Accepted Manuscript

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

Mirian Labrador, María Carmen Rota, Consuelo Pérez-Arquillué, Antonio Herrera, Susana Bayarri

PII: S0956-7135(18)30321-9

DOI: 10.1016/j.foodcont.2018.06.031

Reference: JFCO 6205

To appear in: *Food Control*

Received Date: 26 April 2018

Revised Date: 13 June 2018

Accepted Date: 24 June 2018

Please cite this article as: Labrador M., Rota Marí.Carmen., Pérez-Arquillué C., Herrera A. & Bayarri S., 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, *Food Control* (2018), doi: 10.1016/j.foodcont.2018.06.031.

Title

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

Authors

Mirian Labrador, María Carmen Rota, Consuelo Pérez-Arquillué, Antonio Herrera, Susana Bayarri*

Departamento de Producción Animal y Ciencia de los Alimentos. Facultad de Veterinaria. Instituto Agroalimentario de Aragón -IA2- (Universidad de Zaragoza-CITA), Zaragoza, Spain. C/Miguel Servet 177, 50013 Zaragoza, Spain.

*Corresponding author. Tel.: +34 976 7610 00 Ext. 4135; fax: +34 976 7615 90-1612. E-mail address: sbayarri@unizar.es (S. Bayarri).

Abstract

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

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

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

Keywords

Listeria monocytogenes; detection; rapid methods; RTE meat products

1. Introduction

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

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

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

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

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

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

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

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

2. Materials and methods

The alternative methods were evaluated in comparison with the reference method (ISO 11290-1) following ISO 16140-2:2016 standard (ISO, 2016). Inclusivity, exclusivity, limit of detection 50 (LOD_{50}), relative limit of detection (RLOD), and a sensitivity study of the alternative methods were performed. Additionally, apart from the parameters established by ISO 16140-2:2016, other validation indicators were determined. The alternative methods evaluated were:

- Method A: Pre-enrichment combined with impedance measurement, followed by plating on OCLA (Oxoid Chromogenic Listeria Agar) and Rapid L. mono.
- Method B: Pre-enrichment combined with impedance measurement, followed by RiboFlow® *Listeria Twin* flow assay.
- Method C: Pre-enrichment combined with real-time PCR (iQ-Check® *Listeria monocytogenes* II Kit).

2.1 Description of methods

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

2.1.1 Method A

For the pre-enrichment step, the samples were incubated at 30ºC for 24 h in One Broth Listeria (OB, Oxoid, Hampshire, England). Then, one ml of the pre-enrichment was inoculated in nine ml of OB placed in a specific four-electrode cell (SY-LAB Geräte GmbH, Neupurkersdorf, Austria). The measurement of impedance change (E- value) was monitored using a BacTrac 4300 apparatus (SY-LAB Geräte GmbH). The assay was carried out at 30ºC for a maximum of 24 h. Detection time (DT) was established for an E-value threshold of 5% to 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 37ºC 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) (37ºC/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*

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

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

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

PCR reaction positive and negative controls were included in each assay. The sample was considered positive when the Cq values were ≥10 and ≥ 28 for the target and the IAC, respectively.

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

2.1.4 Reference method (ISO 11290-1)

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

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

2.2 Evaluation parameters following ISO 16140-2:2016

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

2.2.1 Inclusivity and exclusivity tests

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

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

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

In each assay, positive and negative controls were included for each methodology. The positive control was performed by artificial contamination of 225 ml pre-enrichment medium with $2X10^3$ CFU *L. monocytogenes* UZ64, while 225 ml pre-enrichment medium sterile without inoculation was used as negative control. The assays were performed in triplicate.

2.2.2 Limit of detection 50 and relative limit of detection

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

2.2.2.1 Bacterial strain and preparation of inocula

An isolated colony of *L. monocytogenes* UZ64 was incubated at 37ºC/16 h in 10 ml of BHI broth, in order to obtain $2X10^9$ cfu/ml. Serial ten-fold dilutions were performed in peptone water 0.1% to a cell concentration of $2X10^1$ cfu/ml. Colony counting in BHIA (37ºC/24 h) was used in order to obtain the concentration of microorganisms in the inoculum.

