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Validation of standard method EN ISO 11290 - Part 1 - Detection of *Listeria monocytogenes* in food

Nathalie Gnanou Besse[a,*], Bertrand Lombard[a], Laurent Guillier[a], Danièle François[a], Karol Romero[a], Sylvie Pierru[a], Laurence Bouhier[a], Patricia Rollier[b]

[a] Université Paris Est, ANSES, Laboratory for Food Safety, Maisons-Alfort location, F-94701 Maisons-Alfort, France
[b] ACTALIA CECALAIT, F-39800 Poligny, France

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ABSTRACT

The reference method for the detection and enumeration of *L. monocytogenes* in food (Standards EN ISO 11290-1 &2) has been validated by inter-laboratory studies in the frame of the Mandate M381 from European Commission to CEN. In this paper, the collaborative studies led in 2013 on 5 matrices (cold-smoked salmon, milk powdered infant food formula, vegetables, environment, and cheese) to validate the recently revised Standard EN ISO 11290-Part 1 are reported. According to the results obtained, the revised Standard EN ISO 11290-1 can be considered as a good method for the detection of *L. monocytogenes* in foods and food processing environments, in particular for the matrices included in the study. According to the matrices, the sensitivity rate varied from 91.1% to 100%, and the specificity rate varied from 97.6% to 100%. Positive samples were most often detected after 24 h half-Fraser enrichment.

1. Introduction

L. monocytogenes is a Gram-positive bacterium responsible for listeriosis, a severe foodborne illness which may result in meningitis, septicemia, spontaneous abortion, perinatal infections and gastroenteritis. Despite the low incidence of infection in humans, listeriosis is associated with a high lethality, particularly in elderly and immunocompromised individuals (Anonymous, 2000). Moreover, since 2000, an increase in the number of listeriosis cases has been observed in several European countries, but the reasons for this phenomenon still remain unclear (Anonymous, 2007; Anonymous, 2015). In addition, the detection of *L. monocytogenes* in food has important economic consequences, because it can lead to the withdrawal of incriminated products and subsequent decrease of sales.

CEN/TC 275/WG 6,[1] in charge of standardization in microbiology of the food chain at European level, has received a mandate from the European Commission (EC) (Mandate M381 signed in December 2010) to validate by inter-laboratory studies (ILS) and standardize a set of reference methods in food chain microbiology. These methods are or are expected to be cited as the reference methods in the (EC) Regulation 2073/2005 (Anonymous, 2005) on microbiological criteria for foodstuffs. The ILS had to be performed before the end of 2013, and standards had to be published by the end of June 2017. The determined performance characteristics were published in the corresponding CEN ISO standard methods. This validation program included the reference methods for the detection and enumeration of *L. monocytogenes* in food (Standards EN ISO 11290-1&2). The ILS was designed according to a common design elaborated by WG 3 Method Validation and WG 2 Statistics of ISO/TC 34/SC 9,[2] based on the version of Standard EN ISO 16140 on method validation in food microbiology, applicable at the date of the trials (Anonymous, 2003). Since these standards describe horizontal methods, applicable to all food, feed and food processing environment, the ILS had to be performed on 5 matrices. The chosen matrices (cold-smoked salmon, milk powdered infant food formula, vegetables, food processing environment and cheese) were representative of ready-to-eat food categories cited in the EC Regulation 2073/2005 on microbiological criteria for foodstuffs.

In this paper, the results of the inter-laboratory studies to validate Standard EN ISO 11290-Part 1 are reported. The trials, led by ANSES, Laboratory for Food Safety, and by ACTALIA-CECALAIT, in collaboration with TAG 17 (Task Group for *Listeria*) of CEN/TC 275/WG 6, as well as other participants to the trials, were carried out in 2013 (5 matrices, 6 dispatches). TAG 17 of CEN/TC 275/WG 6, in charge of the revision and validation of the standard, made several modifications to

* Corresponding author.
E-mail address: nathalie.gnanou-besse@anses.fr (N. Gnanou Besse).
[1] Working Group 6 "Microbiology of the food chain" of Technical Committee 275 "Food analysis-Horizontal methods" of European Committee for Standardization (CEN).
[2] Sub-Committee 9 "Microbiology" of Technical Committee 34 "Food products" of International Organization for Standardization (ISO).

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Table 1
Strains used in the studies.

Matrices	Cold-smoked salmon			Environmental samples			Ready-to-eat salads			Powdered infant food formulae			Cheese		
Characteristics of the strains	Name	Seroty-pe	Origin	Name	Seroty-pe	Origin	Name	Seroty-pe	Origin	Name	Seroty-pe	Origin	Name	Seroty-pe	Origin
L. monocytogenes	03CHPL82	1/2a	Cold-smoked salmon	10CEB 86Lm	1/2c	Food produc-tion enviro-nment	12CEB 101Lm	4b	Salad	05CEB 631Lm	1/2a	Milk	00004	1/2b	Raw milk
L. innocua				CHPL96		Food produc-tion enviro-nment							93,023		Raw milk
L. welshimeri	02CHPL153		Cold-smoked salmon												
Pseudomonas fragi				09empf71		Food produc-tion enviro-nment									
Bacillus cereus				07HMPL09		Ham									
Staphylococcus epidermidis				10empf131		Food produc-tion enviro-nment									
Lactococcus lactis subsp. *Lactis*													97,009		Dairy product

the earlier version of the standard of1996, amended in 2004. The ILS have been conducted on the basis of the draft revised standard, containing the following modifications for the detection of *L. monocytogenes*: one-hour increase of the primary enrichment in Half-Fraser broth; reduction by 24 h of the secondary enrichment duration in Fraser broth; possible refrigeration of Half-Fraser broth and Fraser broth before transfer or isolation on selective agar; possible refrigeration of isolation plates before reading; simplification of the confirmation step.

