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Validation of standard method EN ISO 11290 - Part 2 for the enumeration of *Listeria monocytogenes* in food

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ABSTRACT

The reference method for the detection and enumeration of *L. monocytogenes* in food (Standards EN ISO 11290-1 &2) have been validated by inter-laboratory studies in the frame of the Mandate M381 from European Commission to CEN. In this paper, the inter-laboratory studies led in 2013 on 5 matrices (cold-smoked salmon, milk powdered infant food formula, vegetables, environment, and cheese) to validate Standard EN ISO 11290-2 are reported. According to the results obtained, the method of the revised Standard EN ISO 11290-2 can be considered as a good method for the enumeration of *L. monocytogenes* in foods and food processing environment, in particular for the matrices included in the study. Values of repeatability and reproducibility standard deviations can be considered satisfactory for this type of method with a confirmation stage, since most of them were below 0.3 $\log_{10}$, also at low levels, close to the regulatory limit of 100 CFU/g.

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, 2007a; 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 between EC and CEN, 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 cited or are expected to be cited as the reference methods in the EC Regulation 2073/2005 (Anonymous, 2005a) 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 method for the detection and enumeration of *L. monocytogenes* in food (Standards EN ISO 11290-1&2). Since these standards describe horizontal methods, applicable to all food, feed and food processing environment, the ILS had to be performed on 5 matrices, according to a common design elaborated by WG 3 Method Validation and WG 2 Statistics of ISO/TC 34/SC 9.[2] 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 collaborative studies to validate Standard EN ISO 11290-Part 2 are reported. The trials, led by ANSES Laboratory for Food Safety, and by ACTALIA-CECALAIT, in collaboration with CEN/TC 275/WG 6/TAG 17 (Task Advisory 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).

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[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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2. Materials and methods

2.1. Design of the trial

Fifteen to 16 laboratories participated to each of the 6 trials and were from 17 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. For each matrix, 4 levels of contamination including a blank (0, ca 100–150, ca 500–1500, ca 5000–15,000 CFU/g), and 2 replicates per level were used. So in total 8 samples per matrix were sent to each laboratory. Environmental samples consisted of gauze pads immerged in 20 ml diluents, and to be homogenous with method's sensitivity for other matrices, contamination levels corresponded of 0, ca 10–15, ca 100–150, ca 1000–1500 CFU/ml of diluent, to be converted in CFU/gauze pad. 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 flora.

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, 2005b), IUPAC protocol (Anonymous, 2006), and/or other internal procedures.

The test materials were shipped to the participants by courier in polystyrene insulated boxes several ice packs to prevent exposure of the test materials to high temperatures during transport. They contained also a temperature monitoring device to follow the temperature 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 2 (1998 version amended in 2004) (Anonymous, 1998; Anonymous, 2004) consisted of spreading decimally diluted samples and further decimal dilutions on selective agar plates. 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 enumeration of *L. monocytogenes*. Following incubation for 24 h and 48 h at 37 °C, typical colonies were purified on a non-selective agar. The confirmation tests for the genus *Listeria* were the Gram staining and the catalase test (as mandatory tests), 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.

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

- Regarding the choice of diluent to prepare the initial suspension, in addition to Buffered Peptone water and Half-Fraser broth base without selective agents, it is now possible to use complete Half-Fraser (with selective agents) and all diluent described in ISO 6887 (Anonymous, 2017a), without restrictions; – The 1 h resuscitation step at ambient temperature was deleted;
- A maximum of 150 colonies per plate remained the rule for the selection of LOA plates, but a note was added, indicating that in case of mixed cultures of blue-green colonies with or without opaque halo, or in case of blue-green colonies with large and overlapping opaque halos, it is preferable to retain the plates containing < 100 characteristic colonies of *Listeria* spp.;
- Microscopic aspect, catalase and CAMP-test became optional for *L. monocytogenes*;
- For haemolysis test or CAMP-test, the composition of blood agar was extended from defibrinated blood of sheep only, to calf or bovine blood. For haemolysis test, blood agar plates have to be inoculated by stabbing, or by streaking (only if positive at purification step).

The general procedure of the draft standard submitted to ILS was the same as the published version of the revised standard EN ISO 11290-2 (Anonymous, 2017b).

Since both detection and enumeration ILS trials were performed at the same time, and given the high level of work, in particular for the confirmation stage, TAG17 *Listeria* of CEN/TC 275/WG 6 decided to provide some guidance to the ILS participants on the maximum dilution to perform, in order to reduce their workload, without giving any precise indication of levels. Two groups of samples were defined: low contaminated samples (including blanks and low contamination levels) from one side, and highly contaminated samples (including the two highest contamination levels) on the other side. Commercial biochemical galleries could be used for confirmation at the condition but all mandatory tests of the standard were performed. Alternative confirmation tests were possible but in addition of mandatory tests. Choice of some media (agars, diluents…) was left free, but in agreement with the revision of EN ISO 11290-2.

2.3. Preparation of test materials

Food samples were inoculated with *L. monocytogenes* either alone or in combination with other *Listeria* species (Table 1).