2.2.2.2 Artificial contamination of dry-cured ham samples used for LOD_{50} and RLOD

Sliced and vacuum-packed dry-cured ham samples were analysed at three levels of contamination: 0 cfu/25 g (Level 1), 0.3 cfu/25 g (Level 2) and 0.9 cfu/25 g (Level 3). For Level 1, 100 g of dry-cured ham were homogenised with 900 ml of each of the three different pre-enrichment media previously described. For Levels 2 and 3, 100 g of dry-cured ham were homogenised with 900 ml of each of the three different pre-enrichment media, and they were inoculated with 60 µl and 180 µl of $2X10^1$ *L. monocytogenes* UZ64 cfu/ml, respectively. After inoculation, additional homogenisation was carried out using a Stomacher® 400 Circulator (Seward Ltd, Worthing, UK) blender (260 rpm/2.5 min). In the case of each methodology, an individual sample was analysed six times.

Positive and negative controls were used for each methodology. For the positive controls, 25 g of dry-cured ham was inoculated with $2X10^3$ cfu *L. monocytogenes* UZ64. The negative controls were the Level 1 samples analysed with each methodology.

2.2.3 Sensitivity study

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

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

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

To carry out this sensitivity study, naturally and artificially contaminated samples were analysed. These samples were different from those used to determine LOD_{50} and RLOD.

2.2.3.1 Bacterial strains and preparation of inocula

The strains assayed were *L. monocytogenes* UZ64, *L. monocytogenes* UZ108, *L. innocua* UZ1, *L. innocua* UZ68, and *L. welshimeri* UZ40. An isolated colony of each strain was incubated overnight at 37ºC in 10 ml of BHI broth to reach $2X10^9$ cfu/ml. Serial ten-fold dilutions of each strain in peptone water 0.1% were

299 carried out in order to obtain concentrations of $2X10^3$ cfu/ml, $2X10^2$ cfu/ml and
300 $2X10^1$ 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 (37ºC/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

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

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

2.3 Statistical analysis

Statistical analysis was performed using Excel software, Version 14.2.0 (Microsoft Corporation, Redmond, WA, USA) and SPSS statistics 22.0.0 software (SPSS. Inc., Chicago, IL, USA). LOD_{50} was calculated using the Spearman & Karber test. The statistical study of detection times was carried out using the t-student test.

3. Results and discussion

3.1 Inclusivity and exclusivity

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

3.2 Limit of detection 50 and relative limit of detection

348 The LOD_{50} 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_{50} 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_{50}. 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

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

3.3 Sensitivity study

The results of 93 analysed samples of dry-cured ham are shown in Table 5.

For methods A and C, SE_{alt}, RT, SP, SE, PPV and NPV were 100 %. FPR values were 0% for both methods, due to absence of false-negative or false-positive results. The limit of acceptability of ND-PD for an unpaired study is set at 3 (ISO, 2016). The ND-PD values were 0 for methods A and C, therefore lying within the limits of acceptability. The Cohen's Kappa index for methods A and C was 1 in each case, thereby indicating very good agreement with the reference method. The pathogen grew on OCLA and Rapid L. mono in the *L. monocytogenes*-positive samples, providing an excellent correlation between those two agars. These results were in concordance with diverse authors, who observed the suitable correctness of Rapid L. mono (Becker et al., 2006).

On the other hand, ten false negatives and one false positive were obtained by method B in comparison with the reference method. As a consequence, a decrease of SE_{alt} (80 %), RT (88.2 %), SP (99.7 %), SE (79.6 %), PPV (97.5

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

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

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

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

However, the use of real-time PCR for this purpose has been studied in meat products. Diverse studies have compared real-time PCR with the reference method to detect *L. monocytogenes*, obtaining results that our similar to our 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*®
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, Piknová, Kaclíková, & 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

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

4. Conclusions

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

Acknowledgements

This work was financed by Spanish MINECO/FEDER (project INNPACTO IPT-2012-0189-060000). The authors wish to thank the Gobierno de Aragón/FEDER (Grupo A06_17R) for financial support. Thanks likewise go to Grupo Jorge, S.L. Author M. Labrador was supported by Grant No. C091/2014 from Gobierno de Aragón/FSE.

References

Alessandria, V., Rantsiou, K., Dolci, P., & Cocolin, L. (2010). Molecular methods to assess *Listeria monocytogenes* route of contamination in a dairy processing plant. *International Journal of Food Microbiology*, *141*, 156–162.