2. Materials and methods

2.1. Design of the trial

Fifteen to 16 laboratories participated to each trial and were from 18 different European countries and USA. Cheese samples were prepared by ACTALIA-CECALAIT, and other matrices by Anses. For practical reasons, samples of each matrix for detection and enumeration trials were prepared and sent together, except cheese. Each food type was inoculated at different inoculum levels with pure *L. monocytogenes* strains. The strains used (Table 1) have been isolated from each matrix and belonged to different serotypes of interest often found in food, in order to better detect potential lack of performance due to serotype. For qualitative studies, according to the common design for the ILS of this CEN Mandate, a blank (0 CFU/25 g) and two levels of contamination (5–10, 50–100 CFU/25 g) with *L. monocytogenes* (were used for each matrix, with 8 blind replicates in each case. So in total 24 samples per matrix were sent to each laboratory. Environmental samples consisted of gauze pads immerged in 20 ml diluent, and to be homogenous with other matrices, contamination levels corresponded of 0, 5–10, 50–100 CFU/sample. In some cases (cold-smoked salmon, environment, and cheese), a competitive background microflora (in particular other *Listeria* species) was artificially added, including in blanks. In addition, environmental samples were contaminated to simulate autochthonous microflora.

All batches were accepted for use in the trials on the basis of achieving satisfactory homogeneity and stability. Homogeneity and stability of samples were evaluated according to ISO 13528 (Anonymous, 2005), XP CEN ISO/TS 22117 (Anonymous, 2010) and IUPAC protocol (Anonymous, 2006).

The test materials were shipped to the participants by courier in polystyrene insulated boxes containing several ice packs to prevent exposure of the test materials to high temperatures during transport, and a temperature monitoring device to follow the temperature fluctuation during transport. Participants provided all media and reagents needed for the collaborative trial, and received from trial leaders a financial contribution for the culture media cost. Test material codes were randomised for each participant to prevent collusion between laboratories. The examination of the test materials was carried out within a specified time period as prescribed in the circular letter containing the information to participants.

2.2. Methods under collaborative trial

The former version of the Standard method EN ISO 11290 part 1 (1996 version amended in 2004) (Anonymous, 1996; Anonymous, 2004a) consisted of a sequential double enrichment in Half-Fraser and Fraser selective broths. The initial incubation in half-Fraser was carried out for 24 h at 30 °C, followed by a second enrichment for 48 h in Fraser broth at 37 °C. After incubation and isolation on selective agars, typical colonies were then submitted to confirmation tests described in the standard. Agar *Listeria* according to Ottaviani and Agosti (LOA agar), a chromogenic selective agar, which distinguishes *L. monocytogenes* and *L. ivanovii* from other species of *Listeria,* was adopted in 2004 by the International Organization for Standardization (ISO) as the standard medium for the detection of *L. monocytogenes*. Following incubation at the fore-mentioned times and temperatures, the enrichment broths

were streaked both on LOA agar and on another selective agar of own choice. Then typical colonies were purified on a non-selective agar. The confirmation tests for the genus *Listeria* are the Gram staining and the catalase test (as mandatory tests), as well as the motility and Henry illumination tests (as optional tests). Additional confirmation towards the species *L. monocytogenes* was achieved via haemolysis test, CAMP test and sugar utilization test.

The *Listeria* TAG 17 of CEN/TC 275/WG 6, in charge of the revision and validation of the standard, made several modifications to the standard. The draft revised standard, on whose basis the ILS trials have been conducted contained the following modifications, compared to the 1996 Standard amended in 2004:

- The primary enrichment in Half-Fraser broth was incubated 25 h ± 1 h (instead of 24 h ± 2 h), as to improve the growth of stressed *Listeria monocytogenes* cells;
- The duration of secondary enrichment in Fraser broth was reduced by 24 h, according to a study of European Union Reference Laboratory for *L. monocytogenes* (Gnanou Besse et al., 2016);
- Half-Fraser broth and Fraser broth may be refrigerated before transfer or isolation on selective agar for a maximum of 72 h;
- Refrigeration of isolation plates was allowed for a maximum of 2 days before reading;
- Catalase test and Camp-test became optional for *L. monocytogenes*.
- Microscopic aspect remained mandatory except an agar allowing distinction of pathogenic *Listeria* spp. was used.
- For haemolysis test or CAMP test, blood agar was extended from defibrinated sheep blood only, to calf or bovine blood. For haemolysis test, blood agar was to be inoculated by stabbing or by streaking (only if positive at purification step).

For the inter-laboratory studies, secondary enrichment duration was set at 24 h, but on a volunteer basis about half of the participants compared isolation agars obtained from 24 h (mandatory) and 48 h Fraser enrichment. For detection, one positive colony per sample was sufficient. Confirmation was performed according to the draft revised Standard. Certain standard tests could be performed with commercial miniaturized galleries, but all mandatory tests were required. Some laboratories may have performed in addition other confirmatory methods, such as PCR protocols. Some media (second isolation agar, diluents, etc.) were left at choice, but in agreement with the draft revision of EN ISO 11290-1. Finally, as it will be detailed later, the inter-laboratory studies were conducted with a pre-enrichment duration of 24 h ± 2 h.

