sensitivity study, along with  
47 lower results for SP (99.7%) and SE (79.6%), due to the occurrence of false  
48 positives and negatives in samples with presence of other *Listeria* spp.

49 Methods A and C were considered to be a thoroughly appropriate control tool  
50 for use in the meat industry to improve the detection of *L. monocytogenes*.

51 Keywords

52 *Listeria monocytogenes*; detection; rapid methods; RTE meat products

## 53 1. Introduction

54 *Listeria monocytogenes* is an important foodborne pathogen with a significant  
55 worldwide impact on public health and the economy. This bacterium causes  
56 listeriosis, a severe disease with a high fatality rate (20-30%) in specific risk  
57 groups such as pregnant women, neonates, the elderly and  
58 immunocompromised people (Zunabovic, Domig, & Kneifel, 2011). A total of  
59 2,206 confirmed human cases of listeriosis with a total of 270 deaths were  
60 reported in the European Union (UE) in 2015 (European Food Safety Authority  
61 (EFSA) and European Centre for Disease Prevention and Control (ECDC),  
62 2016). This was the highest number of deaths observed since 2008,  
63 representing one of the most frequent causes of human death due to foodborne  
64 illness (Cardoen et al., 2009; de Valk et al., 2005).

65 Owing to its elaborate physiological adaptation mechanisms, *L. monocytogenes*  
66 can survive and even proliferate under adverse environmental conditions such  
67 as refrigeration temperatures, low pH, high salinity and the presence of  
68 detergents (Gandhi & Chikindas, 2007; Pricope, Nicolau, Wagner, & Rychli,  
69 2013). It can also adhere to abiotic surfaces and form biofilms, which increase  
70 the possibility of a continuous contamination of the product-processing  
71 environment (Alessandria, Rantsiou, Dolci, & Cocolin, 2010).

72 *L. monocytogenes* has been isolated from a wide variety of "Ready To Eat"  
73 (RTE) products. Such foodstuffs are considered a major risk, since they have a  
74 relative long shelf life and are consumed without any listericidal treatment that  
75 could reduce the *L. monocytogenes* loads before consumption (EFSA/ECDC,

76 2008; Lianou & Sofos, 2007; Zhu, Du, Cordray, & Ahn, 2005). RTE meat  
77 products are very popular around the world due to their high palatability and  
78 convenience, as they need no cooking prior to eating (Awaisheh, 2010). Dry-  
79 cured ham is an important foodstuff in the Mediterranean area, and Spain is  
80 one of the major producers, consumers, and exporters. Dry-cured ham may be  
81 contaminated with *L. monocytogenes* during handling as a consequence of  
82 processing practices such as cutting, slicing, and packaging of finished  
83 products (Lambertz et al., 2012; Myers, Montoya, Cannon, Dickson, &  
84 Sebranek, 2013). Different studies have observed that the prevalence of this  
85 pathogen in this product varies widely: from 2% to 24.3%, although it is also  
86 present at low cell concentration (<100 cfu/g) (Giovannini et al., 2007; Gómez et  
87 al., 2015; López et al., 2008; Mena et al., 2004; Prencipe et al., 2012)

88 The presence of this pathogen requires great care in order to minimize the risk  
89 and improve food safety. The reference method for detection of *L.*  
90 *monocytogenes* is ISO 11290-1 (International Organization for Standardization  
91 (ISO), 2004). It is labour-intensive and lengthy: the time necessary to obtain a  
92 confirmed positive result is up to 7 days. The development of alternative rapid  
93 methods to detect *L. monocytogenes* is essential for food producers. They need  
94 efficient tools to control this pathogen, in order to comply with food safety  
95 regulations while minimizing economic losses. Different rapid methods have  
96 been developed for detection of *L. monocytogenes* such as immunoassays,  
97 fluorescent *in situ* hybridization, amplification methods or impedanciometry (Cho  
98 & Irudayaraj, 2013; Fuchizawa, Shimizu, Ootsubo, Kawai, & Yamazaki, 2009;  
99 Labrador, Rota, Pérez, Herrera, & Bayarri, 2018; Rodriguez-Lazaro, Gonzalez-  
100 Garcia, Gattuso, Gianfranceschi, & Hernandez, 2014). The impedance method

101 is based on the measurement of changes in electrical impedance of a culture  
102 medium due to the growth of microorganisms. This growth-based method  
103 distinguishes between viable and dead cells (Wawerla, Stolle, Schalch, &  
104 Eisgruber, 1999; Yang & Bashir, 2007; Yang, Ruan, & Li, 2007). Commercial  
105 impedance measurement equipment detects *Listeria* spp., so it is necessary to  
106 carry out a subsequent identification of *L. monocytogenes*. Different commercial  
107 alternatives designed to identify *L. monocytogenes* are available such as  
108 ELISA, chromogenic agars, and RNA hybridization kits. Real-time PCR may be  
109 used as an alternative method for rapid and specific identification, as well as  
110 avoiding cross-contamination since no post-PCR steps are needed (Amagliani,  
111 Giammarini, Omiccioli, Brandi, & Magnani, 2007; Fusco & Marina, 2012; Quero,  
112 Santovito, Visconti, & Fusco, 2014).

113 Impedance measurement followed by OCLA with the purpose of detecting *L.*  
114 *monocytogenes* in dry-cured ham has been previously studied in our laboratory  
115 (Labrador, Rota, Pérez, Herrera, & Bayarri, 2018). Excellent values of relative  
116 trueness, specificity and sensitivity were obtained. This paper expands on that  
117 work by shortening the detection time, and it includes a comparison with further  
118 rapid and confirmatory methods.

119 The aim of this study was to evaluate three alternative methods to detect *L.*  
120 *monocytogenes* in sliced dry-cured ham. These were: 1) impedance  
121 measurement followed by identification in chromogenic agars; 2) impedance  
122 measurement followed by RNA hybridization; and 3) the real-time PCR method.  
123 Results from the analysis of naturally contaminated samples of dry-cured ham  
124 could provide further valuable information for the process of risk assessment.