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), plated onto a selective agar and then onto Tryptone Soya Agar Yeast Extract (TSAYE, BIOKAR) for *Listeria* strains, or directly onto Plate Count Agar with milk (mPCA) for lactic acid 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) background flora containing a strain of *Lactococcus lactis* subsp. *Lactis* (internal number 97009) was also added at a level of approximately $5 \cdot 10^3$ 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), 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 curd cheese was stabilized by the addition of an undisclosed bacteriostatic mixture; its effect on bacteriological stability was studied during the pre-essays (see 2.4). The bacteriostatic effect is negated when the sample is diluted during examination; this was demonstrated by internal assays. The inoculum levels for the target organisms were verified using a spiral plating system at a single dilution in TSAYE for

Table 1
Level in cfu/g or ml (and in $\log_{10}$) of inoculation of the samples with *L. monocytogenes* and other *Listeria* species.

Matrices	Cold-smoked salmon				Environmental samples[a,b]			Ready-to-eat salads				Powdered infant food formulae				Cheese			
Contamination levels	Blank	Low	Medium	High	Blank	Low	Medium	High	Blank	Low	Medium	High	Blank	Low	Medium	High	Blank	Low	Medium	High
L. monocytogenes	0	110 (2.03)	1300 (3.12)	13,000 (4.09)	0	14 (1.14)	89 (1.94)	1300 (3.08)	0	140 (2.14)	1600 (3.20)	14,000 (4.14)	0	230 (2.36)	1600 (3.20)	12,000 (4.07)	0	130 (2.11)	650 (2.81)	6500 (3.81)
L. innocua					9.4 (0.94)	1.5 (0.17)	16 (1.20)	175 (2.24)									87 (1.94)	87 (1.94)	440 (2.64)	4400 (3.64)
L. welshimeri	64	130 (2.10)	1100 (3.03)	15,000 (4.17)																

[a] *L. monocytogenes* in cfu/gauze pad ($\log_{10}$): 0, 280 (2.44), 1800 (3.25), 26,000 (4.41) respectively for blank, low, medium and high level of contamination.
[b] *L. innocua* in cfu/gauze pad ($\log_{10}$): 190, (2.27), 30 (1.47), 320 (2.50), 3500 (3.54) respectively for blank, low, medium and high level of contamination.

Listeria strains and in PCAm 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 contaminations, each sample was spiked with 0,1 ml of the appropriate TS dilution of the culture, and gently spread. *Listeria* spp. detection in each purchased matrix was checked, using EN ISO 11290-1 reference method (Anonymous, 2004). Results showed that *Listeria* spp. were not detected in any product used in these studies.

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 10 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. Before and after freezing, total mesophilic aerobic count of the samples were below 1×10^2 CFU/g. 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 inocula. 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. They were divided into 10 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. Before and after freezing, total mesophilic aerobic count of the samples were between 10^4 and 10^5 CFU/g.

Environmental samples consisted of gauze pads immerged in 20 ml diluents (TS containing a bacteriostatic mixture), *L. monocytogenes* serotype 1/2c (10CEB86Lm) isolated from food production environment was used as inoculum. A competitive background microflora, representative of food processing environment (Bagge-Ravn et al., 2003; Chevallier et al., 2006), and at realistic levels, 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. This microflora CFU intends to represent the bacteria recovered from environment after sampling with a gauze pad. The microflora within the sample was stabilized by the addition of a bacteriostatic mixture whose bacteriostatic effect is negated when the sample is diluted during examination (boric acid mixture, Gnanou Besse et al., 2007). Four ml of a solution containing 10 g of boric acid (Sigma, B6768), 2 g of glycerol (Acros Organics, 295600000) and 0.150 g of potassium sorbate (Sigma, Fluka 85520) per 200 ml of distilled water were added to 16 ml of TS diluent.

For powdered infant food formula, lyophilised 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-2M 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. The final powder was divided in 10 g samples. Total mesophilic aerobic counts of uncontaminated PIF samples were between 10^2 and 10^3 CFU/g.

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 common design prepared for all ILS of the CEN Mandate and the usual procedures of the two laboratories preparing them, in particular ISO 13528, CEN ISO/TS 22117 (Anonymous, 2010) and IUPAC protocol. These tests were performed, for qualitative and quantitative studies together, except for cheese samples.

For homogeneity, 10 samples were analysed per batch, with 2 test portions for each sample analysed under repeatability conditions, or 20 samples with 1 test portion. Enumeration results were obtained and studied.

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.

For cheese samples, homogeneity was tested in 10 test samples in duplicate from the higher batch, 2 days after preparation and using a spiral-plating system to inoculate LOA agar plates. Enumeration results (CFU/g) were transformed in $\log_{10}$ before their statistical interpretation by calculating standard deviation of repeatability (S_r) and standard deviation between samples (S_e).

The stability of samples was assessed during the trials under normal transportation and storage conditions at 3 ± 2 °C. The first test was on the day of sample preparation, or the day of shipment of the samples, the end is the last day to analyse the samples with at least 1 or 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 from each batch 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 cheese samples, tolerance limit for stability was $+/-0.1$ $\log_{10}$. This is an internal limit, used for a long time for the proficiency tests organized by ACTALIA-CECALAIT in this type of samples.