2.3. Preparation of test materials

Cheese curd test materials were prepared by ACTALIA-CECALAIT, Poligny, France. The curd cheese was prepared according to an internal procedure, from a "liquid pre-cheese", that is milk enriched in proteins by microfiltration. Then a curd was obtained by addition of rennet, without exudation of whey. The pH value of the curd was about 6,3. All batches were prepared according to the following procedure: stock cultures of strains were maintained frozen at −80 °C using Cryobank tubes (BioMérieux, Combourg, France). Cultures were revived by inoculating Brain Heart Infusion (BHI, BIOKAR, Allonne, France), plated onto a selective agar and then onto Tryptone Soya Agar Yeast Extract (TSAYE, BIOKAR) for *Listeria* strains, or directly onto Plate Count Agar and milk (mPCA, BIOKAR) for lactic acid bacteria strain. A "liquid pre-cheese" was prepared and inoculated with the test strains comprising *L. monocytogenes* strain serotype 1/2b (internal number 00004) and a *L. innocua* strain (internal number 93023), which had been isolated from raw milk. As the natural total flora was low (< 1000 CFU/g), a background microflora containing a strain of *Lactococcus lactis* subsp. *Lactis* (internal number 97009) was also added at a level of approximately 5 10^4 CFU/g. Prior to inoculation, the strains were cultivated in BHI for 18 h at 37 °C for *Listeria* strains, and for 18 h at 30 °C for the strain of *Lactococcus*, as to obtain stationary phase cultures. Then the cultures were diluted in Tryptone Salt solution (TS, BIOKAR), and inoculated in the "liquid pre-cheese". Just after, rennet was added in the inoculated "liquid pre-cheese" that was dispensed in aliquots of 100 g into individual vials and clotted (by the addition of rennet and not by acidification by lactic acid bacteria). The microflora within the cheese was stabilized by the addition of an undisclosed bacteriostatic mixture. The bacteriostatic effect is negated when the sample is diluted during examination. The inoculum levels for the target organisms were verified using a spiral plating system at a single dilution in TSAYE for *Listeria* strains and in mPCA for the lactic acid bacteria strain. Batches of cheese test materials were prepared for the collaborative trial one day prior to dispatch to participants, due to their relatively short stability.

All other matrices were prepared by ANSES Laboratory for food safety, Maisons-Alfort, France. All strains used in the trial studies were isolated and characterized at the laboratory, from the same type of matrix. All batches of cold-smoked salmon, salad and environmental samples were prepared according to the following procedure: stock cultures of test *Listeria* strains were maintained frozen at −80 °C using Cryobank tubes (BioMérieux, Combourg, France). Cultures were revived by plating onto TSAYE and then propagated twice in BHI broth (BioMérieux) at 37 °C before use (stationary phase BHI cultures contain approximately 1×10^9 cfu/ml). To confirm the inoculum level, dilutions of the BHI cultures were enumerated by spreading on TSAYE. All dilutions were made in TS broth (BioMérieux). For artificial contamination, each sample was spiked with 0.1 ml of the appropriate TS dilution of the culture, and gently spread. Absence of *Listeria* spp. in each purchased matrix was previously checked according to the EN ISO 11290-1 reference method (Anonymous, 1996, 2004). Results showed that no product used in these studies was contaminated by *Listeria* spp.

Cold-smoked salmon of a same batch was collected from a French manufacturer and used at the beginning of its shelf-life. It was cut into 2 cm^2 parts and divided into 25 g portions, taking squares from different parts of the salmon sample. Square slices were used to better simulate contamination on the surface of the product. After contamination, portions were stored and sent frozen (−18 °C) before analysis. *L. monocytogenes* serotype 1/2a (03CHPL82) isolated from cold-smoked salmon was used as inoculum. A competitive background microflora consisting of *L. welshimeri* (02CHPL153), isolated from cold-smoked salmon was artificially added, including in blanks; the ratio with *L. monocytogenes* was 1/1.

Ready-to-eat salads, consisting of a mixture of fresh leaves of spinach and iceberg lettuce of a same batch were collected from a French manufacturer and used at the beginning of their shelf-lives. This matrix contained naturally an abundant microflora, able to grow on *Listeria* selective agars. They were divided into 25 g portions, and after contamination, portions were stored and sent frozen (−18 °C) before use. *L. monocytogenes* serotype 4b (12CEB10Lm) isolated from salad was used as inoculum.

Environmental samples consisted of gauze pads immerged in 20 ml diluent (TS containing a bacteriostatic mixture), *L. monocytogenes* serotype 1/2c (10CEB86Lm) isolated from food production environment was used as inoculum. A competitive background microflora was artificially added, including in blanks: *L. innocua* (CHPL96) isolated from food production environment was added at a ratio ¼ with *L. monocytogenes*. Moreover, a mixture of *Staphylococcus epidermidis* (10empf131) isolated from food production environment (20,000 CFU), *Bacillus cereus* (07HMPL09) isolated from ham (2000 CFU) and *Pseudomonas fragi* (09empf71) isolated from food production environment (2000 CFU) was added in each sample. The microflora within the sample was stabilized by the addition of a bacteriostatic mixture. The bacteriostatic effect is negated when the sample is diluted during examination. Four milliliters of a solution containing 10 g of boric acid (Sigma-Aldrich, St. Quentin Fallavier, B6768), 2 g of glycerol (Acros Organics, Thermo Fisher Scientific, Dardilly, 295,600,000) and 0.150 g

of potassium sorbate (Sigma-Aldrich, Fluka 85520) per 200 ml of distilled water were added to 16 ml of TS diluent.