## 125 2. Materials and methods

126 The alternative methods were evaluated in comparison with the reference  
127 method (ISO 11290-1) following ISO 16140-2:2016 standard (ISO, 2016).  
128 Inclusivity, exclusivity, limit of detection 50 (LOD<sub>50</sub>), relative limit of detection  
129 (RLOD), and a sensitivity study of the alternative methods were performed.  
130 Additionally, apart from the parameters established by ISO 16140-2:2016, other  
131 validation indicators were determined. The alternative methods evaluated were:

- 132 - Method A: Pre-enrichment combined with impedance measurement,  
133 followed by plating on OCLA (Oxoid Chromogenic Listeria Agar) and  
134 Rapid L. mono.
- 135 - Method B: Pre-enrichment combined with impedance measurement,  
136 followed by RiboFlow<sup>®</sup> *Listeria Twin* flow assay.
- 137 - Method C: Pre-enrichment combined with real-time PCR (iQ-Check<sup>®</sup>  
138 *Listeria monocytogenes* II Kit).

## 139 2.1 Description of methods

140 The three methods evaluated and the reference method are schematized in  
141 Figure 1.

### 142 2.1.1 Method A

143 For the pre-enrichment step, the samples were incubated at 30°C for 24 h in  
144 One Broth Listeria (OB, Oxoid, Hampshire, England). Then, one ml of the pre-  
145 enrichment was inoculated in nine ml of OB placed in a specific four-electrode  
146 cell (SY-LAB Geräte GmbH, Neupurkersdorf, Austria). The measurement of  
147 impedance change (E- value) was monitored using a BacTrac 4300 apparatus  
148 (SY-LAB Geräte GmbH). The assay was carried out at 30°C for a maximum of  
149 24 h. Detection time (DT) was established for an E-value threshold of 5% to  
150 avoid background noise. The result was considered positive to *Listeria* spp.

151 when a typical impedance curve was observed and the selected threshold was  
152 reached.

153 As soon as the threshold value was attained, an identification step was  
154 performed. The enrichment was plated on OCLA (Oxoid) and Rapid L. mono  
155 agar (Bio-Rad, Marnes-La-Coquette, France), and incubated at 37°C for 24 h  
156 and 24-48 h, respectively.

157 Characteristic colonies of *L. monocytogenes* in OCLA are blue/green  
158 surrounded by an opaque halo. Colonies in Rapid L. mono agar are blue or  
159 greyish-blue without a yellow halo.

160 Presumptive *L. monocytogenes* colonies were confirmed using the Rhamnose  
161 Test (Bio-Rad) (37°C/24 h), since this pathogen is able to ferment that sugar, in  
162 contrast to *L. ivanovii*, which cannot.

#### 163 2.1.2. Method B

164 The pre-enrichment and the impedance measurement steps were the same as  
165 in method A, described in section 2.1.1. For the identification of *L.*  
166 *monocytogenes*, a RiboFlow® *Listeria Twin* kit (SY-LAB Geräte GmbH) was  
167 used. This is a lateral flow assay based on a specific hybridization of a rRNA  
168 target sequence from *L. monocytogenes*.

169 To summarize, after incubation in BacTrac 4300, 0.5 ml of the sample positive  
170 to *Listeria* spp. was centrifuged at 7,000 g/5 min. The supernatant was removed  
171 and the pellet was re-suspended with specific kit reaction buffers, and  
172 incubated at room temperature for 5 min. Then, the mix was placed in the  
173 lateral flow device and incubated for a maximum time of 15 min at 46°C.

#### 174 2.1.3 Method C



175 For the pre-enrichment step, the samples were incubated at 37°C for 25 h in  
176 *Listeria* Special Broth (LSB) (Bio-Rad).

177 The detection of the pathogenic species was performed by an iQ-Check®  
178 *Listeria monocytogenes* II Kit (Bio-Rad) and a Miniopticon® (Bio-Rad)  
179 thermocycler. This kit's method is based in the PCR amplification of a specific  
180 sequence of the *hly* gene of *L. monocytogenes*.

181 For this purpose, 100 µl of the pre-enrichment was mixed with 100 µl of the lysis  
182 reactive, disrupted for 4 min and incubated at 98 °C for 15 min. The samples  
183 were centrifuged at 11,000 g for 4 min and the supernatant was collected. 5 µl  
184 of template DNA and 45 µl of reaction mix were used for the amplification  
185 reaction. A *L. monocytogenes*-specific DNA sequence probe was linked to  
186 fluorophore FAM. An internal amplification control (IAC) linked to fluorophore  
187 HEX was present in each reaction tube. The amplification protocol was: 95°C  
188 for 10 min, followed by 49 cycles of denaturation at 94°C for 15 s, annealing at  
189 58°C for 20 s and extension at 72°C for 30 s.

190 PCR reaction positive and negative controls were included in each assay. The  
191 sample was considered positive when the Cq values were  $\geq 10$  and  $\geq 28$  for the  
192 target and the IAC, respectively.

193 The confirmation step for the positive sample was performed by plating 0.1 ml  
194 of the pre-enrichment onto Rapid L. mono agar, followed by incubation for 24 h  
195 at 37°C.

#### 196 2.1.4 Reference method (ISO 11290-1)

197 This study was performed under ISO 11290-1:1996/Amd 1:2004 (ISO, 2004),  
198 which was in force at that time.

199 For the pre-enrichment step, the samples were incubated in Half Fraser broth  
200 (HF, Oxoid) at 30°C for 24 h. Then, the pre-enrichment was plated on OCLA  
201 and Rapid L. mono agar. The media were incubated under the conditions  
202 previously described. In parallel, 0.1 ml of pre-enrichment was transferred into  
203 10 ml of Fraser broth (enrichment) (Oxoid) and incubated at 37°C for 48 h. The  
204 enrichment was plated on OCLA and Rapid L. mono. The presumptive *L.*  
205 *monocytogenes* colonies were confirmed using a Rhamnose Test (37°C/24 h).

## 206 2.2 Evaluation parameters following ISO 16140-2:2016

207 Following ISO 16140:2016-2 guidelines, different types of samples were  
208 analysed depending on the parameter evaluated. In each section, the samples  
209 used for the determination of the specific parameters are described.