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 temperatures, the expected shipping conditions and their abuse were taken into account. In this case, the stability was studied on only one 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 two abuse temperatures were studied 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 two abuse temperature scenarios were studied: one 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 two abuse temperature scenarios were studied: one night at 8 or 12 °C. For ready-to-eat salads, samples were stored and sent frozen (−18 °C), and two abuse temperature scenarios were studied: one night at 4 or 8 °C. For powdered infant food formula, samples were stored and sent frozen (−18 °C), and two abuse temperature scenarios were studied: one night at 4 or 8 °C. The results were satisfactory for all the matrices.

2.5. Statistical analysis of the data

Results (CFU/g) were transformed in $\log_{10}$, a usual practice in quantitative microbiology to stabilize variance over contamination levels, and data were analysed according to Standard ISO 5725-2 (Anonymous, 1994).

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

We performed recalculation and rounding of enumeration results of each participant according to ISO 7218 (Anonymous, 2007b). The raw data were then $\log_{10}$-transformed before performing calculations. Performance characteristics of the Standard method were then calculated in accordance with ISO 5725- 2 (Anonymous, 1994), using Excel spread-sheets developed according to this standard. It introduces parametric estimators for calculating repeatability and reproducibility standard deviations. The following parameters were calculated to derive the precision data: the mean value ($\log_{10}$ CFU/g), repeatability standard deviation s_r ($\log_{10}$ CFU/g), repeatability limit r, as difference on $\log_{10}$ scale ($\log_{10}$ CFU/g), and as ratio on normal scale (CFU/g), reproducibility standard deviation s_R ($\log_{10}$ CFU/g), reproducibility limit R, as difference on $\log_{10}$ scale ($\log_{10}$ CFU/g), and as ratio on normal scale (CFU/g). The repeatability limit r is defined as the absolute difference between two independent single test results obtained using the same method on identical test material in the same laboratory by the same operator using the same apparatus within shortest feasible time interval. The r value should not be exceeded in > 5% of the cases. If the difference between results within a laboratory exceeds r, the results should be considered suspect. The reproducibility limit R is defined as the absolute difference between two single test results of the two test results on the normal scale obtained using the same method on identical test material in different laboratories with different operators using different apparatus. The R values should not be exceeded in not > 5% of cases. If the difference between results in different laboratories exceeds R, the results should be considered suspect.

In addition, this trial was the opportunity to assess the effect of potential factors impacting enumeration results, such as: laboratory, diluent (BPW, Half Fraser …), diluent ready-to-use vs. agar made from powder, mandatory agar ready-to-use vs. Agar made from powder… In order to test for these effects, we used multi-way analysis of variance. Mixed effect models were fitted for each matrix. Multi-way analysis of variance and mixed effect models allow also characterizing the relationship between more than one explanatory variable and the response variable. Mixed effect models present the advantage of taking into account two kinds of explanatory variables, fixed and random factors. A factor is considered fixed when the values under study are the only values of interest (ex: ready-to-use and powdered LOA). The aim is then to characterize deterministic differences in the response induced by each value of such fixed factors and these differences, named fixed effects, are described by additive single values. On the contrary, a factor is considered random when the values under study are a sample from the population of interest (ex: laboratories, the participants represent a sample of all laboratories that can carry out this analysis). The aim is then to characterize variation in response induced by the different values in the studied sample and to generalize it to the whole population of interest. A mixed linear model (CFU/g ~ firstsusp + formD + formB + …: 1/laboratory) was used for determining the degree of influence of these factors. We fitted mixed

effect models using R software (R: A Language and Environment for Statistical Computing, 2010) with the function "lmer" of "lme4" package (Bates and Maechler, 2010).

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 maximum date for launching the analysis was 10 days, for PIF, it was 9 days. For cheese, all laboratories analysed the samples 1 to 3 days after dispatch. No temperature abuse was recorded.

In the frame of this validation ILS, the performance of the method and its implementation by the laboratories were assessed. It was thus important to send stable and homogenous samples to participants. Pre-assays regarding some matrices allowed us to choose the best way of storage and dispatch of the samples. In particular, for ready-to-eat salads and cold-smoked salmon, freezing was the only way to obtain homogeneous and stable samples. For ready-to-eat salads, we used a mixture of fresh spinach leaves and fresh iceberg lettuce leaves, because of good resistance of the leaves to freezing damage, and high level of consumption in Europe. An abundant and diverse background microflora was obtained both from frozen and unfrozen samples, in particular the presence of some *Bacillus* and of other *Listeria* species. For cheese and environmental samples, the use of a bacteriostatic mixture was the only way to obtain homogeneous and stable samples. Pre-assays showed that the presence of the bacteriostatic mixture did not affect enumeration of *Listeria* species and background microflora. For PIF samples, the use of a bacteriostatic mixture was the only way to obtain homogeneous and stable samples. An abundant and diverse background microflora was obtained both from refrigerated and unrefrigerated samples, in particular the presence of numerous *Bacillus* and Gram positives cocci.

