# PRESENCE OF *LISTERIA MONOCYTOGENES* IN PREPARED FOODS. ANALYSIS OF INFLUENCING FACTORS.

**Short title:** Factors influencing *L. monocytogenes* presence in prepared foods

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**ABSTRACT**

Although food-borne outbreaks of listeriosis are uncommon, they remain a major public health problem. We assessed the prevalence of *L. monocytogenes* in prepared foods and the influence of different factors (including type of food or presence of accompanying bacteria). Results showed that accompanying bacteria cause interference in the sensitivity of the detection method, being half Fraser the enrichment medium of choice. In 2760 samples, the global prevalence of *L. monocytogenes* was 1.4%. In ready-to-eat foods, the highest prevalence (23.3%) was found in products containing fermented or cured ingredients and the prevalence was also higher in foods with high counts of aerobic bacteria and lactose positive *Enterobacteriaceae*, two indicators of process hygiene. We also found that foods stored <0ºC had a higher prevalence (4.1%) of *L. monocytogenes*. In prepared foods, factors favouring the presence of *L. monocytogenes* could be some components of ready-to-eat foods, temperatures <0ºC and presence of accompanying bacteria.

**PRACTICAL APPLICATIONS**

Results are of relevance for practical application since this study was performed in the context of the routine practice of a food control company, with samples collected in different restoration centres from Spain. Results obtained have allowed to identify some factors favouring the presence of *L. monocytogenes*, which should be taken into account in the current practice of the manufacturing centres of prepared foods to avoid contamination by this bacterium. Based on our results, actions aimed at avoiding high numbers of accompanying bacteria as aerobic mesophilic bacteria (AMB) or *Enterobacteriaceae* should be taken into account. Special care should also be taken in the manufacturing of foods containing fermented or cured ingredients, as cheese.

Finally, the manufacturing centres should also consider the risk of *Listeria* contamination due to the use of sophisticated machinery or due to the storage of prepared foods at temperatures < 0ºC.

## INTRODUCTION

*Listeria monocytogenes*, a rod-shaped Gram positive bacterium, is a foodborne pathogen that can cause serious invasive illnesses (Rebagliati *et al*., 2009; Noriega *et al*., 2008). Symptoms can develop at any time from 2 to 70 days after eating contaminated food (Bortolussi, 2008), thus increasing the difficulty of attributing a food vehicle to cases (Rhoades *et al.,* 2009). Moreover, the incidence of listeriosis is difficult to establish, since symptoms may be mistaken for a flu-like illness or gastroenteritis and appropriate cultures not obtained (Bortolussi, 2008). Cases are rare, but mortality from invasive infections is approximately 20 to 30% (Rhoades *et al*., 2009; Perera *et al*., 2015). Countries with surveillance programs have reported rates of infection from 0.6 to 6.2 cases per million.

Although the soils are the main source of *L. monocytogenes*, human transmission is conceived from the environment by animals and contaminated food products during processing (Haley *et al*., 2015). Contaminated meats (such as hot dogs, delicatessen meats and pâté), dairy products and seafood have all been implicated in outbreaks of listeriosis (Rebagliati *et al*., 2009; Rodríguez-López *et al.*, 2015). Epidemiological data obtained in Spain indicate an incidence of foodborne listeriosis of 0.1% between years 2004 and 2007. Although most cases came from restaurants, centres or contributing factors were not always identified (Boletín epidemiológico semanal, 2013). Recently, 3200 packs of cooked pork ears, prepared in Spain and distributed in France, were withdrawn due to contamination by *L. monocytogenes*

(http://institutodeseguridadalimentaria.com). The public health importance of listeriosis is not always recognized, particularly because listeriosis is a relatively rare disease compared with other common foodborne illnesses such as salmonellosis. However, because of its high case fatality rate, listeriosis ranks among the most frequent causes of death due to foodborne illness, ranking second after salmonellosis (Rebagliati *et al*., 2009; Lou *et al*., 2015).

Biofilm formation and persistence, resistance to disinfectants, resistance to *Listeria*-specific viruses, and the ability to replicate at low temperatures are among the attributes contributing to the organism's prevalence and persistence in food processing environments (Elhanafi *et al*., 2010; Rodríguez-López *et al*., 2015). Unlike most other enteric pathogens, *L. monocytogenes* is notable for its ability to grow at refrigeration temperatures (4 - 10°C), commonly used to control pathogens in foods (Pal *et al*., 2008, Rhoades *et al*., 2009, Bortolussi, 2008). This has considerable significance for food safety, as it means that chilling to 4ºC cannot be relied upon to prevent the growth of the organism to dangerous levels. Freezing has also little detrimental effect on the microbe, but it is destroyed by pasteurization or adequate cooking (Rhoades *et al*., 2009). Although pasteurization is sufficient to kill *Listeria*, failure to reach the desired temperature in large packages can allow the organism to survive. Food can also be contaminated after processing by the introduction of unpasteurized material, as happens during the preparation of some cheeses. *Listeria* can also be spread by contact with contaminated hands, equipment and counter tops (Bortolussi, 2008). Since *L. monocytogenes* is one of the most resistant pathogens that is reasonably likely to be present in food, it is considered as an indicator of choice (as vegetative cells) of the efficacy of pasteurization processes by FDA for foods without oxygen limitation (FDA, 2011).