### 210 2.2.1 Inclusivity and exclusivity tests

211 Inclusivity is defined as the ability of the alternative method to detect the target  
212 analyte from a wide range of strains. Exclusivity is the lack of interference in the  
213 alternative method from a relevant range of non-target strains, which are  
214 potentially cross-reactive.

215 In this study, the three pre-enrichment media previously described were  
216 inoculated with one of eleven strains: six strains of *L. monocytogenes*, three  
217 strains of *L. innocua*, one strain of *L. welshimeri*, and *Enterococcus durans*  
218 CECT 411. Table 1 shows the origin of the strains and the level of the inoculum  
219 used. For this purpose, an isolated colony of each strain was incubated  
220 overnight at 37°C in 10 ml of Brain Heart Infusion (BHI) broth. Then, serial ten-  
221 fold dilutions in peptone water 0.1% were carried out. One hundred µl of  
222 selected dilution of each *Listeria* spp. strain were inoculated in 225 ml of the  
223 three different pre-enrichment broths previously described. *E. durans* CECT 411

224 was inoculated in 225 ml of BHI broth, in order not to inhibit the microorganism's  
225 growth, as required by ISO 16140. Colony counts of the selected dilution on  
226 BHIA (37°C/24 h) was used to obtain the concentration of the microorganisms  
227 in the inoculum.

228 In each assay, positive and negative controls were included for each  
229 methodology. The positive control was performed by artificial contamination of  
230 225 ml pre-enrichment medium with  $2 \times 10^3$  CFU *L. monocytogenes* UZ64, while  
231 225 ml pre-enrichment medium sterile without inoculation was used as negative  
232 control. The assays were performed in triplicate.

### 233 *2.2.2 Limit of detection 50 and relative limit of detection*

234 The LOD<sub>50</sub> value estimates the minimum level of contamination (cfu/25 g)  
235 resulting in positive detection in 50% of cases. The RLOD is defined as the  
236 relation between the LOD<sub>50</sub> values of alternative and reference methods. The  
237 assays were carried out for methods A, B, C and the reference method for the  
238 detection of *L. monocytogenes* in artificially contaminated dry-cured ham  
239 samples. The acceptability limits for RLOD was established by ISO 16140-  
240 2:2016

#### 241 *2.2.2.1 Bacterial strain and preparation of inocula*

242 An isolated colony of *L. monocytogenes* UZ64 was incubated at 37°C/16 h in 10  
243 ml of BHI broth, in order to obtain  $2 \times 10^9$  cfu/ml. Serial ten-fold dilutions were  
244 performed in peptone water 0.1% to a cell concentration of  $2 \times 10^1$  cfu/ml.  
245 Colony counting in BHIA (37°C/24 h) was used in order to obtain the  
246 concentration of microorganisms in the inoculum.

#### 247 *2.2.2.2 Artificial contamination of dry-cured ham samples used for LOD<sub>50</sub> and* 248 *RLOD*

249 Sliced and vacuum-packed dry-cured ham samples were analysed at three  
250 levels of contamination: 0 cfu/25 g (Level 1), 0.3 cfu/25 g (Level 2) and 0.9  
251 cfu/25 g (Level 3). For Level 1, 100 g of dry-cured ham were homogenised with  
252 900 ml of each of the three different pre-enrichment media previously  
253 described. For Levels 2 and 3, 100 g of dry-cured ham were homogenised with  
254 900 ml of each of the three different pre-enrichment media, and they were  
255 inoculated with 60  $\mu$ l and 180  $\mu$ l of  $2 \times 10^1$  *L. monocytogenes* UZ64 cfu/ml,  
256 respectively. After inoculation, additional homogenisation was carried out using  
257 a Stomacher® 400 Circulator (Seward Ltd, Worthing, UK) blender (260 rpm/2.5  
258 min). In the case of each methodology, an individual sample was analysed six  
259 times.

260 Positive and negative controls were used for each methodology. For the  
261 positive controls, 25 g of dry-cured ham was inoculated with  $2 \times 10^3$  cfu *L.*  
262 *monocytogenes* UZ64. The negative controls were the Level 1 samples  
263 analysed with each methodology.

### 264 2.2.3 Sensitivity study

265 To perform the sensitivity study of the alternative methods, ISO 16140-2:2016  
266 required the determination of the following parameters: sensitivity of alternative  
267 ( $SE_{alt}$ , the ability of the alternative method to detect the analyte), relative  
268 trueness (RT, the degree of correspondence between the responses obtained  
269 by the alternative and reference methods), false positive rate (FPR), and the  
270 subtraction between negative deviation and positive deviation (ND-PD).

271 The acceptability limits for these parameters were established by ISO 16140-  
272 2:2016. In this study, the limits applied were for unpaired results, since the  
273 alternative and reference methods did not share the pre-enrichment step.

274 Additionally, relative specificity (SP) and sensitivity (SE), positive predictive  
275 value (PPV), and negative predictive value (NPV) were determined for the  
276 alternative methods (Anderson et al., 2011; NordVaL, 2017; Tomás, Rodrigo,  
277 Hernández, & Ferrús, 2009). SP is defined as the alternative method's inability  
278 to detect the analyte when it is not detected by the reference method. SE  
279 determines the ability of the alternative method to detect the analyte when it is  
280 detected by the reference method. PPV and NPV were calculated as the  
281 method's measure of performance by assaying the probability of a sample  
282 being truly positive or negative when the method has a positive or negative  
283 result. The degree of agreement between the alternative methods and the  
284 reference method in dry-cured ham samples was quantified via Cohen's Kappa  
285 index. Kappa values are categorised as follows:  $\leq 0.20$  poor agreement;  
286 between 0.20 and 0.4 fair agreement; between 0.41 and 0.60 moderate  
287 agreement; between 0.61 and 0.80 good agreement;  $\geq 0.81$  very good  
288 agreement. The NordVaL International organization requires SE  $>95\%$  and very  
289 good agreement between alternative and reference methods to obtain a  
290 method's validation (NordVaL, 2017).

291 To carry out this sensitivity study, naturally and artificially contaminated samples  
292 were analysed. These samples were different from those used to determine  
293 LOD<sub>50</sub> and RLOD.