TAG17 *Listeria* considered that the freezing conditions, freeze-drying treatment and desiccation or the addition of a preservative under refrigeration would stress the *Listeria* inoculum. No other stress was then performed prior to inoculation of the samples.

3.2. General results of the trial

A total of 30 laboratories from 17 European 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 diluents used in the first suspension, and in Fig. 2 for LOA agar. They were similar for all matrices. Buffered Peptone Water was the diluent most often used. Most of the participants used dehydrated medium for LOA agar. AES was the manufacturer most often reported for LOA agar.

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

For environmental samples, 1 laboratory was excluded for having received the samples opened (problem during transportation).

3.3. Precision data

In these trials, no result was excluded for a technical reason.

Values of repeatability and reproducibility standard deviations obtained are given in Table 2. They can be considered satisfactory for this type of method with a confirmation stage, in presence for certain matrices of other *Listeria* species (Table 1): they were all below 0.3 $\log_{10}$, also for low levels. It should be underlined that these precision data were obtained with artificially contaminated samples, and that the precision of the method may differ when analyzing naturally contaminated samples. Repeatability and reproducibility standard deviations are very close, which means that variability is mainly due to the method itself, and not to its implementation by laboratories.

Poorer results were explained by the powder stage, since obtained for powders, are not a matrices uneasy to analyse and with a high stress level at desiccation stage, in particular the combined effect of several factors, including (i) hypothesis of micro-aggregates and thus difficulties of powder homogenization at low levels leading to relatively higher dispersion of values between samples than for other matrices contaminated with liquid inocula, and (ii) cells severely stressed by freeze-drying and low contamination levels.

Given the results of this trial, in terms of repeatability, the following values (medians of values per matrix and contamination level) may be used when testing food samples in general (except powdered infant formulae) as a general indication of repeatability limit (r): $r = 0.30$, range [0.19; 0.40] (expressed as a difference between $\log_{10}$-transformed test results), or $r = 1.99$, range [1.55; 2.50] (expressed as a ratio between test results). For example, a test result of 100 or 1.0×10^2 or $\log_{10}$ 2.00 CFU per gram of food product was obtained in a given laboratory. Under repeatability conditions, the difference between $\log_{10}$-transformed results should not be greater than $\pm$ 0.30 $\log_{10}$ units. So the result from a second test result of the same sample should be between 1.70 (2.00–0.30) and 2.30 (2.00 + 0.30) $\log_{10}$ units. For non $\log_{10}$-transformed results, the ratio between the first test result and the second test result from the same sample should not be > 1.99. So the second test result should be between 50 (=100/1.99) and 199 (=100 × 1.99) CFU per gram.

Given the results of this trial, in terms of reproducibility, the following values (medians of values per matrix and contamination level) may be used when testing food samples in general (except powdered infant formulae) as a general indication of reproducibility limit (R): $R = 0.43$, range [0.25; 0.54] (expressed as a difference between $\log_{10}$-transformed test results), or $R = 2.68$, range [1.79; 3.47] (expressed as a ratio between test results). For example, a test result of 100 or 1.0×10^2 or $\log_{10}$ 2.00 CFU per gram of food product was observed in a first laboratory. Under reproducibility conditions, the difference between $\log_{10}$-transformed results should not be greater than $\pm$ 0.43 $\log_{10}$ units. So the results from a second laboratory should be between 1.57 (2.00–0.43) and 2.43 (2.00 + 0.43) log10 units. For non $\log_{10}$-transformed results, the ratio between the test results from this first laboratory and a second laboratory should not be > 2.68. So the result from the second laboratory should be between 37 (=100/2.68) and 268 (=100 × 2.68) CFU per gram. In a second example, a laboratory may want to know the maximum value it may find, which is still in compliance with a pre-set limit (e.g. a limit of 100 or $\log_{10}$ 2). For this, the R value (on the log scale) has to be multiplied by a factor of 0.59. The factor 0.59 reflects the fact that a test with a one-sided 95% interval