For powdered infant food formula (PIF), lyophilized strains were used for artificial contamination: *L. monocytogenes* serotype 1/2a (05CEB63Lm) isolated from milk was used as inoculum. Cultures grown in an equal mixture of BHI and sterile infant milk (follow-on formula from retailer) for 24 h at 37 °C were freeze-dried using the CHRIST LOC-2 M apparatus (Bioblock Scientific, Ile de France, Vanves, France). Contaminated powder was further diluted 1 in 100 in PIF intended for infants below 6 months of age, and stored at refrigeration temperature before use. This resulted in highly contaminated milk powders. The required contamination levels were prepared by mixing these powders with sterile milk powder in several steps (to optimize homogeneity of the test material) until the required contamination level was reached. This milk powder matrix contained naturally some *Bacillus* and Gram-positive cocci, able to grow on *Listeria* selective agars.

2.4. Homogeneity and stability of the test materials

Acceptance of all production batches for use in the trials was made on the basis of achieving satisfactory homogeneity and stability. The homogeneity and stability of the test materials were tested according to the usual procedures of the two laboratories preparing them. These tests were performed, for qualitative and quantitative studies together, except for cheese samples.

For homogeneity, 10 or 20 samples were analysed per batch or at the lower contamination level (cheese), with 2 test portions for each of 10 samples analysed under repeatability conditions, or 1 test portion of 20 samples. Enumeration (except for cheese samples) or detection results were obtained and studied for quantitative and qualitative studies respectively. For the milk powder samples, the homogeneity was tested with T1-T2 test. Ten samples per level were examined in duplicate. The samples were analysed to determine the variation between duplicate counts, i.e. within sample variation (T1 test) and also the variation between test materials (T2 test), as described by Schulten et al. (2000). A value for $T2/(I-1) < 2$ (where I is the number of test material tested), is regarded as good homogeneity.

The stability of samples was assessed during the trials under normal transportation and storage conditions at 3 ± 2 °C. These tests were performed before to launch the trial, for quantitative and qualitative studies together for all matrices except cheese. For cheese, stability was tested by a quantitative method on a batch at a higher contamination (at a level easy to count): The first test was on the day of sample preparation, or the day of shipment of the samples, the end was the last day to analyse the samples, with at least 2 additional tests.

At each time, 3 samples were analysed in duplicate, or 6 samples tested once, according to ISO 13528. The counts obtained were $\log_{10}$-transformed before their interpretation. For all matrices except cheese, data were analysed using a linear regression to determine the regression coefficient and corresponding t-value, which was used to determine whether or not the regression coefficient differed significantly from zero. For qualitative studies, qualitative results on the batch at the low contamination level were studied. For cheese samples, tolerance limit for stability was ± 0.1 log.

Additionally, before launching the trial, stability tests were carried out at elevated temperatures to estimate the effect of temperature abuse. For the combinations of time and temperature, the expected shipping conditions and their abuse were taken into account. These tests were performed for quantitative and qualitative studies together, except for cheese. For the latter, the stability was studied on only 1 batch for quantitative studies (at a level that was easy to count).

For cheese samples containing a bacteriostatic mixture, samples were stored and sent under a refrigeration temperature, and 2 abuse temperatures were studied: package at room temperature (22–28 °C) during 3 days and a temperature of 15 °C during one day. For cold-smoked salmon, samples were stored and sent frozen (−18 °C), and 2 abuse temperature scenarios were studied: 1 night at 4 or 12 °C. For environmental samples, samples were stored and sent under a refrigeration temperature, with the addition of a bacteriostatic mixture, and 2 abuse temperature scenarios were studied: 1 night at 8 or 12 °C. For ready-to-eat salads, samples were stored and sent frozen (−18 °C), and 2 abuse temperature scenarios were studied: 1 night at 4 or 8 °C. For powdered infant food formula, samples were stored and sent frozen (−18 °C), and 2 abuse temperature scenarios were studied: 1 night at 4 or 8 °C. The results were satisfactory for all the matrices.

2.5. Statistical analysis of the data of inter-laboratory studies

For the performance of a qualitative method, specificity rate and sensitivity rate were calculated as follows.

Specificity rate (*SP*):

$$SP = \frac{N_-}{N}$$

where *N*- is the number of negative results at blank level and *N* is the total number of results at this level.

Sensitivity rate:

$$SE = \frac{N_+}{N}$$

where *N*+ is the number of positive results at a low/high contamination level and N is the total number of results at each level.

Results were excluded from further analyses according to the following criteria: a) the test materials were exposed to temperature or time abuse during shipment; b) the laboratory has deviated from the specified instructions for the collaborative study.

3. Results and discussion

3.1. Stability and homogeneity of the test materials

All the test materials were considered stable and homogeneous for the trial. For salmon, environmental samples and salads, the period between dispatch of samples and limit date for launching the analysis was 10 days, for PIF it was 9 days. For cheese samples all laboratories analysed the samples the same day, 5 days after dispatch. No temperature abuse was recorded.