Data on *L. monocytogenes* prevalence are considerably fewer in certain types of food as prepared and ready-to-eat foods, particularly at the production and abattoir processing stages. In raw products such as minced beef and retail cuts, *L. monocytogenes* is more frequently isolated than *Salmonella* and *E. coli* O157 in the surveys available (Rhoades *et al*., 2009). In Europe, the number of listeriosis outbreaks associated with dairies is now about half of the total outbreak and most of them have been linked to raw milk intake or products manufactured with it, as mellow cheeses (Rebagliati *et al*., 2009).

According to the European Union summary report of the European Food Safety Authority (EFSA), human listeriosis has experienced an increasing EU trend in 2009-2013, although in ready-to-eat foods *Listeria* was seldom detected above the legal safety limit (EFSA, 2013; EFSA, 2015).

Importantly, it has been shown that in prepared foods and, in particular, in ready-to-eat foods, presence of *L. monocytogenes* can depend on numerous factors such as the initial contamination of raw food, product characteristics, ingredients, preparation and storage conditions and the presence of other hygiene indicator bacteria (FAO/WHO, 2004; Rhoades *et al*., 2009; Al-Zeyara *et al*., 2011). In this context, the present study was designed to obtain recent epidemiological data in Spain about the prevalence of *L. monocytogenes* in a large sample of prepared foods, including ready-to-eat foods, and to establish relationships between the presence of *L. monocytogenes* and different factors as storage temperature, type of food, treatment or presence of accompanying bacteria. Results obtained are of relevance for practical application since this study was performed in the context of the routine practice of a food control company, with samples collected in different restoration centres from Spain. Of note, detection of *L. monocytogenes* in prepared foods is still a controversial issue, in which

European regulations have not established its absence in all processes (manufacturing and marketing), maybe due to the impossibility to obtain its total eradication.

Therefore, results obtained provide new insights into the food safety focused to avoid *Listeria* contamination during the manufacturing process and preservation of prepared foods.

## MATERIALS AND METHODS

### Study objectives

To assess the prevalence of *L. monocytogenes* in Spain from a sample of prepared foods, including ready-to-eat foods, and to establish relationships between the presence of *L. monocytogenes* and different factors as storage temperature, type of food, treatment or presence of accompanying bacteria

### Sample collection

Samples were collected according to regulation requirements (EU Commission Regulation, No 2073/2005 and subsequent amendments). During a period of 5 months (May to October 2013) samples (n = 2763) were collected in different centres from Catalonia (Spain). Each sample (75-100 g approximately) was collected in sterile plastic bags, labelled and additional data for the microbiological report were recorded. For the analysis, prepared food samples were classified depending on the type of ingredients according to the Spanish regulation during the study (Real Decreto 3484/2000), and also depending on their capacity to support *L. monocytogenes* growth in agreement to European regulations (EU Regulation 2073/2005 and as amended in 1441/2007).

### Sample transport and storage (according to UNE-EN ISO 7218:2008)

Transport time was reduced at most and transport temperature was between 1-8ºC for unstable products and between 18-27ºC for stable products at room temperature. Until analysis the storage temperature was: 3 ± 2ºC for fresh foods, below -18ºC for frozen foods and 18 – 27ºC for stable foods.

**Plate count method for *L. monocytogenes* detection**

The plate count method was performed according with UNE-EN ISO 11290 2:2000/A1:2005. Briefly, each sample (25 g) was weighed into buffered peptone water (225 ml), blended for 1 min and incubated for 20 - 30 min at 20°C. The mixture (0.1 ml) was distributed on the surface of PALCAM medium (Reactivos para diagnóstico SL, Spain) in spiral (Eddy Jet IUL Instruments SA, Spain) and incubated at 37 ± 1ºC for 48 h. Results were expressed as CFU (colony forming units)/g of food. PCA (Plate count agar, Reactivos para diagnóstico SL) medium was used to prepare pure cultures (at 37 ± 1ºC for 24 h) and Agar Colorex Listeria medium (Reactivos para diagnóstico SL) for confirmation purposes (at 37ºC for 24 h).