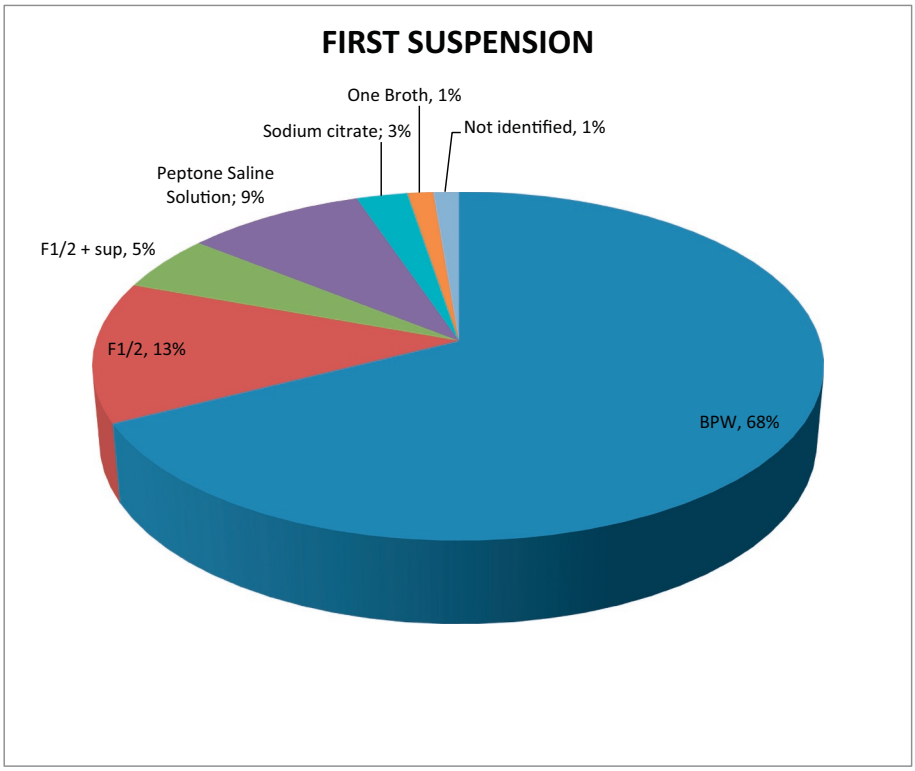

Fig. 1. Diluent used by participants for all matrices for first suspension.

is used to test whether the limit is exceeded; it is obtained from the following formula:

$$0.59 = \frac{1.64}{1.96 \times \sqrt{2}}$$

The maximum value is 0.25 (0.43 × 0.59) as a difference between $\log_{10}$-transformed test results or 1.78 ($10^{0.25}$) as a ratio between test results. So results up to $\log_{10}$ 2.25 ($\log_{10}$ 2 + $\log_{10}$ 0.25) or 178 (100 × 1.78) comply with the limit.

Since the impact of the presence of other *Listeria* species on enumeration of *L. monocytogenes* is well known (Gnanou Besse et al., 2005, 2010, 2016; Loncarevic et al., 2008), some food samples were inoculated with *L. monocytogenes* in combination with other *Listeria* species (Table 1). However, even in these cases, precision values were considered correct, since they were all < 0.3 $\log_{10}$. In fact, the specificity of the method has been improved since 2004 with the introduction of the specific agar, agar *Listeria* according to Ottaviani and Agosti (LOA agar), which distinguishes *L. monocytogenes* from other species of *Listeria* and thus better identifies the *L. monocytogenes* colonies for confirmation.

An inter-laboratory validation study of EN ISO 11290-2 in its initial version (without its amendment which replaced PALCAM agar with LOA agar) was funded by the European Commission (Standards, Measurement and Testing Fourth Framework Programme Project SMT4-CT96-2098). The objective was to determine the precision of the method in terms of repeatability (r) and reproducibility (R) using different sample types (meat, cheese, dried egg powder and reference material) inoculated at different levels. The overall repeatability was $r = \log_{10}$ 0.58, and the overall reproducibility was $R = \log_{10}$ 0.81 (Scotter et al., 2001). This means that for a sample having a true contamination of 100 CFU/g, a laboratory could find a result as low as 15 CFU/g, or as high as 646 CFU/g.

The impact of the presence of other *Listeria* species on enumeration of *L. monocytogenes* has also been demonstrated by the validation study of the revised Nordic Committee on Food Analysis (NMKL) method no. 136 "*Listeria monocytogenes*. Detection and enumeration in foods" (Loncarevic et al., 2008), which is very similar to the current EN ISO 11290-2 Standard method. The precision of the NMKL method in terms of repeatability and reproducibility using different food sample types (Brie cheese made from pasteurized milk, hot-smoked salmon and cooked vacuum-packed ham) was determined. For a contamination level close to 100 CFU/g (2.2 $\log_{10}$ CFU/g), the overall repeatability of the method for these three food types was respectively r = 0.44, 0.91, 0.66 and the overall reproducibility was respectively R = 0.48, 1.08, 0.54 (Loncarevic et al., 2008). In the presence of *L. innocua*, these values reached respectively r = 0.76, 0.52, 0.70 and R = 0.87, 0.68, 0.87. This means that for a sample having a true contamination level of 100 CFU/g, if R = 0.87 a laboratory may find a result as low as 13 CFU/g, or as high as 741 CFU/g.

3.4. Potential factors impacting enumeration results

Potential factors impacting enumeration results that were studied are laboratory, diluent (type and presentation -ready-to-use vs. dehydrated), LOA agar (ready-to-use vs. dehydrated, manufacturer). We tested these effects by multi-way analysis of variance. Mixed effect models were fitted for each matrix. Main results show that the variance explained by laboratories is small (i.e. few difference between laboratories), and there is no significant effect of the factors studied. However, for milk powder, there is a significant but limited effect of the first diluent: half-Fraser with supplement led to lower enumeration results (−0.18 $\log_{10}$ CFU/g). However this concerned only one laboratory and the low level (Fig. 3).