3.2. General results of the trial

A total of 30 laboratories from 17 countries in Europe and USA participated in the collaborative trials. A total of 15 to 16 laboratories participated in each trial. Media used by participants for each matrix are described in Fig. 1 for enrichment broths, and in Fig. 2 for selective agars. Most of the participants used dehydrated medium both for enrichment broths and LOA agar. Oxoid, Biolife, BioMérieux were the manufacturers most often reported for enrichment broths, and bioMérieux (AES) was the manufacturer most often reported for LOA agar. The second selective agar mostly used was PALCAM, followed by Oxford, and then by RAPID'L.mono. These trends are identical for all matrices.

On a volunteer basis, about half of the participants (7, 8, 8, 4 and 4 laboratories for respectively cold-smoked salmon, environmental samples, ready-to-eat salads, powdered infant food formulae and cheese) each time compared isolations obtained after 24 h (mandatory) and 48 h Fraser enrichment, and found no differences between results. Previously, we suggested that 24 h incubation in Fraser broth was sufficient to attain the maximum population, in contrast to the former practice of 48 h (Gnanou Besse et al., 2005; Gnanou Besse et al., 2010; Gnanou Besse et al., 2016); a finding which we have confirmed here. This reduces the total duration of the standard detection method by 24 h, which represents a significant improvement in its practicability.

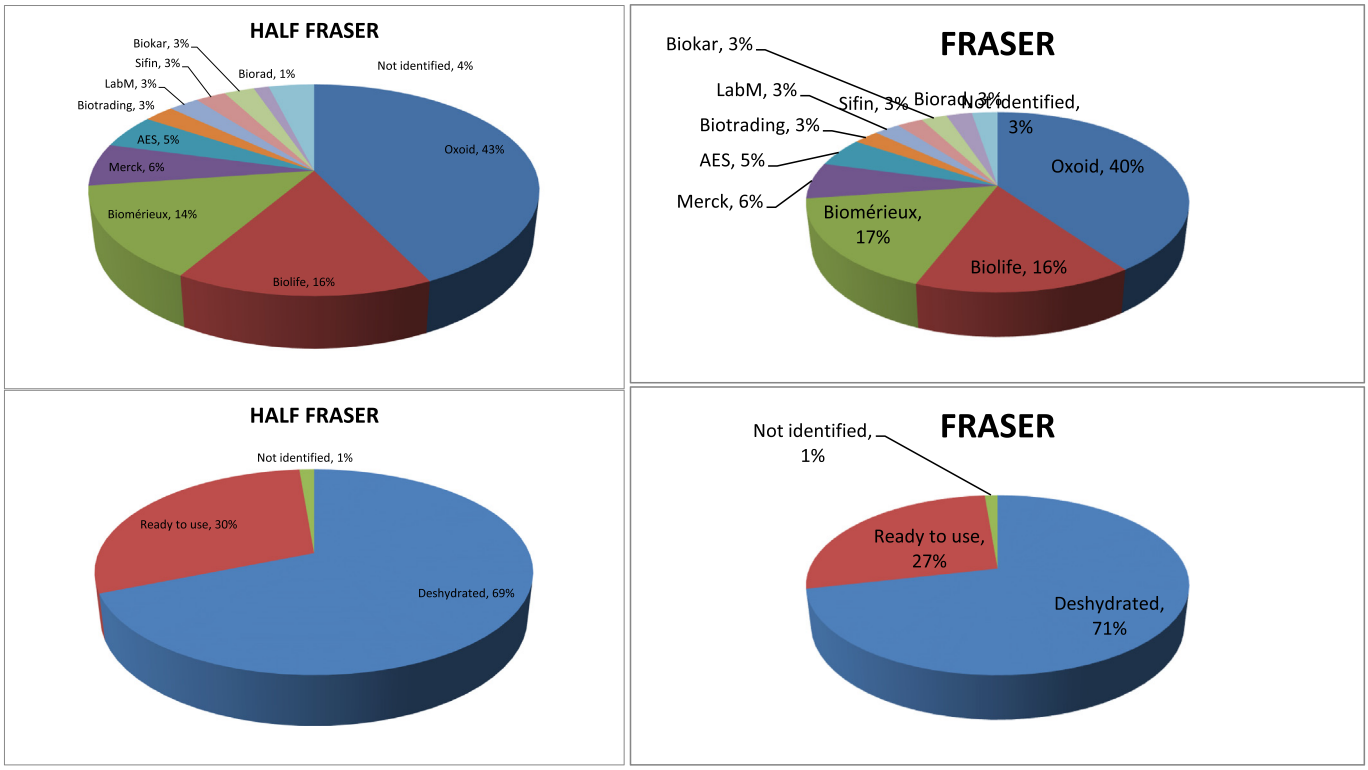

Fig. 1. Media used by participants for all matrices for enrichment broths: manufacturer and presentation.

The inter-laboratory studies were conducted with a pre-enrichment duration of 24 h ± 2 h, but this duration was modified afterwards in the revised standard to 25 h ± 1 h. The number of laboratories that performed < 24 h pre-enrichment, for each trial was respectively: 3 for cold-smoked salmon, 2 for environmental samples, 5 for ready-to-eat salads, 1 for powdered infant food formula, and 6 for cheese. The results obtained confirmed that this change has no impact on performance characteristics of the method, and it was adopted in order to allow growth of target bacteria to a higher level in pre-enrichment broth.