**PCR for the detection of *L. monocytogenes***

In the present study, *L. monocytogenes* was detected by the commercial kit Listerfast Complet (Microbial, Spain). A real-time quantitative polymerase chain reaction (PCR) assay targeting *hly*, a gene encoding the hemolysin listeriolysin O, was used (Rodríguez-Lázaro *et al*., 2004). The procedure used was according to the UNE-EN ISO 11290-2:2000/A1:2005. Briefly, 25 g of each sample was diluted in 225 ml of half Fraser broth, homogenized and incubated at 37ºC for 24 ± 2 h. After 5 min centrifugation at 10,000g, pellets were resuspended with the lysis buffer DNAready

(200 µl) to enable cell breakdown and the stabilization of the genetic material released. One microliter of the lysate was added to the PCR reagent (Listerfast Reaction Mix®, Microbial Systems, Spain) that included an internal amplification control (IAC), whose detection indicated the absence of inhibitors. Reactions were run on a Stratagene Mx3000P qPCR System apparatus (Agilent Technologies, USA) with the following program: 10 min at 95°C and 50 cycles of 15 s at 95°C and 1 min at 60°C. Finally, to determine the positive results, the amplified DNAs were automatically analysed by fluorescent signals by the specific amplicons, as already described (Rodríguez-Lázaro *et al*., 2004).

**Recovery of *L. monocytogenes* depending on the pre-enrichment media and accompanying microbiota**

Different concentrations of *L. monocytogenes* and accompanying microbiota (*Salmonella enterica* and *Escherichia coli*) and different pre-enrichment media: Buffered peptone water (Reactivos para diagnóstico SL) and half Fraser both (Reactivos para diagnóstico SL) were used. Before inoculation of bacteria, food (tuna fish product) was autoclaved at 121ºC for 10 minutes. Overnight pure cultures of reference strains of *L. monocytogenes* (ATCC 13932), *S. enterica* (CECT 4300) and *E. coli* (CECT 434) were obtained in PCA after incubation at 37ºC for 24 hours. Liquid bacterial suspensions in saline solution were prepared and adjusted to $10^8$ CFU/ml (corresponding to an absorbance of 0.33 at 620 nm). Food was divided into 12 samples of 25 g each (Table 1). In the first 6 experiments, only *L. monocytogenes* was inoculated (at 1000, 100 and 10 CFU/25 g), while in the 6 last experiments, *S. enterica* and *E. coli* were also added at 100 CFU/25 g to each sample. After inoculation, samples were left

to stand for 30 minutes. All 12 samples were analysed by PCR for the detection of *L. monocytogenes*, as detailed above.

**Method of determining the count of aerobic mesophilic bacteria (AMB) (Plate count method)**

Enumeration of mesophilic aerobes was performed according the Spiral Streak Plate Method (norm AFNOR NF V08-100). Briefly, each sample (25 g) was weighed into 225 ml buffered peptone water, was blended for 1 min and subsequently plated in spiral (0.1 ml) onto PCA. Plates were incubated at 30ºC for 72h and CFU/g of food were obtained.

**Method of determining the count of lactose positive Enterobacteriaceae (Plate count method)**

Enumeration of lactose positive *Enterobacteriaceae* was performed according Spiral Streak Plate Method (norm AFNOR NF V08-100). Briefly, each sample (25 g) was weighed into 225 ml buffered peptone water, blended for 1 min and subsequently plated in spiral (0.1 ml for foods containing heat untreated ingredients and 1ml for foods containing heat treated ingredients) onto VRBL (Violet red bile lactose) agar. Plates were incubated at 30ºC for 24h. CFU/g of food were obtained.

**Statistical analysis**

The statistical analysis was performed using Statistical Package for the Social Sciences (SPSS, version 18). Frequency distributions using contingency tables were analyzed statistically by the chi-squared test and the level of significance was set at $p <$

0.05. Pearson correlation coefficient (r) was calculated to assess the association between variables. Dependence between variables was analysed using linear regression models.

## RESULTS

### Detection of *L. monocytogenes* in function of accompanying bacteria and enrichment media.

The influence of the accompanying microbiota and the enrichment medium in the sensitivity of the detection method was evaluated by adding *L. monocytogenes* at 10, 100 and 1000 CFU/25 g, and *S. enterica* and *E. coli* at 100 CFU/25 g (Table 1). After a single inoculation (only in presence of *L. monocytogenes*), both enrichment media were equally useful to detect *L. monocytogenes* using PCR. In the samples co-inoculated with *L. monocytogenes, S. enterica* and *E. coli*, detection of *L. monocytogenes* using PCR was positive using half Fraser medium at all tested concentrations. However, in presence of accompanying bacteria, buffered peptone water medium was not efficient to favour the growth of *L. monocytogenes* at the lowest concentrations of the bacteria (100 and 10 CFU/25 g). Using this medium, the bacteria detection was only possible using the highest concentrated inoculum of *L. monocytogenes* (1000 CFU/25 g) (sample 10, Table 1).