4. Conclusions

The method of the revised Standard EN ISO 11290-2 can be considered as a satisfactory method for the enumeration of *L. monocytogenes* in foods and food processing environments, in particular for the matrices included in the study. Values of repeatability and

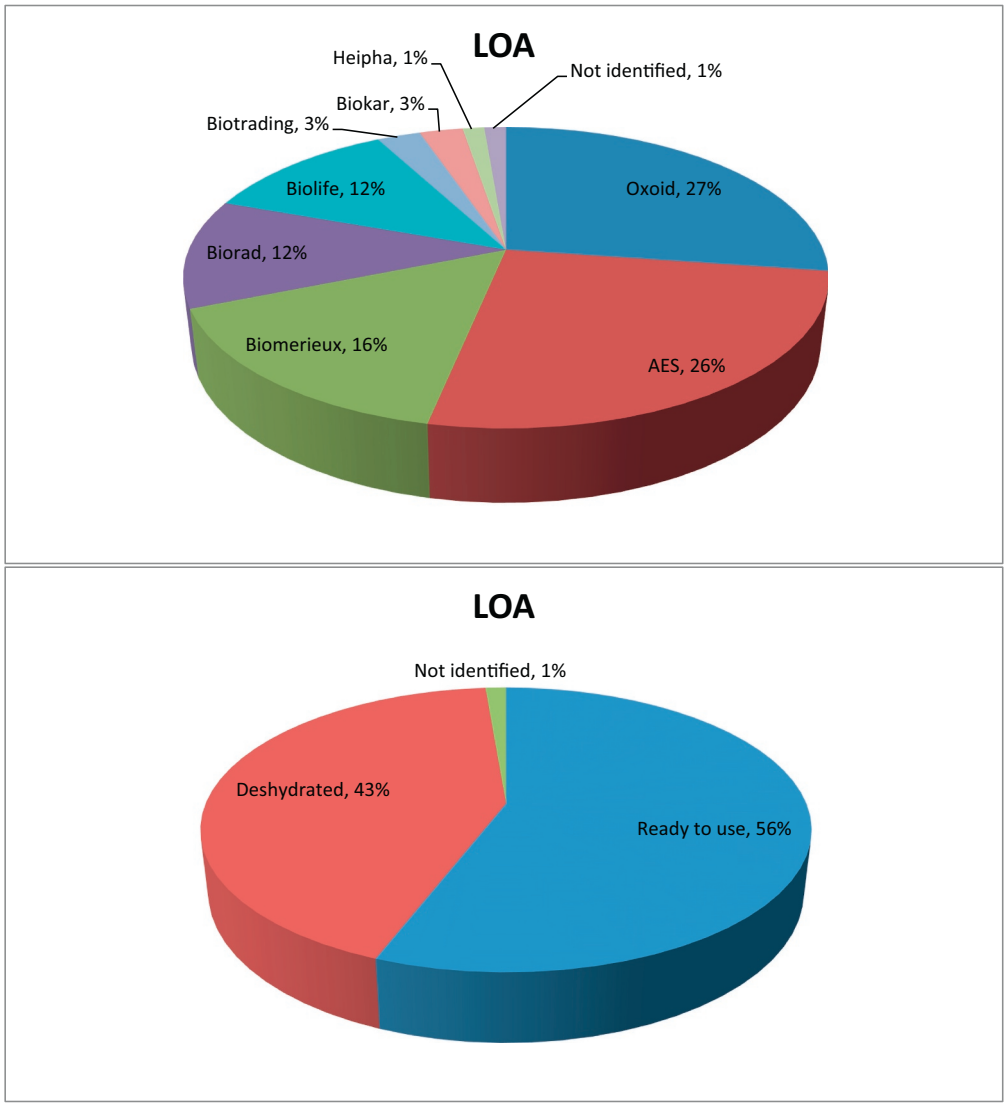

Fig. 2. Media used by participants for all matrices for enumeration agar (LOA agar): manufacturer and presentation.

reproducibility standard deviations can be considered satisfactory for this type of method with a confirmation stage, since most of them were below 0.3 $\log_{10}$, also at low levels. The method described in the revised version of the standard (2017) has been improved, in comparison to the method in the former version of the standard, which had been validated for the European Commission (Standards, Measurement and Testing Fourth Framework Programme Project SMT4-CT96-2098, Scotter et al., 2001). In fact, PALCAM agar, prescribed in the former version of the standard (1998) before being amended, could not distinguish *L. monocytogenes* from other *Listeria* spp. colonies. It dramatically enlarged method variability. A significant number of false-negative results were obtained when large numbers of *L. innocua* or other *Listeria* species were present in the food sample. *L. innocua* could mask small numbers of colonies of *L. monocytogenes* on PALCAM agar. Due to these problems, Scotter et al. (2001) recommended to ISO to launch a revision of the standard to improve the enumeration of low numbers of *L. monocytogenes* in foods. The specificity of the standard method has been improved in 2004 with the introduction of a more specific agar, *Listeria* according to Ottaviani and Agosti agar (LOA agar), which distinguishes *L. monocytogenes* from other species of *Listeria* and thus better targets the *L. monocytogenes* colonies for confirmation. LOA agar was adopted by ISO and CEN as the standard medium for *L. monocytogenes* enumeration method in Amendment 1 (2004) to EN ISO 11290-2. This medium performs well, as demonstrated in terms of productivity ratio (at least 0.95), selectivity and detection ratio (Vlaemynck et al., 2000).