Amagliani, G., Giammarini, C., Omiccioli, E., Brandi, G., & Magnani, M. (2007). Detection of *Listeria monocytogenes* using a commercial PCR kit and different DNA extraction methods. *Food Control*, *18*(9), 1137–1142.

Anderson, A., Pietsch, K., Zucker, R., Mayr, A., Müller-Hohe, E., Messelhäusser, U., … Huber, I. (2011). Validation of a Duplex Real-Time PCR for the Detection of Salmonella spp. in Different Food Products. *Food Analytical Methods*, *4*(3), 259–267.

Awaisheh, S. S. (2010). Incidence and contamination level of L*isteria monocytogenes* and other *Listeria* spp. in ready-to-eat meat products in Jordan. *Journal of Food Protection*, *73*(3), 535–540.

Barros, M. D. A. F., Nero, L. A., Manoel, A. V.-B., D'Ovídio, L., Da Silva, L. C., Franco, B. D. G. D. M., & Beloti, V. (2007). *Listeria* spp. associated to different levels of autochthonous microbiota in meat, meat products and processing plants. *Brazilian Journal of Microbiology*, *38*(4), 603–609.

573 Becker, B., Schuler, S., Lohneis, M., Sabrowski, A., Curtis, G., & Holzapfel, W.
574 (2006). Comparison of two chromogenic media for the detection of *Listeria*
575 *monocytogenes* with the plating media recommended by EN/DIN 11290-1.
576 *International Journal of Food Microbiology*, *109*(1–2), 127–131.
577 Besse, N. G., Barre, L., Buhariwalla, C., Vignaud, M. L., Khamissi, E.,
578 Decourseulles, E., … Kalmokoff, M. (2010). The overgrowth of *Listeria*
579 *monocytogenes* by other *Listeria* spp. in food samples undergoing
580 enrichment cultivation has a nutritional basis. *International Journal of Food*
581 *Microbiology*, *136*, 345–351.
582 Cardoen, S., Van Huffel, X., Berkvens, D., Quoilin, S., Ducoffre, G.,
583 Saegerman, C., … Dierick, K. (2009). Evidence-Based Semiquantitative
584 Methodology for Prioritization of Foodborne Zoonoses. *Foodborne*
585 *Pathogens and Disease*, *6*(9), 1083–1096.
586 Chaitiemwong, N., Hazeleger, W. C., Beumer, R. R., & Zwietering, M. H.
587 (2014). Quantification of transfer of *Listeria monocytogenes* between
588 cooked ham and slicing machine surfaces. *Food Control*, *44*, 177–184.
589 Cho, I.-H., & Irudayaraj, J. (2013). Lateral-flow enzyme immunoconcentration
590 for rapid detection of *Listeria monocytogenes*. *Analytical and Bioanalytical*
591 *Chemistry*, *405*(10), 3313–3319.
592 de Valk, H., Jacquet, C., Goulet, V., Vaillant, V., Perra, A., Simon, F., … Listeria
593 Surveillance Feasibility Study Participants. (2005). Surveillance of listeria
594 infections in Europe. *Euro Surveillance : Bulletin Europeen Sur Les*
595 *Maladies Transmissibles = European Communicable Disease Bulletin*,
596 *10*(10), 251–255.
597 Delibato, E., Gattuso, A., Minucci, A., Auricchio, B., De Medici, D., Toti, L., …