### Prevalence of *L. monocytogenes* in food samples analysed by PCR or plate count

For the analysis of those foods more susceptible to be contaminated by *L. monocytogenes* (highest risk), a high sensitivity is required and thus a PCR assay is used as a routine method, following current guidelines (according which PCR is justified in foods at high risk of *Listeria* proliferation with specifications at release of absence/25 g; while foods at low risk of proliferation require the use of the plate count method to

demonstrate a limit of < 100 CFU/g during the shelf-life) (Health Protection Agency, 2009; European Regulation 1441/2007). The criteria used in this study to use the PCR method were: foods with a shelf life longer than 5 days, foods more susceptible to be contaminated (meat products and cooked offal, natural bowel, pasteurized ice creams, baking pastries and seafood) and matured cheese (made from milk or serum and treated at high temperature or pasteurized). A total of 2760 samples were analyzed, 78.7% (n = 2171) by the plate count method and 21.3% (n = 589) by PCR. Overall, the prevalence of *L. monocytogenes* in all analyzed samples was 1.4%. As shown in Table 2, using the plate count method, only a positive sample for *L. monocytogenes* was found, representing a prevalence of –0.05%, while using PCR, 39 positive samples were detected, representing a prevalence of 6.6% (Table 2), being this difference statistically significant ($p < 0.05$). These results were expected since samples were analyzed according to the risk and confirm that the criteria applied were adequate and correct, thus confirming the sensitivity of PCR method to detect *Listeria* in samples with low concentrations of this bacterium

Ready-to-eat low risk products, with pH ≤ 4.4 or $a_w \leq 0.92$, products with pH ≤ 5.0 and $a_w \leq 0.94$, and products with a shelf-life of less than five days, shall be automatically considered to belong to this category (Regulation1441/2007 amending Regulation No 2073/2005 on microbiological criteria for foodstuffs) and were analysed by plate count. Ready-to-eat foods included raw vegetables (as lettuce, tomatoes and carrots), fruits and/or eggs (sauce) were classified in four groups. Group A: foods containing heat untreated ingredients, group B: foods containing heat treated ingredients, group AIFC: foods containing fermented or cured ingredients and group D: ready-to-eat vegetables, fruits and derived products, while non-ready-to-eat foods

included dairy products (as cheese), cakes, pasteurized ice creams, cooked or cured meat products.

**Prevalence of *L. monocytogenes* in foods at high risk of *Listeria* contamination using PCR.**

A total of 589 ready-to-eat samples, considered at high risk of *Listeria* contamination, were analysed using real time PCR (Table 2). The presence of *L. monocytogenes* in different types of ready-to-eat foods, groups: A, B, AIFC, and D (described above) was assessed. Among 589 samples, 43 (7.3%), belonged to group A, 471 (80.0%) to group B, 60 (10.2%) to group AIFC, and 15 (2.5%) to group D. *L. monocytogenes* was detected in 4.7% and 4.9% of samples of groups A and B, respectively, and no positive samples were reported in group D. The highest percentage of positive samples of each type of ready-to-eat food was observed in group AIFC (23.3%, n = 14), which included foods with fermented or cured products as ingredients (Table 2). Significant differences were found between groups ($p < 0.001$), and the prevalence of *L. monocytogenes* in the AIFC group was significantly higher. In a thorough analysis of positive samples belonging to group AIFC, cheese was the common ingredient in most of them (Table 2).

**Influence of temperature of food storage on contamination by *L. monocytogenes.***

From a total of 2684 food samples with temperature records available, the influence of storage temperature on contamination by *L. monocytogenes*, regardless of the detection method used, was assessed. Samples were grouped according to the storage temperature in 3 different groups: 1) < 0ºC, freezing; 2) 0 – 10ºC, refrigeration;

and 3) > 10ºC. Most samples belonged to group > 10ºC (63.5%), followed by group 0 - 10ºC (32.0%) and group < 0ºC (4.5%). As shown in Table 3, *L. monocytogenes* was detected in all temperature ranges. The highest prevalence (4.1%) was observed in those foods stored below 0ºC. Foods stored between 0 - 10ºC had a prevalence of 3.3%, while foods stored at temperatures higher than 10ºC had the lowest prevalence (0.3%), being these differences among groups statistically significant (Table 4).

**Influence of sample collection centres on presence of *L. monocytogenes***

All samples (n = 2760) belonged to 505 manufacturing centres randomly selected. The presence of *L. monocytogenes* was more common in certain centres and, based on this, we grouped centres depending on the frequency of detection of *L. monocytogenes*. The first group included five centres and was defined to have two or more positive samples, while the second group included the remaining 500 centres, defined to have less than 2 positive samples. 87.5% of all positive samples for *L. monocytogenes* belonged to the 5 centres of the first group, while the remaining 500 centres of the second group accounted only for the 12.5% of positive samples.

In common, centres of the first group (5 centres) were big establishements, with a central kitchen where products with a long shelf-life are prepared.