#### 294 2.2.3.1 Bacterial strains and preparation of inocula

295 The strains assayed were *L. monocytogenes* UZ64, *L. monocytogenes* UZ108,  
296 *L. innocua* UZ1, *L. innocua* UZ68, and *L. welshimeri* UZ40. An isolated colony  
297 of each strain was incubated overnight at 37°C in 10 ml of BHI broth to reach  
298  $2 \times 10^9$  cfu/ml. Serial ten-fold dilutions of each strain in peptone water 0.1% were

299 carried out in order to obtain concentrations of  $2 \times 10^3$  cfu/ml,  $2 \times 10^2$  cfu/ml and  
300  $2 \times 10^1$  cfu/ml.

301 Subsequently, ten mixtures of *Listeria* spp. strains at proportions of 1:1 and  
302 1:100 (*L. monocytogenes*:other *Listeria* specie) were performed to inoculate the  
303 dry-cured ham samples.

304 Colony counting of the inoculum on Rapid L. mono agar (37°C/24 h) was used  
305 in order to ascertain the concentration of microorganisms in the inoculum.

#### 306 2.2.3.2 Dry-cured ham samples used for the sensitivity study

307 A total of 93 samples of 25 g of sliced and vacuum-packed dry-cured ham were  
308 analysed by the methods A, B, C, and by the reference method. Forty-four of  
309 those samples were naturally contaminated. For assays using artificial  
310 contamination, 49 samples of 25 g were used: 20 were contaminated with *L.*  
311 *monocytogenes* and 29 were co-contaminated with mixes formed by *L.*  
312 *monocytogenes* and *Listeria innocua* or *Listeria welshimeri*, in the different  
313 proportions described above. The Log cfu of *Listeria* spp. in 25 g of dry-cured  
314 ham for each group of samples is described in Table 2.

315 For the analysis of naturally contaminated samples, 75 g of dry-cured ham were  
316 homogenised with 75 ml of sterile distilled water using a Stomacher® 400  
317 Circulator blender (260 rpm/2.5 min). With this step, a homogeneous paste was  
318 obtained, which allowed the subdivision of the sample into three portions of 50  
319 g each. Each portion of 50 g was mixed with 200 ml of each pre-enrichment (10  
320 % more concentrated) to obtain 25 g of dry-cured ham in 225 ml of medium.

321 The samples were analysed by the A, B, C and reference methods. For  
322 artificially contaminated samples, 100 g of dry-cured ham were homogenised

323 with 100 ml sterile distilled water, following the same procedure described  
324 above.

325 The sample was subdivided into four portions of 50 g each. Subsequently, three  
326 of them were inoculated with *Listeria* spp. inoculum, following the guidelines of  
327 ISO 16140:2016-2. After the inoculation, each portion was homogenised in a  
328 blender (260 rpm/2.5 min) with 200 ml of selected pre-enrichment medium (10  
329 % more concentrated), and analysed by the A, B, C and reference methods.  
330 The remaining portion was analysed by reference method as a negative control.  
331 All samples were analysed in triplicate using all four methods.

### 332 2.3 Statistical analysis

333 Statistical analysis was performed using Excel software, Version 14.2.0  
334 (Microsoft Corporation, Redmond, WA, USA) and SPSS statistics 22.0.0  
335 software (SPSS. Inc., Chicago, IL, USA). LOD<sub>50</sub> was calculated using the  
336 Spearman & Karber test. The statistical study of detection times was carried out  
337 using the t-student test.

## 338 3. Results and discussion

### 339 3.1 Inclusivity and exclusivity

340 All the strains tested gave the expected results with methods A, B, and C in the  
341 inclusivity and exclusivity tests (Table 3). Discriminating *L. monocytogenes* from  
342 the other *Listeria* species is a challenge, since they are phylogenetically and  
343 phenotypically closely related. The three alternative methods evaluated were  
344 adequate due to the absence of cross-reaction with all the strains tested,  
345 including *L. innocua*, which is closely related to *L. monocytogenes* (Quero et al.,  
346 2014; Schmid et al., 2005).

### 347 3.2 Limit of detection 50 and relative limit of detection

348 The LOD<sub>50</sub> were similar for the three methods studied and for the reference  
349 method, showing low values (<1 cfu of *L. monocytogenes*/25 g) for all, as  
350 displayed in Table 4. The acceptability limit of RLOD for unpaired studies is 2.5  
351 (ISO, 2016). The RLODs for methods A and B were 1.265. The RLOD for the  
352 method C was 1.000. Thus, the RLODs of the three evaluated alternative  
353 methods complied with the established limits. Achieving a low limit of detection  
354 is an important challenge, since the concentration of *L. monocytogenes* in foods  
355 is usually low (< 100 cfu/g), and the cell may have suffered sub-lethal injury due  
356 to heat, drying or the presence of antimicrobial compounds (Wu, 2008). The  
357 alternative methods should be able to resuscitate *L. monocytogenes* and  
358 support its replication up to adequate levels for detection. Pre-enrichment is a  
359 crucial step in order to assure this fact prior to exposure to selective agents  
360 (Delibato et al., 2009; Oravcová, Kuchta, & Kaclíková, 2007; Oravcová,  
361 Trnčíková, Kuchta, & Kaclíková, 2008; Rodriguez-Lazaro et al., 2014). If this is  
362 not possible, false-negative results can appear and contaminated products can  
363 reach the consumer, increasing the risk for public health as well as economic  
364 losses.

365 In the literature, the limits of detection are determined and presented from  
366 different approaches. In the present study, the LOD<sub>50</sub> were determined following  
367 the guidelines established in ISO 16140-2:2016. It might be of interest to point  
368 out that the limit of detection was calculated in a standardized way in order to  
369 compare results among methods. Portanti et al., (2011) developed and  
370 validated an ELISA method to detect *L. monocytogenes* in food, obtaining 5-10  
371 cfu/g for LOD<sub>50</sub>. Following the same trend, Ruiz-Rueda, Soler, Calvó, & García-  
372 Gil, (2011) established a limit of detection of 5 cfu/25 g analysing 22 different



373 matrices (eight times for each sample) via the real-time PCR method.  
374 Differences in the limits of detection among different types of foods were  
375 observed by Rossmanith, Krassnig, Wagner, & Hein, (2006), combining real-  
376 time PCR with enrichment (24 h). These authors obtained a limit of detection of  
377 7.5 cfu/25 ml and 1-9 cfu/15 g in artificially contaminated raw milk, and salmon,  
378 pâté and cheese, respectively. Also, O'Grady et al., (2009) observed a limit of  
379 detection of 1-5 cfu/25 g analysing 175 samples (meat, fish, dairy products, and  
380 desserts) combining enrichment (24h) and real-time PCR. Therefore, as is  
381 reflected in ISO 16140-2:2016, it would be necessary to evaluate the detection  
382 method for each category of food analysed.