598 Gianfranceschi, M. V. (2009). PCR experion automated electrophoresis
599 system to detect *Listeria monocytogenes* in foods. *Journal of Separation*
600 *Science*, *32*(21), 3817–3821.
601 European Food Safety Authority (EFSA) and European Centre for Disease
602 Prevention and Control (ECDC). (2008). Request for updating the former
603 SCVPH opinion on *Listeria monocytogenes* risk related to ready-to-eat
604 foods and scientific advice on different levels of *Listeria monocytogenes* in
605 ready-to-eat foods and the related risk for human illness - Scientific
606 Opinion. *EFSA Journal*, *6*(1), 599.
607 European Food Safety Authority (EFSA) and European Centre for Disease
608 Prevention and Control (ECDC). (2016). The European Union summary
609 report on trends and sources of zoonoses, zoonotic agents and food-borne
610 outbreaks in 2015. *EFSA Journal*, *14*(12), 4634.
611 Fuchizawa, I., Shimizu, S., Ootsubo, M., Kawai, Y., & Yamazaki, K. (2009).
612 Specific and Rapid Quantification of Viable *Listeria monocytogenes* Using
613 Fluorescence *in situ* Hybridization in Combination with Filter Cultivation.
614 *Microbes and Environments*, *24*(3), 273–275.
615 Fusco, V., & Marina, G. (2012). Nucleic Acid-Based Methods to Identify, Detect
616 and Type Pathogenic Bacteria Occurring in Milk and Dairy Products. In
617 *Structure and Function of Food Engineering*. InTech.
618 Gandhi, M., & Chikindas, M. L. (2007). Listeria: A foodborne pathogen that
619 knows how to survive. *International Journal of Food Microbiology*, *113*(1),
620 1–15.
621 Garrido, A., Chapela, M. J., Román, B., Fajardo, P., Lago, J., Vieites, J. M., &
622 Cabado, A. G. (2013). A new multiplex real-time PCR developed method

for *Salmonella* spp. and *Listeria monocytogenes* detection in food and environmental samples. *Food Control*, *30*(1), 76–85.

Giovannini, A., Migliorati, G., Prencipe, V., Calderone, D., Zuccolo, C., & Cozzolino, P. (2007). Risk assessment for listeriosis in consumers of Parma and San Daniele hams. *Food Control*, *18*(7), 789–799.

Gómez, D., Iguácel, L. P., Rota, M. C., Carramiñana, J. J., Ariño, A., & Yangüela, J. (2015). Occurrence of *Listeria monocytogenes* in Ready-to-Eat Meat Products and Meat Processing Plants in Spain. *Foods*, *4*(3), 271–282.

Hoorfar, J., Malorny, B., Abdulmawjood, A., Cook, N., Wagner, M., & Fach, P. (2004). Minireview Practical Considerations in Design of Internal Amplification Controls for Diagnostic PCR Assays. *Journal Of Clinical Microbiology*, *42*(5), 1863–1868.

Hospital, X. F., Hierro, E., Arnau, J., Carballo, J., Aguirre, J. S., Gratacós-Cubarsí, M., & Fernández, M. (2017). Effect of nitrate and nitrite on Listeria and selected spoilage bacteria inoculated in dry-cured ham. *Food Research International*, *101*, 82–87.

International Organization for Standardization. (2004). ISO 11290-1/Amd 1:2004. Microbiology of food and animal feeding stuffs -- Horizontal method for the detection and enumeration of Listeria monocytogenes -- Part 1: Detection method. Modification of the isolation media and the haemolysis test, and inclusion of precision data.

International Organization for Standardization. (2016). ISO 16140-2:2016. Microbiology of the food chain -- Method validation -- Part 2: Protocol for the validation of alternative (proprietary) methods against a reference

method.

Keys, A. L., Dailey, R. C., Hitchins, A. D., & Derike Smiley, R. (2013). Postenrichment Population Differentials Using Buffered Listeria Enrichment Broth: Implications of the Presence of *Listeria innocua* on *Listeria monocytogenes* in Food Test Samples. *Journal of Food Protection*, *76*(11), 1854–1862.

Krascsenicsová, K., Piknová, L., Kaclíková, E., & Kuchta, T. (2008). Detection of Salmonella enterica in food using two-step enrichment and real-time polymerase chain reaction. *Letters in Applied Microbiology*, *46*(4), 483–487.

Labrador, M., Rota, M. C., Pérez, C., Herrera, A., & Bayarri, S. (2018). Evaluation of a Method for Rapid Detection of *Listeria monocytogenes* in Dry-Cured Ham Based on Impedanciometry Combined with Chromogenic Agar. *Journal of Food Protection*, *81*(5), 705–712. https://doi.org/10.4315/0362-028X.JFP-17-380

Lambertz, S. T., Nilsson, C., Brådenmark, A., Sylvén, S., Johansson, A., Jansson, L.-M., & Lindblad, M. (2012). Prevalence and level of *Listeria monocytogenes* in ready-to-eat foods in Sweden 2010. *International Journal of Food Microbiology*, *160*(1), 24–31.

Leistner, L. (2000). Basic aspects of food preservation by hurdle technology. *International Journal of Food Microbiology*, *55*, 181–186.