**Relationship between presence of *L. monocytogenes* and aerobic mesophilic bacteria (AMB)**

Quality indicator bacteria, as AMB, can help to assess centre functioning and procedures for internal control in the manufacturing of prepared foods. Foods of groups as described before were analyzed and were classified according to AMB in 3 count

levels: < $10^2$ CFU/g, $10^2$ – $10^4$ CFU/g and > $10^4$ CFU/g. A total of a 2301 samples had AMB counts in any of the indicated levels. The AMB counts of < $10^2$ CFU/g, $10^2$ – $10^4$ CFU/g and > $10^4$ CFU/g had frequencies of 36.9%, 32.5% and 30.6%, respectively. 24 samples were positive for *L. monocytogenes,* had AMB counts between $10^2$ - $10^4$ and >$10^4$ CFU/g, and corresponded to 60% of *Listeria* positive foods of this study. In foods with AMB counts lower than $10^2$ CFU/g, *L. monocytogenes* was not detected in any sample. For AMB count between $10^2$ – $10^4$ CFU/g, a 29.2% (n = 7) of *Listeria* positive foods were found, while AMB counts higher than $10^4$ CFU/g corresponded to 70.8% (n = 17) of *Listeria* positive samples. As shown in Table 4, these results indicate a tendency to a relationship between *L. monocytogenes* presence and AMB counts. Prevalence of *L. monocytogenes* for AMB counts $10^2$ – $10^4$ CFU/g was 0.9 %, while for counts higher than $10^4$ CFU/g, prevalence was 2.4 %, with a correlation coefficient (*r*) value of 0.984.

Regarding AMB counts in foods of group A (containing some heat untreated ingredients), 40 samples were analysed by PCR. We found a high number of samples (n = 26) with AMB counts higher than $10^4$ CFU/g and the number of samples with AMB counts between $10^2$ and $10^4$ CFU/g was also considerable (n = 12), corresponding all these samples to 95% (n = 38) of all tested samples (Table 4). Most of these samples, however, were within the specifications established for these type of products considered satisfactory if ≤ $10^5$ CFU/g with a maximum of $10^6$ CFU/g. In the analysis by PCR a low prevalence of *L. monocytogenes* was found. Only 2 positive results (7.7%) were obtained in samples with AMB counts higher than $10^4$ CUF/g, showing a poor association between both analyses ($p$ = 0.567).

Regarding foods of group B (containing heat treated ingredients), a total of 383 samples were analyzed. As shown in Table 4, in most samples AMB counts were low: 50.1% had AMB counts lower than $10^2$ CFU/g, 31.3% counts were between $10^2$ and $10^4$ CFU/g and 18.5% counts were higher than $10^4$ CFU/g. 19 positive results for *Listeria* (18.0%) were obtained in samples with AMB counts higher than $10^4$. Thus, AMB counts in foods of group B were positively correlated with the presence of *L. monocytogenes* ($r = 0.97$).

**Relationship between presence of *L. monocytogenes* and lactose positive *Enterobacteriaceae***

Lactose positive *Enterobacteriaceae* are indicator bacteria of food contamination due to unhygienic practices during food manipulation and processing. For this reason, we assessed the relationship between presence of *L. monocytogenes* and lactose positive *Enterobacteriaceae* in a total of 2181 samples of foods classified in groups A (containing some heat untreated ingredients) and B (containing heat treated ingredients). Results, expressed as $\leq 3 \times 10^3$ CFU/g and $> 3 \times 10^3$ CFU/g, showed that 1882 samples (86.3%) had counts lower than $3 \times 10^3$ CFU/g, while the remaining 299 samples had counts higher than $3 \times 10^3$ CFU/g. Prevalence of *L. monocytogenes* was 0.8% in samples with counts lower than $3 \times 10^3$ CFU/g and 2.7% in samples with counts higher than $3 \times 10^3$ CFU/g (Table 5), with a positive correlation and statistically significant differences ($p = 0.003$), thus indicating that in foods with high counts of lactose positive *Enterobacteriaceae*, the presence of *L. monocytogenes* is high.

According to current regulations, allowed limits of lactose positive *Enterobacteriaceae* for prepared foods are $\leq 10^3$ CFU/g for group A and $\leq 10$ CFU/g

for group B. The analysis in samples of group A (n = 40) showed that 15% of samples had counts of lactose positive bacteria ≤ $10^3$ CFU/g (Table 5) and 85% of samples would be out of specifications. Of note, in samples with counts of lactose positive bacteria lower than $10^3$ CFU/g, no positive samples for *L. monocytogenes* were found and the prevalence in samples with counts higher than $10^3$ CFU/g was 5.9% (Table 5), without statistically significant differences ($p = 0.527$).