### 383 3.3 Sensitivity study

384 The results of 93 analysed samples of dry-cured ham are shown in Table 5.  
385 For methods A and C,  $SE_{alt}$ , RT, SP, SE, PPV and NPV were 100 %. FPR  
386 values were 0% for both methods, due to absence of false-negative or false-  
387 positive results. The limit of acceptability of ND-PD for an unpaired study is set  
388 at 3 (ISO, 2016). The ND-PD values were 0 for methods A and C, therefore  
389 lying within the limits of acceptability. The Cohen's Kappa index for methods A  
390 and C was 1 in each case, thereby indicating very good agreement with the  
391 reference method. The pathogen grew on OCLA and Rapid L. mono in the *L.*  
392 *monocytogenes*-positive samples, providing an excellent correlation between  
393 those two agars. These results were in concordance with diverse authors, who  
394 observed the suitable correctness of Rapid L. mono (Becker et al., 2006).  
395 On the other hand, ten false negatives and one false positive were obtained by  
396 method B in comparison with the reference method. As a consequence, a  
397 decrease of  $SE_{alt}$  (80 %), RT (88.2 %), SP (99.7 %), SE (79.6 %), PPV (97.5

398 %), and NPV (81.1 %) was observed. The FPR value (2 %) was higher than for  
399 methods A and C. The ND-PD of method B was 9: this value was above the  
400 limit of acceptability as prescribed by the ISO 16140-2:2016 standard. Also, SE  
401 value was lower than the limit established by NordVaL (NordVaL, 2017). The  
402 Cohen's Kappa index for method B was 0.7, which can be considered to be in  
403 good agreement with the reference method, but did not comply with the limit  
404 (kappa >0,80) established by the guidelines of NordVal International for  
405 validation of alternative methods (NordVaL, 2017).

406 Obtaining high values (>95%) for SE<sub>alt</sub>, RT, SP, SE, PPV, NPV and, ND-PD  
407 value within the limits of acceptability is an important fact, since these  
408 parameters determine whether the developed method is suitable for analysing  
409 the target in the matrix.

410 In a previous study carried out by our research group, an impedance  
411 measurement combined with OCLA to detect *L. monocytogenes* in dry-cured  
412 ham offered excellent values for RT, SP and SE (Labrador, Rota, Pérez,  
413 Herrera, & Bayarri, 2018), but the impedance measurement time was longer  
414 than in the present study (40 h vs 24 h).

415 As far as we know, no existing studies regarding the use of impedance  
416 measurement followed by RNA hybridization, for the detection of *L.*  
417 *monocytogenes* in dry-cured ham, since we are dealing here with a research  
418 novelty.

419 However, the use of real-time PCR for this purpose has been studied in meat  
420 products. Diverse studies have compared real-time PCR with the reference  
421 method to detect *L. monocytogenes*, obtaining results that our similar to our  
422 study. Garrido et al., (2013) developed a new multiplex real-time PCR method

423 to detect *L. monocytogenes* (*hly* gene) and *Salmonella* spp. (*invA* gene) in  
424 diverse categories of food, finding values of 100% for SP, SE, and RT in meat  
425 products. A slightly lower value for RT (<90%) was observed by Delibato et al.,  
426 (2009), who used conventional PCR with classical gel electrophoresis to detect  
427 *L. monocytogenes* in meat products. The detection of *L. monocytogenes* using  
428 the *hly* gene as a target has been evaluated or validated in several food  
429 matrices. Rodriguez-Lazaro et al., (2014) analysed 100 samples of meat via the  
430 real-time PCR (*hly* gene-IAC) and the reference method. These authors  
431 achieved a high RT value ranging between 100% and 113.6%. Similarly to our  
432 study, the real-time PCR method used was based on the co-amplification of a  
433 specific region of the *L. monocytogenes hly* gene and IAC. The positive IAC  
434 signal confirms that the negative result is not due to an inhibition during  
435 amplification, thus reducing the false-negative rate (Hoorfar et al., 2004). The  
436 need to reduce the occurrence of false negatives is a specific public health  
437 concern, since batches of food contaminated with *L. monocytogenes* would  
438 reach consumers.

439 In the present study, dry-cured ham samples were artificially contaminated with  
440 *L. monocytogenes* and co-contaminated with *L. innocua* or *L. welshimeri* in  
441 order to reproduce as faithfully as possible the scenario that occurs in food  
442 samples (Sauders et al., 2012; Simmons et al., 2014; Vongkamjan et al., 2016).  
443 Method B were affected by the presence of other species. All the false-  
444 negative results obtained in method B corresponded to samples artificially co-  
445 contaminated with the mixes of *Listeria* spp., *L. innocua* and *L. welshimeri* were  
446 present in seven and three of false negatives observed. Among them, 90% of  
447 the false negatives were obtained from samples co-contaminated with mixes at