Morphological and traditional biochemical tests, for the confirmation of *L. monocytogenes,* including optional tests for some laboratories, were performed according to the draft revised standard method. In addition, other tests have been used and lead to concordant results: the Voges Proskauer test was used by some laboratories: 1 for cold-smoked salmon, 1 for environmental samples, 1 for ready-to-eat salads, 2 for powdered infant food formula, and 2 for cheese; the Henry illumination test was used by 1 laboratory for cold-smoked salmon, for environmental samples, for ready-to-eat salads and for cheese; additional Glucose, Salicin, Mannitol and methyl α-D-mannoside test was used by 1 laboratory for environmental samples; API *Listeria* biochemical gallery (Biomérieux) was used by 4 laboratories for powdered infant food formula, 4 for smoked salmon, 3 for environmental samples, 5 for ready-to-eat salads, and 5 for cheese. Further isolation on RAPID'L.mono (Bio-Rad) was performed by 1 laboratory for cold-smoked salmon, environmental samples, ready-to-eat salads and powdered infant food formula; nucleic acid probe ACCUPROBE (Biomérieux) was used by 1 laboratory for ready-to-eat salads and cheese; a PCR test for confirmation, according to Bubert et al. (1999) was indicated by 1 laboratory for cold-smoked salmon.

Concerning environmental samples, the results of 1 laboratory were excluded for having received the samples opened (problem during transportation). For cheese samples, the results of 1 laboratory were excluded for having received the samples too late.

3.3. Sensitivity and specificity

In these trials, no result was excluded for a technical reason (deviation from the protocol).

In most cases, the standard method revealed to be highly sensitive in the detection of *L. monocytogenes.* Food samples were inoculated with *L. monocytogenes* either alone or in combination with other *Listeria* species (Table 2). After the two-step enrichment procedure, the sensitivity rate varied from 91.1% to 100%, and the specificity rate varied from 97.6% to 100% (Table 3).

For cold-smoked salmon, both sensitivity and specificity rates were equal to 100%.

For environmental samples, sensitivity rate was equal to 99.1 and 100%, respectively for the low and high levels of contamination. One laboratory (different for low and high levels) detected *L. monocytogenes* in 7 samples out of 8. In fact, characteristic *L. monocytogenes* colonies were seen but overgrown by *L. innocua*: this did not allow isolation and purification for further confirmation. Specificity was equal to 100%.

For ready-to-eat salads, sensitivity rate for both low and high levels of contamination was equal to 100%. Specificity rate was equal to 97.6%. Three false positives have been obtained in 3 different laboratories (1 sample out of 8). Possible reasons for them may be (i) cross-contamination at the organizer's laboratory, (ii) cross-contamination at the participating laboratories, (iii) very low and heterogeneous contamination of the matrix undetected by preliminary detection tests performed both by the organizer and by the sample producer, and unlikely to affect quality results of the trial.

For powdered infant food formula, sensitivity rate for low and high levels of contamination was respectively equal to 99.2% (1 laboratory has detected *L. monocytogenes* in 7 samples out of 8) and 100%. Specificity rate was equal to 100%.

For cheese samples, sensitivity rate for the low level of contamination was equal to 91.1%: Three laboratories have detected *L. monocytogenes* in 7 samples out of 8 and 1 laboratory in 1 sample out of 8. For the latter with very low performance, no technical reason could