Finally, in prepared foods of group B (heat treated) (n = 384), 80.5% of samples had counts of lactose positive bacteria ≤ 10 CFU/g and 19.5% had counts higher than 10 CFU/g (Table 5), thus showing the efficacy of heat treatment to decrease the load of lactose positive bacteria in foods. Prevalence of *L. monocytogenes* in foods of group B was 5.2%. The prevalence of *L. monocytogenes* was 1.9% in foods with low counts and 18.7% in foods with high counts, being this difference statistically significant ($p < 0.05$) and showing a positive correlation between presence of *L. monocytogenes* and high counts of lactose positive *Enterobacteriaceae*.

## DISCUSSION

Although food-borne outbreaks of listeriosis remain a major public health problem (Bortolussi, 2008), no enough epidemiological data are available in different countries as Spain or in different types of foods, such as prepared foods, which can may become contaminated due to their origin or their manufacturing process (Luo *et al.*, 2015; Bortolussi, 2008).

Nowadays, the rapid, cost-effective, and automated detection of *L. monocytogenes* throughout the food chain continues to be a major concern (Traunsek *et al.*, 2011). Diagnosis of listeriosis and detection of *L. monocytogenes* is commonly

based on classical culturing which are laborious and time-consuming (Liu and Busse, 2009; Traunsek *et al*., 2011; Jasson *et al*., 2010).

Results obtained in the present study highlight that PCR and the enrichment medium half Fraser allows the detection of *L. monocytogenes*, even at low concentrations of *L. monocytogenes* (10 and 100 CFU/25 g) and in the presence of accompanying bacteria (as *S. enterica* and *E. coli*). This is, in fact, the medium currently recommended by International Organization Standardisation (ISO) (UNE-EN ISO 11290-2:2000) for the enrichment of *Listeria*.

Our results are also of relevance for practical application, since most samples taken in along the food chain are contaminated with low numbers of pathogens (usually less than 100 CFU/g) and even hygiene indicators (10 to max $10^4$ CFU/g) (Jasson *et al*., 2010).

As we have reported in this study, presence of *L. monocytogenes* is frequently correlated with presence of accompanying microbiota as AMB and lactose positive *Enterobacteriaceae*, thus demonstrating the usefulness of this detection strategy (PCR plus half Fraser) in real scenarios.

Overall, the prevalence of *L. monocytogenes* in all analyzed samples was low (1.4%), in comparison with studies performed in other countries (3.6% in ChileNoriega *et al*., 2008) or even in certain Spanish regions (2.7% in commercially available fruits and raw and ready-to-eat vegetables (Badosa et al., 2008)6.2% in ready-to-eat products, ranging from 0.8% for vacuum-packed pâté to 25% for smoked trout; Garrido *et al*., 2009). As we have also reported, products that received heat treatment (deli meat products and pâté) showed a smaller pathogen incidence than smoked products (Garrido *et al*., 2009).

The positive samples found in our study (whipped cream, pork blood sausages and stew of calf´s foot and cheek) are dairy and meat products, which are considered at risk of *L. monocytogenes* contamination (Garrido *et al*., 2009; Rebagliati *et al*., 2009; Luo *et at.,* 2015;AESAN, 2010, Rebagliati *et al*., 2009). Of note, all samples of ice cream in our study were negative.

Current regulations only determine the detection of *L. monocytogenes* in ready-to-eat foods and for this reason not many data on the prevalence of *Listeria* exist on prepared foods of meat, raw fish, fruit, etc. In this regard, a recent outbreak of listeriosis has occurred in USA associated to the intake of melon (http://www.bbc.co.uk/news/world-us-canada-15086103), thus showing the need of control of *Listeria* also in whole fruits.

Of note, we found a high prevalence (23.3%) of *L. monocytogenes* in foods containing fermented or cured ingredients, . It is usual that in manufactured cheeses from pasteurized milk, contamination occurs in the manufacturing facilities, during transport or storage and also certain strains can even survive pasteurization temperatures (Marzano and Balzaretti, 2011). Regarding the influence of storage temperature, a higher prevalence when foods were stored below 0ºC was observed, suggesting that the presence of *L. monocytogenes* can be limited as temperature increases. It is known the capacity of this bacterium to maintain its viability at freezing temperatures over long periods of time (in ice creams, frozen foods, etc.) (Rebagliati *et al*., 2009), maybe due to the lower competence with other microorganisms below 0ºC, since *L. monocytogenes* is a psychrotrophic poor competitor bacterium that can supersede mesophilic bacteria only at low temperature (Carpentier and Cert, 2011).

Among centres with higher frequencies of detection of *L. monocytogenes*, we found different factors that could explain this. For example, all centres had several

production lines, with a wide range of final products and, therefore, receiving an equally high number of raw materials. They were also characterized to have a central kitchen and to prepare products with a long shelf-life and, in some cases, meat ready-to-eat products. All have well-conditioned cold rooms, where a part of the production process is performed. In this context, it seems logical that a competitive bacteria in cold atmospheres as *Listeria* could enter in the production of some raw materials in these cold rooms and have more probability to contaminate and proliferate in ready-to-eat foods, which have received more manipulation.