448 a proportion of 1:100 (*L. monocytogenes*:other *Listeria* spp.). In the current  
449 study, false-negatives may be caused by a possible low sensitivity of *RiboFlow*<sup>®</sup>  
450 *Listeria Twin* or/and by the negative influence of the presence of other species  
451 of *Listeria* on the growth of *L. monocytogenes* strains. Different studies have  
452 highlighted the possibility that the presence of other, more competitive species  
453 of *Listeria*, or the production of inhibitory substances during selective  
454 enrichment, could produce a decrease in the growth of *L. monocytogenes*. This  
455 fact could lead researchers or testing personnel to underestimate their  
456 presence and thereby increase the risk of listeriosis due to the non-detection of  
457 this pathogen in food (Besse et al., 2010; Keys, Dailey, Hitchins, & Smiley,  
458 2013; Oravcová et al., 2008; Zitz, Zunabovic, Domig, Wilrich, & Kneifel, 2011).  
459 The detection of *L. monocytogenes* could be influenced by the food's intrinsic  
460 and extrinsic characteristics. Dry-cured ham matrix is complex, with high NaCl  
461 and fat content, and possesses abundant background flora that could affect the  
462 detection of the pathogen (Barros et al., 2007; O'Grady et al., 2009; Suh &  
463 Knabel, 2001). In a previous study carried out by our research group, the  
464 pathogen was subjected to stressful conditions before inoculation in dry-cured  
465 ham. The detection of *L. monocytogenes* was not influenced by previous stress  
466 (Labrador, Rota, Pérez, Herrera, & Bayarri, 2018). This demonstrated that pre-  
467 enrichment media allowed the recovery and multiplication of the pathogen. In  
468 the current study, the alternative methods A and C were not affected by the  
469 food matrix, which allowed the detection of a low number of *L. monocytogenes*.  
470 Moreover, Prencipe et al. (2012) observed that the drying of the ham surface  
471 decreased the contamination levels, but the pathogen was able to survive and  
472 could be detected by the reference method. Similarly, Hospital et al. (2017)

473 determined that the pH,  $a_w$  and temperature conditions during the entire  
474 experimental process of dry-cured ham elaboration would indeed allow the  
475 growth of *Listeria* and its detection.

#### 476 3.4 Suitability of the evaluated methods

477 With respect to the impedance measurement carried out for the methods A and  
478 B, no signal was observed for samples that did not contain *Listeria* spp.

479 In all the *Listeria* spp. positive samples, the signal was due to the growth of the  
480 *Listeria* spp. present. Since for impedance measurement, the concentration of  
481  $10^6$ - $10^7$  cfu/ml is required for the typical curve to reach the threshold and for the  
482 DT to appear (Yang & Bashir, 2007), the DTs observed were shorter in the  
483 samples co-contaminated with *Listeria* spp. ( $p \leq 0.05$ ). In the case of the co-  
484 contaminated samples, the signal produced by *L. monocytogenes* was added to  
485 that generated by the strains of *L. innocua* or *L. welshimeri*. Concretely, DTs  
486 obtained for samples contaminated with the pathogen species alone, were from  
487 7.19 to 14.80 h, while, for co-contaminated samples, the DTs ranged from 0.64  
488 to 13.95 h. Globally, the DTs obtained for samples with a presence of *L.*  
489 *monocytogenes* were from 0.64 to 14.80 h.

490 The impedance measurement proved to be an excellent screening for *Listeria*  
491 spp.-negative samples, since the absence of this species was obtained in 2  
492 days. This is one of the few growth-based methods for detection of bacteria  
493 capable of differentiating dead cells from live cells, thereby significantly  
494 improving food safety.

495 In the case of method A, OCLA and Rapid L. mono offered the same results  
496 with respect to the parameters evaluated for identification *L. monocytogenes*.  
497 Rapid L. mono is faster, since the incubation time was 24 h compared to OCLA

498 (48 h). Method A followed by Rapid L. mono agar was selected, since it  
499 required 2.5-4 days to obtain a *L. monocytogenes*-positive confirmed result.  
500 However, the alternative method A is not always more rapid than the reference  
501 method, because the impedance measurement can take a few hours to one full  
502 day, depending on the cell concentration. Despite this, workflow was improved  
503 since this alternative method permitted the simultaneous analysis of multiple  
504 samples.

505 Method B was faster than the reference method and enabled the obtaining of a  
506 positive confirmed result in 48 h. However, the quality values obtained in the  
507 sensitivity study were not satisfactory.

508 Method C based on real-time PCR allowed the obtaining of negative- and  
509 positive-confirmed results in 26 and 48 h, respectively. Generally, nucleo-acid  
510 based methods are very specific and sensible, since they target a single  
511 specific sequence. The main drawback of PCR is that it generates false-positive  
512 results due to the fact that it can not distinguish between dead and live cells.  
513 However, the pre-enrichment step prior to PCR is used to reduce false  
514 positives, because this also involves diluting the sample and thus reducing the  
515 concentration of dead cells (Krascsenicsová, Píknová, Kačíková, & Kuchta,  
516 2008).

517 Attractively, real-time PCR can be monitored and automatized, improving the  
518 workflow and reducing the costs compared with the reference method  
519 (Rodriguez-Lazaro et al., 2014; Välimaa, Tilsala-Timisjärvi, & Virtanen, 2015).

### 520 *3.5 Presence of L. monocytogenes in dry-cured ham commercial samples*

521 In our study, 44 samples of sliced and packed dry-cured ham were analysed,  
522 and *L. monocytogenes* was not detected in any of them. Usually, the presence

523 of the pathogen in this product is low. Giovannini et al., (2007) found 4% of  
524 prevalence of the pathogen analysing 490 samples of de-boned dry-cured ham,  
525 and Mena et al., (2004) detected 2 % of positive samples, analysing 44  
526 samples of dry-cured ham. However, Gómez et al., (2015) analysed 37 samples  
527 of dry-cured ham by the reference method. These authors detected the  
528 pathogen's presence in 24.3% of the samples at day 0, while the percentage  
529 decreased to 2.7 % throughout the whole shelf-life of the product. The authors  
530 provided a partial explanation with the theory of metabolic exhaustion and  
531 stress response in hurdle technology applied to the manufacturing and storage  
532 of RTE meat products (Leistner, 2000). The presence of *L. monocytogenes* in  
533 dry-cured ham may be produced by a cross-contamination through operations  
534 such as deboning, slicing and packing (Chaitiemwong, Hazeleger, Beumer, &  
535 Zwietering, 2014; Myers et al., 2013; Ortiz et al., 2010). *L. monocytogenes* can  
536 be present in dry-cured ham, but its growth may be difficult due to the  
537 physicochemical characteristics of the product (low water activity, presence of  
538 nitrates, and high salinity). Thus, the concentration of the pathogen was usually  
539 low, never exceeding 100 cfu/g at the end of the shelf-life (Giovannini et al.,  
540 2007; Gómez et al., 2015).