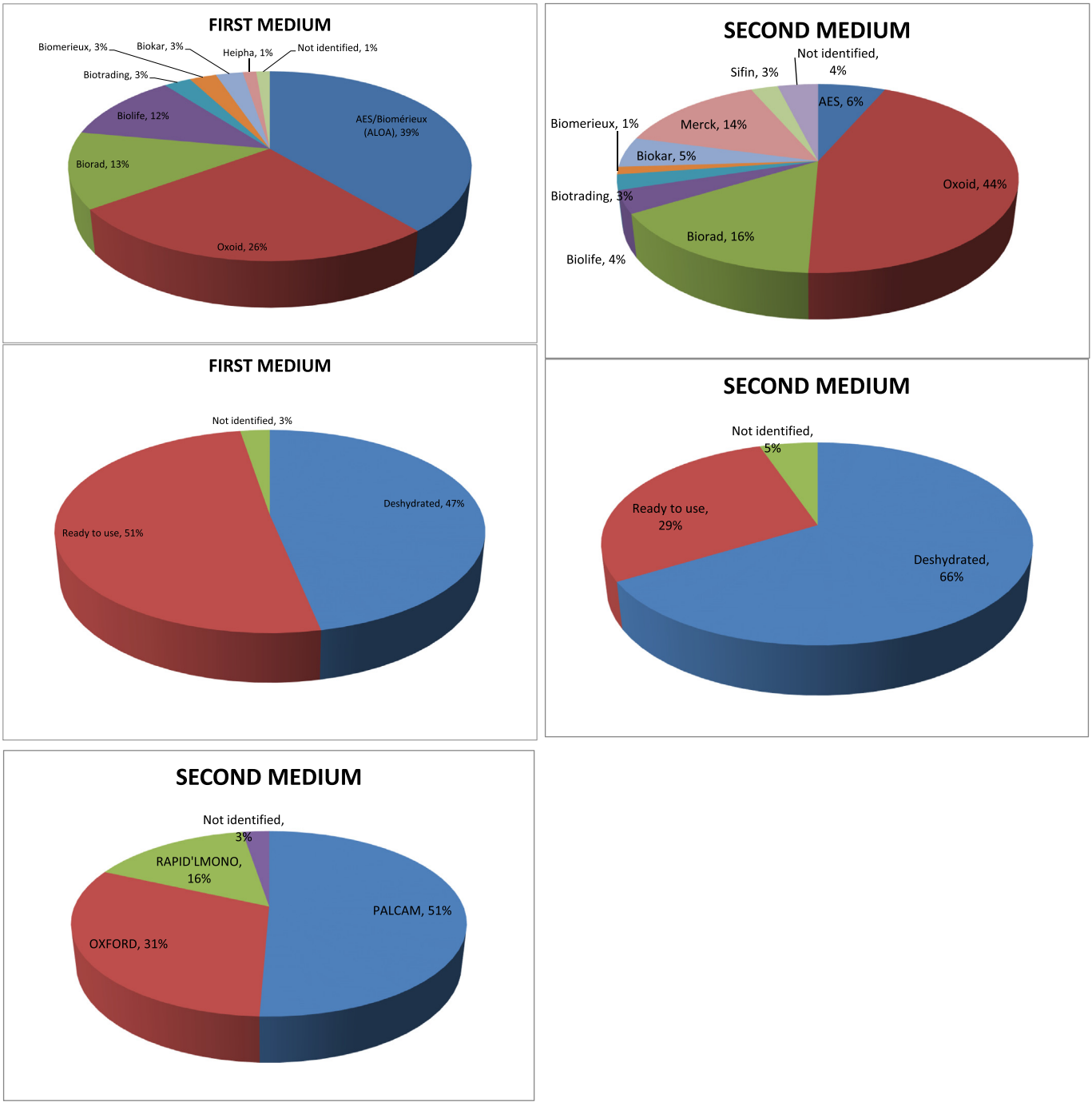

Fig. 2. Media used by participants for all matrices for selective agars: manufacturer and presentation.

Table 2
Level (cfu/25 g or cfu/gauze pad) of inoculation of the samples with *L. monocytogenes* and other *Listeria* species.

Matrices	Cold-smoked salmon			Environmental samples			Ready-to-eat salads			Powdered infant food formulae			Cheese		
Contamination levels	Blank	Low	High	Blank	Low	High	Blank	Low	High	Blank	Low	High	Blank	Low	High
L. monocytogenes	0	8	74	0	11	100	0	9	90	0	11	91	0	6.9	78
L. innocua				100	2.7	25							6.2	6.2	62
L. welshimeri	40	5	76												

Table 3
The overall sensitivities and specificities in the detection of *L. monocytogenes* from food and environmental samples.

Matrices	Cold-smoked salmon			Environmental samples			Ready-to-eat salads			Powdered infant food formulae			Cheese		
Contamination levels	Blank	Low	High	Blank	Low	High	Blank	Low	High	Blank	Low	High	Blank	Low	High
Number of participating collaborators	15	15	15	15	15	15	16	16	16	16	16	16	15	15	15
Number of collaborators retained after evaluation of the data	15	15	15	14[a]	14[a]	14[a]	16	16	16	16	16	16	14[e]	14[e]	14[e]
Number of samples	8 (120)	8 (120)	8 (120)	8 (120)	8 (120)	8 (120)	8 (128)	8 (128)	8 (128)	8 (128)	8 (128)	8 (128)	8 (120)	8 (120)	8 (120)
Number of samples retained after evaluation of the data	8 (120)	8 (120)	8 (120)	8 (112)	8 (112)	8 (112)	8 (128)	8 (128)	8 (128)	8 (128)	8 (128)	8 (128)	8 (112)	8 (112)	8 (112)
Accuracy (sensitivity), %		100	100		99.1[b]	100		100	100		99,2[d]	100		91,1[f]	100
Accuracy (specificity), %	100			100			97,6[c]			100			100		

[a] 1 laboratory excluded for having received samples opened (problem during transportation).

[b] 1 laboratory (different for low and high levels) has detected *L. monocytogenes* in 7 samples out of 8. In fact characteristic *L. monocytogenes* colonies were seen but overgrown by *L. innocua*: this did not allow isolation and purification for further confirmation.

[c] 3 false positives have been obtained in 3 different laboratories (1 sample out of 8). Possible reasons may be: (i) cross-contamination at organizer's level, (ii) cross-contamination at the participating laboratories' level, (iii) very low and heterogeneous contamination of the matrix undetected by preliminary detection tests performed both by the organizer and by the sample producer, and unlikely to affect quality results of the trial.

[d] 1 laboratory has detected *L. monocytogenes* in 7 samples out of 8.

[e] 1 laboratory excluded for having received samples too late.

[f] 3 laboratories have detected *L. monocytogenes* in 7 samples out of 8 and 1 laboratory in 1 sample out of 8.

be found to exclude these data from statistical interpretation. For the high level, the sensitivity rate was equal to 100% and 100% for specificity.