Moreover, most of these centres had sophisticated machinery that is often difficult to clean and that can favour biofilm formation. Of note, we observed that the centres with high frequency of detection had a tool in common, the blast chiller, thus suggesting that it could be a point for contamination in the manufacturing process, although further studies, including the localization of the samples, should be performed to confirm this.

Of note, we observed a trend to anassociation between incidence of *L. monocytogenes* and AMB counts. As already reported, *Listeria* is able to form ecological niches with other bacteria as *Pseudomonas*, thus allowing its persistence in the environment and making its eradication difficult (Pérez-Rubiano *et al*., 2008, Carpentier and Cerf, 2011).

In samples of group A (containing some heat untreated ingredients), we found a high number of samples with AMB counts > $10^4$ CFU/g and the number of samples with AMB counts between $10^2$ and $10^4$ CFU/g was also considerable. These elevated counts could be due to the absence of heat treatment, thus allowing AMB colonization of the food, and also due to the presence of thermoduric microorganisms that can resist heat treatments. This highlights the importance of refrigeration to minimize

multiplication of microorganisms. In raw foods, AMB counts can be up to $10^9$ CFU/g, although in most non-fermented foods, AMB counts can range between $10^4$ and $10^6$ CFU/g (De Giusti *et al*., 2010).

As expected, in samples of group B (including heat treated ingredients), AMB counts were low (< $10^2$ CFU/g) and, in general, were within limits established by current regulations (> $10^5$ CFU/g). We used group A and group B samples to compare the effect of the presence/absence of heat treatment in the ingredients and according the requirements of current guidelines. Only in case of counts > $10^6$ CFU/g, a thorough study of influencing factors in the food chain and implementation of corrective measures should be indicated. In this type of foods, contamination could be occurred in the raw materials, supporting cooking temperatures, or by cross-contamination after the cooking process. AMB growth mainly corresponds to thermoduric bacteria (probably including *Listeria* among AMB), which can survive after heat treatment and, of note, in the presence of low populations of competitors. This can explain the positive correlation between AMB counts and incidence of *L. monocytogene*s we observed.

An association between presence of lactose positive *Enterobacteriaceae* and *L. monocytogenes* was observed. Interestingly, in foods of group A, most samples (85%) were out of specifications, while most samples of group B (80%) were within established specifications, thus showing the efficacy of heat treatment to decrease the number of lactose positive bacteria. These results are also in line with previous works performed in fresh vegetables (as lettuce, carrot, cabbage and scallion), in which a high concentrations of coliforms (87.5% of samples with counts > $10^3$ CFU/g) and presence of *Listeria* were concomitantly detected (Martino *et al*., 2008). In a study in 120 samples of minimally processed ready-to-eat vegetables, *E. coli* was detected in approximately 30.3% and the genus *Listeria* was evidenced in 25% of the samples (30%

corresponded to *L. monocytogenes*), while other Gram negative bacteria as *Shigella* spp and *Vibrio cholerae* were not isolated (De Curtis *et al*., 2002).

According to results obtained, it can be concluded that in cases of high counts of accompanying bacteria as AMB and lactose positive *Enterobacteriaceae*, it is indicated to test the presence of *L. monocytogenes*. Further studies, however, should be needed to assess the scope of the reported associations, testing other types of foods and also obtaining quantitative data for *Listeria* detection. Finally, factors favouring the presence of *L. monocytogenes* could be some components in ready-to-eat foods, temperatures below 0ºC and the presence of accompanying bacteria.

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## TABLES

TABLE 1.
Influence of the pre-enrichment medium and the presence of *S. enterica* and *E. coli* on the detection of *L. monocytogenes*.

| | **Medium** | | **Inoculum** | | | **Results** | |
|---|---|---|---|---|---|---|---|
| **Nº (*)** | **Buffered peptone water (total mL)** | **Half Fraser (total mL)** | ***L. monocytogenes* (CFU/25g)** | ***S. enterica* (CFU/25g)** | ***E. coli* (CFU/25g)** | ***L. monocytogenes* count detection (72 h)** | ***L. monocytogenes* PCR detection** |
| 1 | - | 250 | 1000 | - | - | Positive | Positive |
| 2 | - | 250 | 100 | - | - | Positive | Positive |
| 3 | - | 250 | 10 | - | - | Positive | Positive |
| 4 | 250 | - | 1000 | - | - | Positive | Positive |
| 5 | 250 | - | 100 | - | - | Positive | Positive |
| 6 | 250 | - | 10 | - | - | Positive | Positive |
| 7 | - | 250 | 1000 | 100 | 100 | Positive | Positive |
| 8 | - | 250 | 100 | 100 | 100 | Positive | Positive |
| 9 | - | 250 | 10 | 100 | 100 | Positive | Positive |
| 10 | 250 | - | 1000 | 100 | 100 | Negative | Positive |
| 11 | 250 | - | 100 | 100 | 100 | Negative | Negative |
| 12 | 250 | - | 10 | 100 | 100 | Negative | Negative |

(*) All prepared samples (25 g) were left to stand for 30 min.