Lianou, A., & Sofos, J. N. (2007). A review of the incidence and transmission of *Listeria monocytogenes* in ready-to-eat products in retail and food service environments. *Journal of Food Protection*, *70*(9), 2172–2198.

López, V., Villatoro, D., Ortiz, S., López, P., Navas, J., Dávila, J. C., & Martínez-

673 Suárez, J. V. (2008). Molecular tracking of *Listeria monocytogenes* in an
674 Iberian pig abattoir and processing plant. *Meat Science*, *78*(1–2), 130–134.
675 Mena, C., Almeida, G., Carneiro, L., Teixeira, P., Hogg, T., & Gibbs, P. A.
676 (2004). Incidence of *Listeria monocytogenes* in different food products
677 commercialized in Portugal. *Food Microbiology*, *21*(2), 213–216.
678 Myers, K., Montoya, D., Cannon, J., Dickson, J., & Sebranek, J. (2013). The
679 effect of high hydrostatic pressure, sodium nitrite and salt concentration on
680 the growth of *Listeria monocytogenes* on RTE ham and turkey. *Meat*
681 *Science*, *93*(2), 263–268.
682 NordVaL. (2017). NordVal International Protocol for the validation of
683 microbiological alternative (proprietary) methods against a reference
684 method, (September 8th), 1–29.
685 O´Grady, J., Ruttledge, M., Sedanobalbas, S., Smith, T., Barry, T., & Maher, M.
686 (2009). Rapid detection of *Listeria monocytogenes* in food using culture
687 enrichment combined with real-time PCR. *Food Microbiology*, *26*(1), 4–7.
688 Oravcová, K., Kuchta, T., & Kaclíková, E. (2007). A novel real-time PCR-based
689 method for the detection of *Listeria monocytogenes* in food. *Letters in*
690 *Applied Microbiology*, *45*(5), 568–573.
691 Oravcová, K., Trnčíková, T., Kuchta, T., & Kaclíková, E. (2008). Limitation in the
692 detection of *Listeria monocytogenes* in food in the presence of competing
693 *Listeria innocua*. *Journal of Applied Microbiology*, *104*(2), 429–437.
694 Ortiz, S., López, V., Villatoro, D., López, P., Dávila, J. C., & Martínez-Suárez, J.
695 V. (2010). A 3-year Surveillance of the genetic diversity and persistence of
696 Listeria monocytogenes in an Iberian pig slaughterhouse and processing

plant. *Food Pathogens and Disease*, *7*(10), 1177–1184. https://doi.org/10.1089/fpd.2010.0535

Portanti, O., Febo, T. Di, Luciani, M., Pompilii, C., Lelli, R., & Semprini, P. (2011). Development and validation of an antigen capture ELISA based on monoclonal antibodies specific for *Listeria monocytogenes* in food. *Veterinaria Italiana Vet Ital*, *47*(473).

Prencipe, V. A., Rizzi, V., Acciari, V., Iannetti, L., Giovannini, A., Serraino, A., … Caporale, V. (2012). *Listeria monocytogenes* prevalence, contamination levels and strains characterization throughout the Parma ham processing chain. *Food Control*, *25*(1), 150–158.

Pricope, L., Nicolau, A., Wagner, M., & Rychli, K. (2013). The effect of sublethal concentrations of benzalkonium chloride on invasiveness and intracellular proliferation of *Listeria monocytogene*s. *Food Control*, *31*(1), 230–235.

Quero, G. M., Santovito, E., Visconti, A., & Fusco, V. (2014). Quantitative detection of *Listeria monocytogenes* in raw milk and soft cheeses: Culture-independent versus liquid- and solid-based culture-dependent real time PCR approaches. *LWT - Food Science and Technology*, *58*(1), 11–20.

Rodriguez-Lazaro, D., Gonzalez-Garcia, P., Gattuso, A., Gianfranceschi, M. V., & Hernandez, M. (2014). Reducing time in the analysis of *Listeria monocytogenes* in meat, dairy and vegetable products. *International Journal of Food Microbiology*, *184*, 98–105.

Rossmanith, P., Krassnig, M., Wagner, M., & Hein, I. (2006). Detection of *Listeria monocytogenes* in food using a combined enrichment/real-time PCR method targeting the prfA gene. *Research in Microbiology*, *157*(8), 763–771.