#### 541 4. Conclusions

542 The evaluation of the three methods assayed in this study showed that the  
543 impedance method followed by Rapid L. mono and real-time PCR method (iQ-  
544 Check *Listeria monocytogenes* II kit) were reliable, easy to use, and time-  
545 saving. Furthermore, the handling of multiple samples and the avoidance of  
546 cross contamination are attractive tools to help improve the routine control of *L.*  
547 *monocytogenes* in the meat industry.

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768 ready-to-eat foods and manufacture environments - A review. *LWT - Food*  
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- 770



771 Table 1. Target and non- target strains used for inclusivity and exclusivity tests

Strain	Origin	Level of inoculum <sup>a</sup> (Log cfu/225 ml pre-enrichment)
<i>L. monocytogenes</i> UZ22	Fresh longaniza	1.05±0.19
<i>L. monocytogenes</i> UZ64	Dry-cured ham	1.46±0.13
<i>L. monocytogenes</i> UZ102	Fresh longaniza	1.38±0.08
<i>L. monocytogenes</i> UZ104	Fresh longaniza	1.37±0.12
<i>L. monocytogenes</i> UZ106	Dry-cured ham	1.03±0.30
<i>L. monocytogenes</i> UZ108	Cured longaniza	1.33±0.10
<i>L. innocua</i> UZ1	Fresh longaniza	2.37±0.08
<i>L. innocua</i> UZ65	Surfaces from RTEMP	1.81±0.14
<i>L. innocua</i> UZ68	Cheese	2.17±0.14
<i>L. welshimeri</i> UZ40	Cured longaniza	2.33±0.08
<i>E. durans</i> CECT 411	Dried milk	7.81±0.09

772 <sup>a</sup>Log cfu mean ± standard deviation from six replicates obtained by colony count on BHI agar

773 Table 2. Distribution of dry-cured ham samples analysed by the three methods  
 774 evaluated and reference method to detect *L. monocytogenes* (n=93).

Type of sample	Log cfu/25 g of dry-cured ham <sup>a</sup>		Number of samples
Commercial dry-cured ham	Not contaminated		44
Dry-cured ham contaminated with <i>L. monocytogenes</i> UZ64	0.58±0.01		20
Dry-cured ham contaminated with <i>L. monocytogenes</i> UZ64 and <i>L. innocua</i> UZ1 <sup>b</sup>	<i>L. monocytogenes</i> UZ64	0.42±0.07	3
	<i>L. innocua</i> UZ1	0.32±0.09	
	<i>L. monocytogenes</i> UZ64	0.42±0.07	3
	<i>L. innocua</i> UZ1	2.32±0.09	
Dry-cured ham contaminated with <i>L. monocytogenes</i> UZ64 and <i>L. innocua</i> UZ68 <sup>b</sup>	<i>L. monocytogenes</i> UZ64	0.42±0.07	3
	<i>L. innocua</i> UZ68	0.19±0.13	
	<i>L. monocytogenes</i> UZ64	0.42±0.07	3
	<i>L. innocua</i> UZ68	2.19±0.13	
Dry-cured ham contaminated with <i>L. monocytogenes</i> UZ64 and <i>L. welshimeri</i> UZ40 <sup>b</sup>	<i>L. monocytogenes</i> UZ64	0.42±0.07	3
	<i>L. welshimeri</i> UZ40	0.40±0.06	
	<i>L. monocytogenes</i> UZ64	0.42±0.07	3
	<i>L. welshimeri</i> UZ40	2.40±0.06	
Dry-cured ham contaminated with <i>L. monocytogenes</i> UZ108 and <i>L. innocua</i> UZ1 <sup>b</sup>	<i>L. monocytogenes</i> UZ108	0.29±0.12	2
	<i>L. innocua</i> UZ1	0.32±0.09	
	<i>L. monocytogenes</i> UZ108	0.29±0.12	3
	<i>L. innocua</i> UZ1	2.32±0.09	
Dry-cured ham contaminated with <i>L. monocytogenes</i> UZ108 and <i>L. welshimeri</i> UZ40 <sup>b</sup>	<i>L. monocytogenes</i> UZ108	0.29±0.12	3
	<i>L. welshimeri</i> UZ40	0.40±0.06	
	<i>L. monocytogenes</i> UZ108	0.29±0.12	3
	<i>L. welshimeri</i> UZ40	2.40±0.06	

775 <sup>a</sup>Mean ± standard deviation from six replicates obtained by of colony count on Rapid *L. mono*

776 agar

777 <sup>b</sup>Two different proportions *L. monocytogenes*:other *Listeria* specie (1:1 and 1:100) were

778 assayed for each pair of microorganisms

779 Table 3. Inclusivity and exclusivity test of methods assayed.

	Method A <sup>a</sup>	Method B <sup>b</sup>	Method C <sup>c</sup>
<i>L. monocytogenes</i> UZ22	+	+	+
<i>L. monocytogenes</i> UZ64	+	+	+
<i>L. monocytogenes</i> UZ102	+	+	+
<i>L. monocytogenes</i> UZ104	+	+	+
<i>L. monocytogenes</i> UZ106	+	+	+
<i>L. monocytogenes</i> UZ108	+	+	+
<i>L. innocua</i> UZ1	-	-	-
<i>L. innocua</i> UZ65	-	-	-
<i>L. innocua</i> UZ68	-	-	-
<i>L. welshimeri</i> UZ40	-	-	-
<i>E. durans</i> CECT 411	-	-	-

780 Each microorganism was assayed in triplicate (n=3)

781 <sup>a</sup>Method A: Impedance measurement followed by OCLA and Rapid L. mono782 <sup>b</sup>Method B: Impedance measurement followed by RiboFlow<sup>®</sup> *Listeria Twin*783 <sup>c</sup>Method C: Real time PCR (iQ-Check<sup>®</sup> *Listeria monocytogenes* II Kit)

784

785 Table 4. Limit of detection 50 (LOD<sub>50</sub>) and relative limit of detection (RLOD) of  
 786 three methods evaluated and reference method for detection of *L.*  
 787 *monocytogenes*.