The good results obtained can be explained by the fact that the method chosen for this revision of the standard has been improved, in comparison (i) to the method of the previous version of the standard (1996), formerly validated for the European Commission (Standards, Measurement and Testing Fourth Framework Programme Project SMT4-CT96-2098, Scotter et al., 2001), or (ii) to the very similar NMKL method (Johansson et al., 2000; Loncarevic et al., 2008). In fact, PALCAM agar, included in these former reference methods, cannot distinguish *L. monocytogenes* from other *Listeria* spp. colonies. In these methods, five typical colonies had to be confirmed per plate, and these five colonies may not be *L. monocytogenes*. This dramatically decreased method sensitivity. A significant number of false-negative results were obtained when large numbers of *L. innocua* or other *Listeria* species were present. *L. innocua* tends to dominate *L. monocytogenes* during the selective enrichment stages and thus masked small numbers of colonies of *L. monocytogenes* on the isolation media. Due to the problem of false-negative results with the method, Scotter et al. (2001) recommended ISO to launch a revision of the standard to improve the detection of low numbers of *L. monocytogenes* in foods. The specificity and sensitivity had been improved since 2004 with the introduction of a more specific agar, agar *Listeria* according to Ottaviani and Agosti (LOA), which distinguishes *L. monocytogenes* from other species of *Listeria* and thus better identifies the *L. monocytogenes* colonies for confirmation. LOA agar was adopted by ISO and CEN in Amendment 1 (2004) to EN ISO 11290-1 (1996)/, as the standard medium for *L. monocytogenes* detection. This medium has good performance characteristics in terms of productivity ratio (at least 0.95), selectivity and detection ratio (Vlaemynck et al., 2000).

Already after one step enrichment in half-Fraser broth, the sensitivity for detection was high. While most of the samples displayed LOA plates with characteristic *L. monocytogenes* colonies following the primary enrichment, the secondary enrichment resulted in more samples showing typical colonies on the mandatory agar. These results underline the importance of the secondary enrichment step. In fact, 41 additional “positive” samples were recovered on a total of 1336 positive samples with the second enrichment (Table 4): respectively 1, 16, 1, 19, 4 more “positive” sample for cold-smoked salmon, environmental samples, ready-to-eat salads, powdered infant food formulae and cheese.

While the majority of *L. monocytogenes* are detected following the primary enrichment in half-Fraser broth (Johansson et al., 2000; Scotter et al., 2001; Gnanou Besse et al., 2005; Oravcova et al., 2008; Loncarevic et al., 2008; Gnanou Besse et al., 2016), the secondary enrichment in Fraser broth results in more *L. monocytogenes* positive samples, particularly when the original food sample contains low levels of *L. monocytogenes* or high levels of competing microflora (Johansson et al., 2000; Scotter et al., 2001; Loncarevic et al., 2008). This was also the case in this trial where 16 and 19 more “positive” samples were recovered with the second enrichment step for products with high levels of competing microflora such as environmental samples, or products with highly stressed cells such as powdered infant food formulae. With a second enrichment, some *L. monocytogenes* cells could be lost due to various reasons, for example an overgrowth by other *Listeria* species (Johansson et al., 2000; Scotter et al., 2001; Loncarevic et al., 2008); this was also the case for 3 samples in our trial (Table 4).

3.4. Level of detection

The level of detection at 50% ($LOD_{50\%}$) was not estimated in the frame of these interlaboratory studies, by lack of a low contamination level yielding to fractional positive results. However, mean $LOD_{50\%}$ values of the method of the former amended version of EN ISO 11290-1 Standard (2004), available from AFNOR Certification validation studies of alternative commercial methods, where the amended version of EN ISO 11290-1 was taken as reference method, are given in Annex F.2 of the revised standard for several food categories or environmental samples. TAG 17 of CEN/TC 275/WG 6 considered that technical modifications of Standard EN ISO 11290-1 do not have significant impact on $LOD_{50\%}$ values, which can thus characterise the method in the revised standard.

Mean $LOD_{50\%}$ values range from 0.5 to 1 cfu/25 g, depending on the matrix type. These values are satisfactory for a method expected to detect at least one cell in 25 g, the regulatory criterion in EC Regulation 2073/2005 and in other regulatory texts.

Table 4
Number of samples (in comparison to total samples number) displaying "agar *Listeria* according to Ottaviani and Agosti" plates with characteristic *L. monocytogenes* colonies.

Matrices	After half-Fraser enrichment isolation	After Fraser enrichment isolation	After Fraser enrichment (−): lost samples displaying characteristic colonies (+): more samples displaying characteristic colonies	After all isolation steps (Half-Fraser + Fraser enrichment)
Cold-smoked salmon	239/240	240/240	+1	240/240
Environmental samples	207/224	223/224	+16	223/224
Ready-to-eat salads	255/256	254/256	−2 + 1	256/256
Powdered infant food formulae	236/256	255/256	+19	255/256
Cheese	345/360	348/360	−1 + 4	349/360
Total	1282/1336	1320/1336	−3 + 41	1323/1336

4. Conclusions

The revised Standard EN ISO 11290–1, published in 2017 (Anonymous, 2017) describes an efficient method for the detection of *L. monocytogenes* in foods and food production environments, in particular for the matrices included in the study. Positive samples were most often detected after 24 h half-Fraser enrichment, which further shortens the analysis time by one day compared to the former version of the standard. Secondary enrichment had to be kept, because samples with high levels of competing microflora and with injured *L. monocytogenes* cells, need secondary enrichment, for an efficient detection of *L. monocytogenes*.

In addition, values of level of detection for the method were found in the literature and are satisfactory for a method expected to detect one cell in 25 g.

The outcome of this study was presented at the joint annual meeting of CEN/TC275/WG6-ISO/TC 34/SC 9 (Washington, June 2014). CEN/TC 275/WG 6 TAG 17 (Technical Advisory Group for *Listeria*) has decided to include the precision data generated by this study and the results of inter-laboratory studies in Annex F of the revised standard.

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