The outcome of this study was presented at the annual meeting of CEN/TC275/WG6 (Washington, June 2014) which decided, based upon the recommendation of its TAG 17 *Listeria*, to include the precision data generated by this study in the revised standard and the detailed results of the inter-laboratory study in an informative annex of the standard.

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Table 2
The overall precision data in the enumeration 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	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High
Number of participating collaborators	15	15	15	15	15	15	16	16	16	16	16	16	16	16	16
Number of collaborators retained after evaluation of the data	15	15	15	14[a]	14[a]	14[a]	16	16	16	16	16	16	16	16	16
Number of samples	2 (30)	2 (30)	2 (30)	2 (30)	2 (30)	2 (30)	2 (32)	2 (32)	2 (32)	2 (32)	2 (32)	2 (32)	2 (32)	2 (32)	2 (32)
Number of samples retained after evaluation of the data	2 (30)	2 (30)	2 (30)	2 (28)	2 (28)	2 (28)	2 (32)	2 (32)	2 (32)	2 (32)	2 (32)	2 (32)	2 (32)	2 (32)	2 (32)
Mean value Σ*a* (log10 cfu/g)	1.85	3.04	3.95	2.42	3.15	4.37	2.16	3.14	4.17	2.34	3.21	4.09	2.06	2.78	3.83
Repeatability standard deviation sr (log_{10} cfu/g)	0.13	0.10	0.12	0.14	0.10	0.12	0.11	0.07	0.10	0.25[b]	0.06	0.04	0.11	0.11	0.07
Repeatability limit r:															
- as difference on log_{10} scale (log_{10} cfu/g)	0.37	0.28	0.32	0.40	0.29	0.33	0.32	0.19	0.28	0.70	0.17	0.12	0.30	0.30	0.20
- as ratio on normal scale (cfu/g)	2.33	1.92	2.10	2.50	1.96	2.11	2.08	1.55	1.89	4.96	1.49	1.31	1.99	2.01	1.58
Reproducibility standard deviation sR (log_{10} cfu/g)	0.18	0.19	0.19	0.19	0.19	0.13	0.13	0.09	0.11	0.25[b]	0.10	0.07	0.11	0.17	0.10
Reproducibility limit r:															
- as difference on log_{10} scale (log_{10} cfu/g)	0.51	0.53	0.54	0.54	0.54	0.36	0.37	0.25	0.31	0.70	0.28	0.19	0.31	0.49	0.29
- as ratio on normal scale (cfu/g)	3.26	3.37	3.44	3.47	3.45	2.28	2.33	1.79	2.02	4.96	1.91	1.55	2.03	3.12	1.94

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

[b] The high values of repeatability and reproducibility for the low level may be due to the combined effect of several factors, including (i) hypothesis of micro-aggregates and thus difficulties of powder homogenization at low levels leading to relatively higher dispersion of values between samples than for other matrices contaminated with liquid inocula, and (ii) cells severely stressed by freeze-drying and low contamination levels.

France: Anses LSAl, Agnes Chamoin, Annie Beaufort, Sylvie Rudelle, Anne laure Lardeux; France: Anses Boulogne, Graziella Bourdin; France: Actilait-LARF, Michèle Bruna-Rosso; France: Silliker France, Christophe Dufour, Annabelle Defer; France, ISAE, Catherine Loysance-Paroux, Chantal Monfort; Germany: Muva Kempten, Monika Knödlseder, Ursula Hartmann; Germany: Federal Institute for Risk Assessment, NRL for *Listeria monocytogenes*, Sylvia Kleta; Germany: Fachtierarzt für Lebensmittelhygiene, Institut für Lebensmittelhygiene, Veterinärmedizinische Fakultät der Universität Leipzig, Thiemo Albert, Martina Ludewig; Greece: Agricultural University of Athens, Eleftherios Drosinos, Spiros Paramithiotis; Greece: Aristotle University of Thessaloniki, Apostolos Angelidis; Ireland: Dairy Science Laboratory, Bernadette Hickey, Regina Heslin, Damien O Meara; Norway: Norwegian Veterinary Institute, Taran Skjerdal, Semir Loncarevic; Poland: National Veterinary Research Institute, Jacek Osek, Jolanta Rola, Kinga Wieczorek, Katrzyna Dmowska; Romania: Institute for Hygiene and Veterinary Public Health, Iuliana Mihai; Slovakia: State Veterinary and Food Institute Dolny Kubin, Olga Skuntova, Miriam Revallova, Lenka Cabanova; Slovakia: NRC of environmental Microbiology, PHA, Zuzana Sirotna; Spain: AINIA, David Tomas, Amparo De Benito; Spain: Agencia de Salud Publica de Barcelona, Mercedes De Simon; Spain: AESAN CNA Servicio de Microbiología Alimentaria, Maria del Carmen Blanco Vidal; Switzerland: Swiss Federal Research Station for Animal Production and Dairy Products, Joerg Hummerjohann; The Netherlands RIVM-LZO, Wilma Jacobs, Wendy van Overbeek; The Netherlands: NVWA, food and Consumer Product Safety Authority, Karin Geurts, Els Biesta Peters; United Kingdom: Microtech Wessex, Melody Greenwood, Roger Hutson; United States, Food and Drug Administration (FDA), Center for Food Safety and Applied Nutrition, Yi Chen. The authors also wish to thank the members of CEN/TC 275/WG 6 TAG 17 (Technical Advisory Group for *Listeria*):