722 Ruiz-Rueda, O., Soler, M., Calvó, L., & García-Gil, J. L. (2011). Multiplex Real-
723 time PCR for the Simultaneous Detection of *Salmonella* spp. and *Listeria*
724 *monocytogenes* in Food Samples. *Food Analytical Methods*, *4*(2), 131–138.
725 Sauders, B. D., Overdevest, J., Fortes, E., Windham, K., Schukken, Y., Lembo,
726 A., & Wiedmann, M. (2012). Diversity of Listeria species in urban and
727 natural environments. *Applied and Environmental Microbiology*, *78*(12),
728 4420–4433. https://doi.org/10.1128/AEM.00282-12
729 Simmons, C., Stasiewicz, M. J., Wright, E., Warchocki, S., Roof, S., Kause, J.
730 R., … Oliver, H. F. (2014). *Listeria monocytogenes* and *Listeria* spp.
731 Contamination Patterns in Retail Delicatessen Establishments in Three
732 U.S. States. *Journal of Food Protection*, *77*(11), 1929–1939.
733 Schmid, M. W., Ng, E. Y. W., Lampidis, R., Emmerth, M., Walcher, M., Kreft, J.,
734 … Schleifer, K. H. (2005). Evolutionary history of the genus Listeria and its
735 virulence genes. *Systematic and Applied Microbiology*, *28*(1), 1–18.
736 Suh, J. H., & Knabel, S. J. (2001). Comparison of different enrichment broths
737 and background flora for detection of heat-injured *Listeria monocytogenes*
738 in whole milk. *Journal of Food Protection*, *64*(1), 30–36.
739 Tomás, D., Rodrigo, A., Hernández, M., & Ferrús, M. A. (2009). Validation of
740 Real-Time PCR and Enzyme-Linked Fluorescent Assay-Based Methods for
741 Detection of Salmonella spp. in Chicken Feces Samples. *Food Analytical*
742 *Methods*, *2*(3), 180–189. https://doi.org/10.1007/s12161-009-9082-3
743 Välimaa, A.-L., Tilsala-Timisjärvi, A., & Virtanen, E. (2015). Rapid detection and
744 identification methods for *Listeria monocytogenes* in the food chain – A
745 review. *Food Control*, *55*, 103–114.
746 Vongkamjan, K., Fuangpaiboon, J., Turner, M. P., & Vuddhakul, V. (2016).

747 Various Ready-to-Eat Products from Retail Stores Linked to Occurrence of
748 Diverse *Listeria monocytogenes* and *Listeria* spp. Isolates. *Journal of Food*
749 *Protection*, *79*(2), 239–245.
750 Wawerla, M., Stolle, A., Schalch, B., & Eisgruber, H. (1999). Impedance
751 Microbiology: Applications in Food Hygiene. *Journal of Food Protection*,
752 *62*(12), 1488–1496.
753 WU, V. (2008). A review of microbial injury and recovery methods in food. *Food*
754 *Microbiology*, *25*(6), 735–744.
755 Yang, L., & Bashir, R. (2007). Electrical/electrochemical impedance for rapid
756 detection of foodborne pathogenic bacteria.
757 Yang, L., Ruan, C., & Li, Y. (n.d.). Detection of viable *Salmonella typhimurium*
758 by impedance measurement of electrode capacitance and medium
759 resistance. *Biosensors and Bioelectronics*, *19*(5), 495–502.
760 Zhu, M., Du, M., Cordray, J., & Ahn, D. U. (2005). Control of *Listeria*
761 *monocytogenes* Contamination in Ready-to-Eat Meat Products.
762 *Comprehensive Reviews in Food Science and Food Safety*, *4*(2), 34–42.
763 Zitz, U., Zunabovic, M., Domig, K. J., Wilrich, P.-T., & Kneifel, W. (2011).
764 Reduced Detectability of *Listeria monocytogenes* in the Presence of
765 *Listeria innocua*. *Journal of Food Protection*, *74*(8), 1282–1287.
766 Zunabovic, M., Domig, K. J., & Kneifel, W. (2011). Practical relevance of
767 methodologies for detecting and tracing of *Listeria monocytogenes* in
768 ready-to-eat foods and manufacture environments - A review. *LWT - Food*
769 *Science and Technology*, *44*(2), 351–362.
770