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TABLE 2.
Detection of *L. monocytogenes* food samples at low risk (analysed using the plate count method) and at high risk (analysed by real-time PCR).

| **Code** | **Type of food** | **Number of positive results for *L. monocytogenes* detection (%)** | **Total of samples analysed (%)** |
|---|---|---|---|
| **Low risk** (analysed by plate count) | | | |
| A * | Ready-to-eat products with some heat-untreated ingredient | - | 389(100) |
| AIFC** | Ready-to-eat foods with some fermented and/or cured ingredient | - | 298(100) |
| B | Ready-to-eat products containing heat treated ingredients | 1 (0.07) | 1459(100) |
| D | Ready-to-eat vegetables, fruits and derived products | - | 25(100) |
| | Total samples plate count | 1 (0.05) | 2171 (100) |
| **High risk** (analysed PCR) | | | |
| A | Ready-to-eat products with some heat-untreated ingredient | 2 (4.7) | 43(100) |
| AIFC | Ready-to-eat foods with some fermented and/or cured ingredient | 14 (23.3) | 60(100) |
| B | Ready-to-eat products containing heat treated ingredients | 23 (4.9) | 471(100) |
| D | Ready-to-eat vegetables, fruits and derived products | - | 15(100) |
| | Total samples PCR | 39 (6.6) | 589 (100) |
| | TOTAL SAMPLES | 40 (1.4) | 2760 (100) |

*Foods classified according to Spanish regulations (RD 3484/2005): Groups A (containing heat untreated ingredients), B (heat treated prepared foods), AIFC (containing fermented or cured ingredients) and D (ready-to-eat vegetables, fruits and derived products).

**AIFC foods included 10 samples containing cheese and 4 samples containing other fermented and/or cured ingredients.

TABLE 3.
Presence of *L. monocytogenes* depending on the storage temperature.

| **Temperature ranges** | **Number of samples with *L. monocytogenes* (%)** | **Total number of samples studied (%)** |
|---|---|---|
| < 0ºC | 5 (4.1) | 122 (100) |
| 0-10ºC | 28 (3.3) | 858 (100) |
| >10ºC | 5 (0.3) | 1704 (100) |
| Total | 38 (1.4) | 2684 (100) |

TABLE 4.

Correlation between prevalence of *L. monocytogenes* and AMB counts on ready-to-eat foods.

| **Total ready-to-eat-foods analysed (plate count and PCR)** | | |
|---|---|---|
| **AMB range (CFU/g)** | ***L. monocytogenes* detection (%)** | **Number of samples analysed for counts of aerobic mesofil bacteria (%)** |
| $\leq 10^2$ | - | 849 (100) |
| $10^2$-$10^4$ | 7 (0.9) | 748 (100) |
| $>10^4$ | 17 (2.4) | 704 (100) |
| Total | 24 (1.0) | 2301 (100) |
| **A: Ready-to-eat-foods containing some heat untreated ingredients (PCR)** | | |
| $\leq 10^2$ | - | 2 (100) |
| $10^2$-$10^4$ | - | 12 (100) |
| $>10^4$ | 2 (7.7) | 26 (100) |
| Total | 2 (5) | 40 (100) |
| **B: Ready-to-eat-foods containing heat treated ingredients (PCR)** | | |
| $\leq 10^2$ | - | 192 (100) |
| $10^2$-$10^4$ | 1 (0.8) | 120 (100) |
| $>10^4$ | 19 (18) | 71 (100) |
| Total | 20 (5.2) | 383 (100) |

TABLE 5.

Correlation between frequency of *L. monocytogenes* and counts of lactose positive *Enterobacteriaceae* on ready-to-eat foods

| **Total ready-to-eat-foods analysed (plate count and PCR)** | | |
|---|---|---|
| **Limit of lactose positive *Enterobacteriaceae* (CFU/g)** | ***L. monocytogenes* detection (%)** | **Number of samples analysed for counts of lactose positive *Enterobacteriaceae* (%)** |
| $\leq 3x10^3$ | 15 (0.8) | 1882 (100) |
| $>3x10^3$ | 8 (2.7) | 299 (100) |
| Total | 23 (1.1) | 2181 (100) |
| **A: Ready-to-eat-foods containing some heat untreated ingredients (PCR)** | | |
| $\leq 3x10^3$ | 0 (0) | 6 (100) |
| $>3x10^3$ | 2 (5.9) | 34 (100) |
| Total | 2 (5) | 40 (100) |
| **B: Ready-to-eat-foods containing heat treated ingredients (PCR)** | | |
| $\leq 10$ | 6 (1.9) | 309 (100) |
| >10 | 14 (18.7) | 75 (100) |
| Total | 20 (5.2) | 384 (100) |