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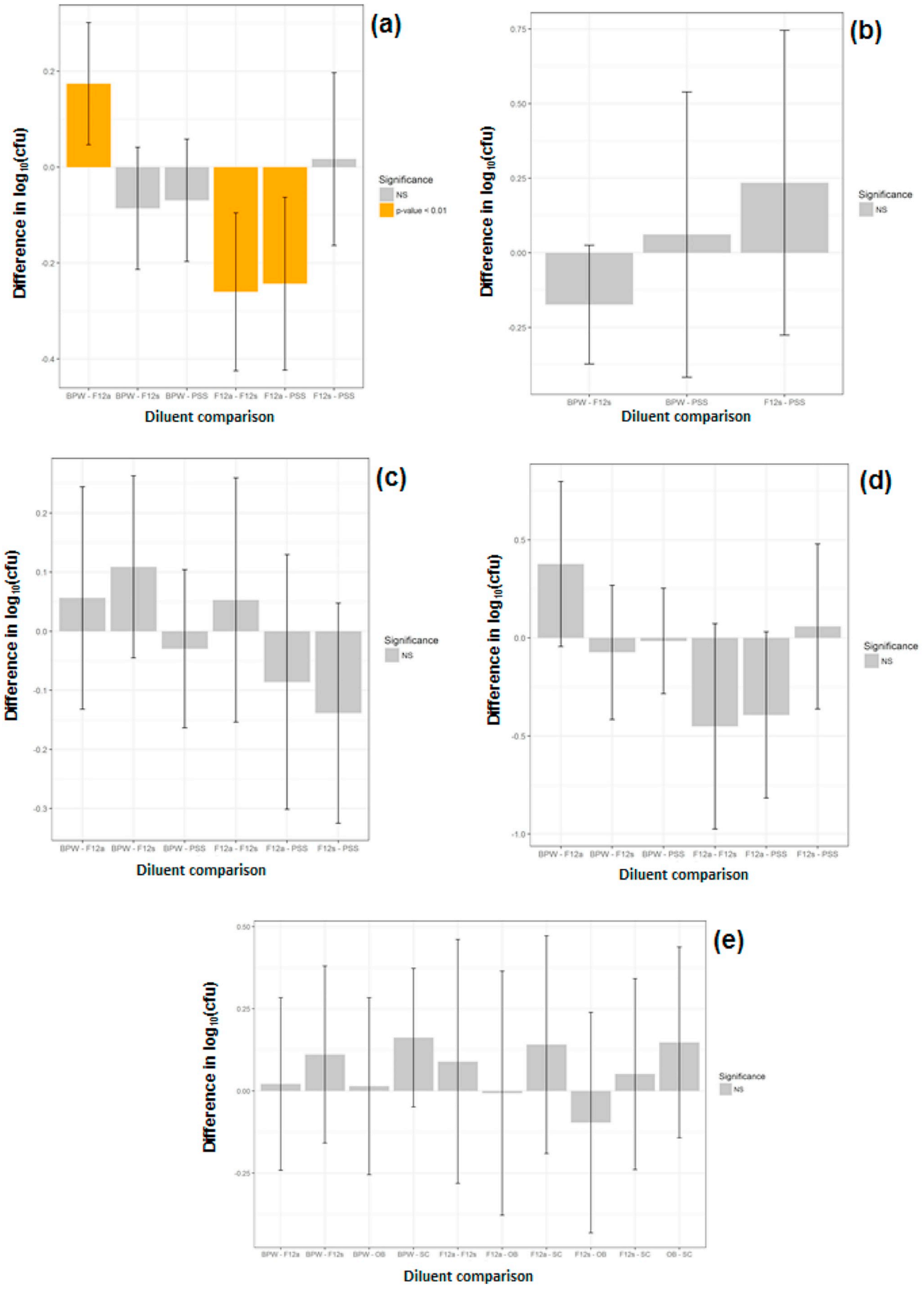

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Fig. 3. Effect of diluent on enumeration results: (a) milk powder, (b) salad, (c) salmon, (d) environment, (e) cheese. (BPW: Buffered Peptone Water; F12a: Half Fraser with supplements; F12s: Half Fraser without supplements; PSS: peptone saline solution; OB: One Broth; SC: Sodium Citrate).

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Appendix A. Supplementary data

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