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

Strain	Origin	Level of inoculum[a] (Log cfu/225 ml pre-enrichment)
*L. monocytogenes UZ*22	Fresh longaniza	1.05±0.19
L. monocytogenes UZ64	Dry-cured ham	1.46±0.13
L. monocytogenes UZ102	Fresh longaniza	1.38±0.08
L. monocytogenes UZ104	Fresh longaniza	1.37±0.12
L. monocytogenes UZ106	Dry-cured ham	1.03±0.30
L. monocytogenes UZ108	Cured longaniza	1.33±0.10
L. innocua UZ1	Fresh longaniza	2.37±0.08
L. innocua UZ65	Surfaces from RTEMP	1.81±0.14
L. innocua UZ68	Cheese	2.17±0.14
L. welshimeri UZ40	Cured longaniza	2.33±0.08
E. durans CECT 411	Dried milk	7.81±0.09

772 [a]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[a]		Number of samples
Commercial dry-cured ham	Not contaminated		44
Dry-cured ham contaminated with *L. monocytogenes* UZ64	0.58±0.01		20
Dry-cured ham contaminated with *L. monocytogenes* UZ64 and *L. innocua* UZ1[b]	*L. monocytogenes UZ*64	0.42±0.07	3
	*L. innocua UZ*1	0.32±0.09	
	*L. monocytogenes UZ*64	0.42±0.07	3
	*L. innocua UZ*1	2.32±0.09	
Dry-cured ham contaminated with *L. monocytogenes* UZ64 and *L. innocua* UZ68[b]	*L. monocytogenes UZ*64	0.42±0.07	3
	*L. innocua UZ*68	0.19±0.13	
	*L. monocytogenes UZ*64	0.42±0.07	3
	*L. innocua UZ*68	2.19±0.13	
Dry-cured ham contaminated with *L. monocytogenes* UZ64 and *L.welshimeri* UZ40[b]	*L. monocytogenes UZ*64	0.42±0.07	3
	*L. welshimeri UZ*40	0.40±0.06	
	*L. monocytogenes UZ*64	0.42±0.07	3
	*L. welshimeri UZ*40	2.40±0.06	
Dry-cured ham contaminated with *L. monocytogenes* UZ108 and *L. innocua* UZ1[b]	*L. monocytogenes UZ*108	0.29±0.12	2
	*L. innocua UZ*1	0.32±0.09	
	*L. monocytogenes UZ*108	0.29±0.12	3
	*L. innocua UZ*1	2.32±0.09	
Dry-cured ham contaminated with *L. monocytogenes* UZ108 and *L.welshimeri* UZ40[b]	*L. monocytogenes UZ*108	0.29±0.12	3
	*L. welshimeri UZ*40	0.40±0.06	
	*L. monocytogenes UZ*108	0.29±0.12	3
	*L. welshimeri UZ*40	2.40±0.06	

775 [a]Mean ± standard deviation from six replicates obtained by of colony count on Rapid L. mono
776 agar
777 [b]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[a]	Method B[b]	Method C[c]
L. monocytogenes UZ22	+	+	+
L. monocytogenes UZ64	+	+	+
L. monocytogenes UZ102	+	+	+
L. monocytogenes UZ104	+	+	+
L. monocytogenes UZ106	+	+	+
L. monocytogenes UZ108	+	+	+
L. innocua UZ1	-	-	-
L. innocua UZ65	-	-	-
L. innocua UZ68	-	-	-
L. welshimeri UZ40	-	-	-
E. durans CECT 411	-	-	-

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

781 [a]Method A: Impedance measurement followed by OCLA and Rapid L. mono

782 [b]Method B: Impedance measurement followed by *RiboFlow® Listeria Twin*

783 [c]Method C: Real time PCR (iQ-Check® *Listeria monocytogenes* II Kit)

784

785 Table 4. Limit of detection 50 (LOD_{50}) and relative limit of detection (RLOD) of
786 three methods evaluated and reference method for detection of *L.*
787 *monocytogenes*.