	Signal ratio <sup>b</sup>			LOD <sub>50</sub> <sup>d</sup>	RLOD <sup>e</sup>
	0 <sup>c</sup>	0.3 <sup>c</sup>	0.9 <sup>c</sup>		
Method A <sup>a</sup>	0/6	1/6	6/6	(0.3-0.7)	1.265
Method B <sup>a</sup>	0/6	1/6	6/6	(0.3-0.7)	1.265
Method C <sup>a</sup>	0/6	2/6	6/6	(0.2-0.6)	1.000
Reference method	0/6	2/6	6/6	(0.2-0.6)	1.000

788 <sup>a</sup>Method A: Impedance measurement followed by OCLA and Rapid L. mono; Method B: Impedance  
 789 measurement followed by *RiboFlow*<sup>®</sup> *Listeria Twin*; Method C: Real time PCR (*iQ-Check*<sup>®</sup> *Listeria*  
 790 *monocytogenes* II Kit).

791 <sup>b</sup>Positive results of 6 replicates.

792 <sup>c</sup>cfu/25 g

793 <sup>d</sup>Limit of detection (LOD<sub>50</sub>) was calculated as a confidence interval of 95%.

794 <sup>e</sup>Relative limit of detection (RLOD)

795

796 Table 5. Detection of *L. monocytogenes* by the three methods evaluated and  
 797 reference method in dry-cured ham samples.

	Reference method +	Reference method -
Method A +	PA 49	PD 0
Method A -	ND 0	NA 44
Method B +	PA 39	PD 1
Method B -	ND 10	NA 43
Method C +	PA 49	PD 0
Method C -	ND 0	NA 44

798 PA: Positive Accordance; PD: Positive Deviation; ND: Negative Deviation; NA: Negative Accordance

799 Method A: Impedance measurement followed by OCLA and Rapid L. mono; Method B: Impedance  
 800 measurement followed by *RiboFlow*<sup>®</sup> *Listeria Twin*; Method C: Real time PCR (*iQ-Check*<sup>®</sup> *Listeria*  
 801 *monocytogenes* II Kit).

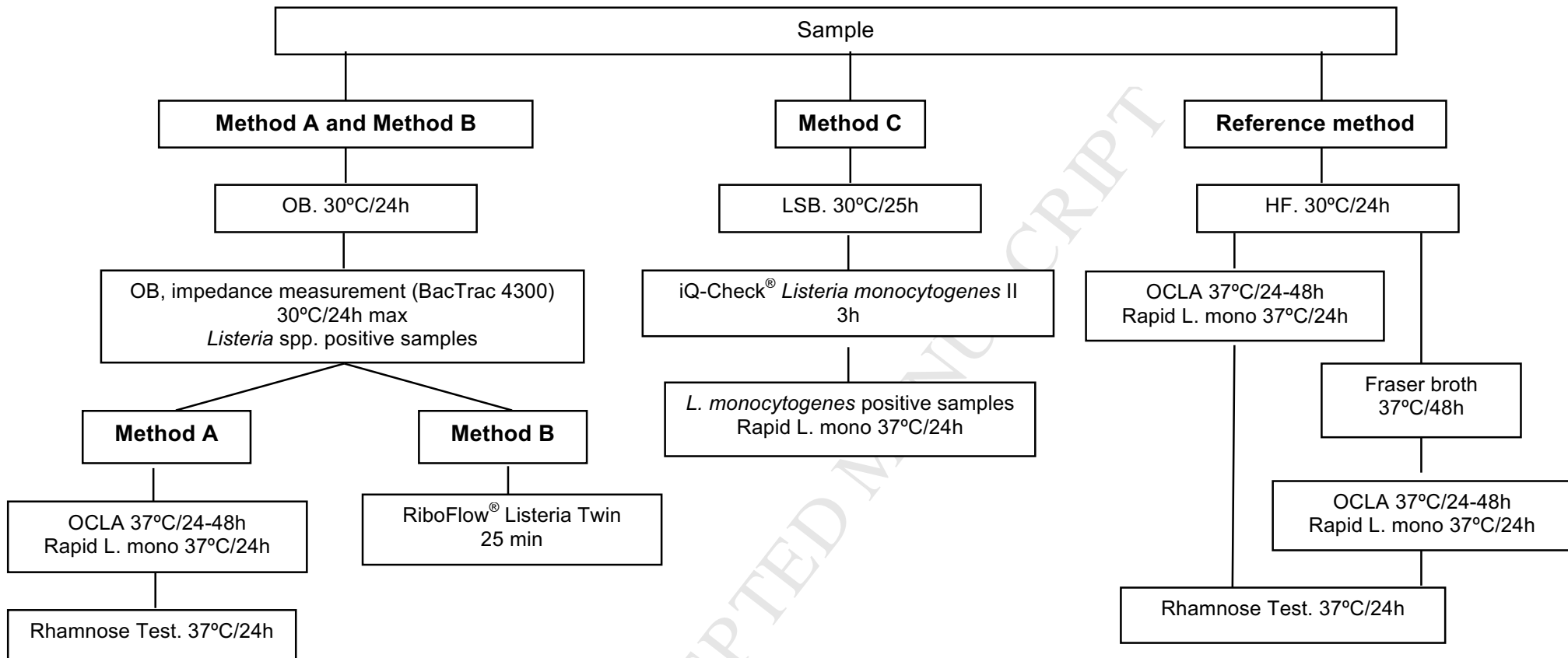


Figure 1. Flow diagram of the four assayed methodologies for the detection *L. monocytogenes*: Method A: Impedance measurement followed by OCLA and Rapid L. mono; Method B: Impedance change measurement followed by *RiboFlow*® *Listeria Twin*; Method C: Real time PCR (iQ-Check® *Listeria monocytogenes* II Kit). OB: One Broth Listeria; LSB: Listeria Special Broth; HF: Half Fraser; OCLA (Oxoid Chromogenic Listeria Agar).

## Highlights

- Three alternative methods 1-3 days faster than the ISO standard
- Excellent results for inclusivity, exclusivity and RLOD were obtained for the three methods.
- Two of the three methods presented very good agreement with the reference method
- Impedance measurement followed by RNA hybridization showed lower relative trueness