	Signal ratio[b]				
	0[c]	0.3[c]	0.9[c]	LOD_{50}[d]	RLOD[e]
Method A[a]	0/6	1/6	6/6	(0.3-0.7)	1.265
Method B[a]	0/6	1/6	6/6	(0.3-0.7)	1.265
Method C[a]	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 [a] Method A: Impedance measurement followed by OCLA and Rapid L. mono; Method B: Impedance
789 measurement followed by *RiboFlow® Listeria Twin;* Method C: Real time PCR (iQ-Check® *Listeria*
790 *monocytogenes* II Kit).
791 [b]Positive results of 6 replicates.
792 [c]cfu/25 g
793 [d]Limit of detection (LOD_{50}) was calculated as a confidence interval of 95%.
794 [e]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*® *Listeria Twin;* Method C: Real time PCR (iQ-Check® *Listeria*
801 *monocytogenes* II Kit).

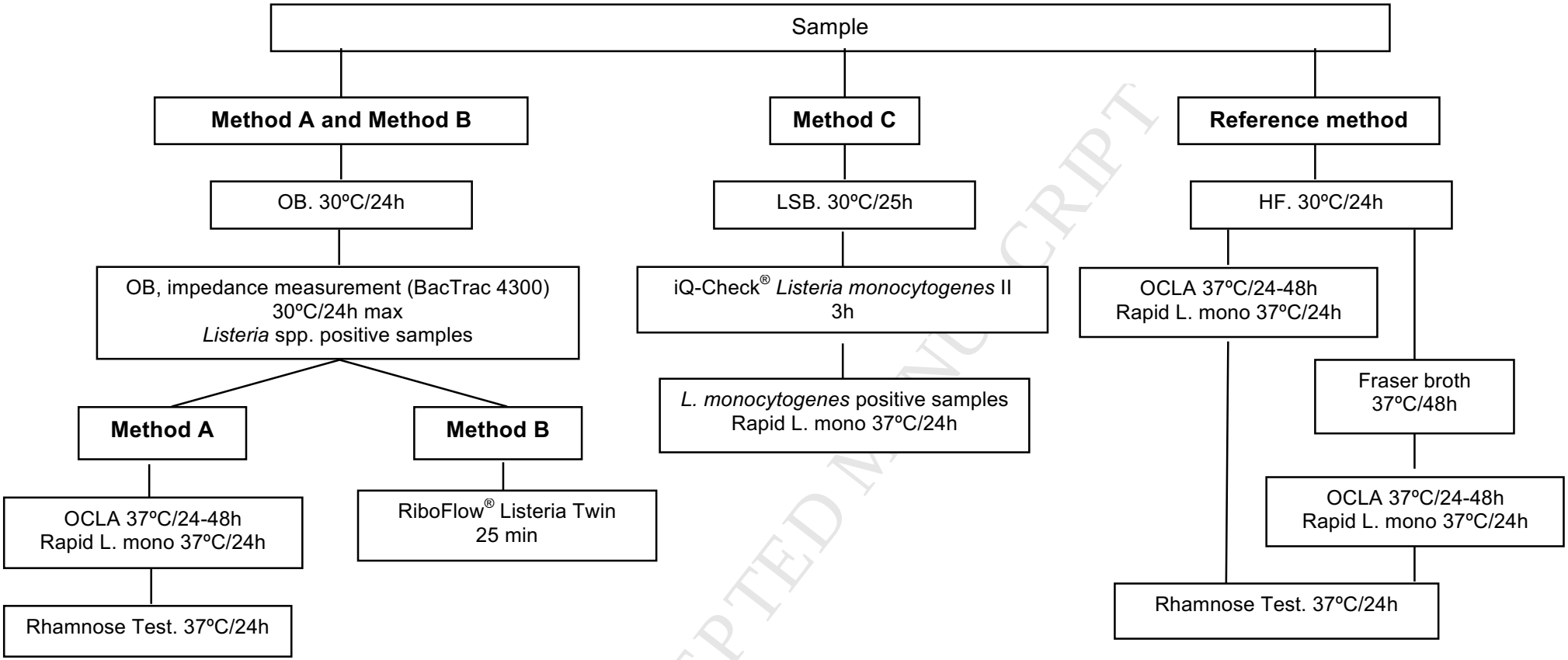

Figure 1. Flow diagram of the four assayed methodologies for the detection *L. monocytogenes*: Method A: Impedance measurement followed by OCLA and Rapid L. mono; Method B: Impedance change measurement followed by *RiboFlow® Listeria Twin;* Method C: Real time PCR (iQ-Check® *Listeria monocytogenes* 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