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Detection of *Listeria* spp. and *L. monocytogenes* in pooled test portion samples of processed dairy products

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ABSTRACT

Listeria monocytogenes is a major foodborne pathogen. Testing multiple portions of the same final product is often required to verify the effectiveness of a food safety management system. Therefore, it will be advantageous to the laboratories to combine these test portions and process as one sample. However, combining samples for analysis, i.e., pooling, can be done only if there is no negative impact on the result. The objective of this study was to validate pooling of test portions for the detection of *L. monocytogenes* and *Listeria* spp. in dairy products as no scientific evidence currently exists to support this practice. Six representative matrices, namely, pudding, yogurt, brie cheese, 2% milk, ice cream and infant formula were spiked separately with stressed *L. monocytogenes* and *Listeria* spp. in 25 g and pooled test portions (375 g/250 g/125 g). Two methods, namely, ISO-11290-1:1996 Amd1:2004 and a validated alternative method Rapid'L.Mono were used for sample testing. Performance of a method in pooled test portions was considered to be satisfactory if the relative limit of detection (RLOD$_{50}$; LOD$_{50}$ [pooled test portion]/LOD$_{50}$ [25 g test portion]) and limit of detection (LOD$_{50}$) obtained was ≤ 2.5 and 1 CFU or MPN, respectively. Results obtained from *L. monocytogenes* and *Listeria* spp. trials were given equal weightage to decide on the impact of pooling. Acceptable RLOD$_{50}$ and LOD$_{50}$ values were consistently obtained in *L. monocytogenes* and *Listeria* spp. inoculation experiments when test portions were pooled up to 125 g for all matrices tested with both methods. While there was a slight delay for the primary enrichment of the pooled test portions to reach the desired incubation temperature when compared to the 25 g test portions, it did not negatively impact the outcome when samples were pooled up to 125 g. Background organisms were in general present at low concentrations and did not seem to adversely impact the recovery of the target organism in 125 g samples. Thus, pooling of test portions to up to 125 g for the detection of *L. monocytogenes* and *Listeria* spp. by two culture methods in processed dairy products has been validated.

1. Introduction

Listeriosis remains an important foodborne disease with 2206 confirmed cases reported from 28 member states in Europe alone in 2015 (EFSA and ECDC, 2016). *Listeria monocytogenes* (LM) is the etiological agent of human listeriosis, a disease characterised by abortions in pregnant women and high mortality rates in susceptible individuals in an invasive infection (Vazquez-Boland et al., 2001). LM is mostly transmitted to humans through the consumption of contaminated high moisture ready-to-eat (RTE) and non-ready-to-eat (NRTE) foods manufactured in wet and cold processing facilities. Major source of infection includes contaminated RTE meat, fish and dairy products, though other foods of plant origin such as cantaloupes have been involved in some outbreaks. Food items from the processed dairy food category such as soft cheese are an important contributor of LM infections (EFSA and ECDC, 2016) and in the recent past a novel vector from this category, ice cream, has been incriminated in an outbreak in the United States (Rietberg et al., 2016). Though LM is mostly associated with high moisture products manufactured in wet and cold environment, it is also considered a significant pathogen in low moisture foods targeted for sensitive population such as infants and pregnant women.

Control of LM in finished products is achieved by a combination of stringent raw material microbiological specifications, application of validated kill steps, good manufacturing practices, hygienic design of facility and equipment, zoning, cleaning and sanitation programmes during manufacturing process and product handling steps in the

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downstream supply chain. Testing raw materials, environmental samples and finished products for LM and or its indicator *Listeria* spp. often verify the efficiency of these measures to control LM.

Depending on the product type and the regulatory framework in place, LM should be absent in finished products (absence in 25 g) or up to 100 colony forming units (CFU)/g is permissible in products, which do not support the growth of LM. For example, European Commission regulation no. 2073/2005 on microbiological criteria for foods requires absence of LM in 10 samples of 25 g ($n = 10$) for RTE foods intended for infants and special medical purposes and 5 samples of 25 g ($n = 5$) for RTE foods which supports the growth of LM. The legislation permits levels up to 100 CFU/g in RTE foods unable to support the growth of LM or if it can be demonstrated by the manufacturer that levels will not exceed 100 CFU/g throughout the shelf life of a product where growth can occur. The legislation also allows a manufacturer to set low intermediate limits during the process to ensure that the limit of 100 CFU/g is not exceeded at the end of the shelf life in products allowing LM growth (EC, 2005). Consequently, a typical sampling plan for LM qualitative (presence or absence) analysis requires testing of multiple 25 g test portions for products, which can result in extensive testing for the same product type. Therefore, for the qualitative testing of LM in multiple samples, strategies such as compositing and pooling can be used to reduce the associated analytical costs and to increase the laboratory efficiency.

When testing a composited sample, only a portion of the original laboratory sample is tested (Anonymous, 2017a) (Fig. 1). Compositing may have the greatest reduction on both analyst time and consumable cost, but it will also have a considerable impact on the reliability of the analytical result. Pooling is an alternative strategy for testing multiple samples and it comprises of two approaches: pooling of test portions (“dry pooling”) and pooling of (pre-) enriched test portions (“wet pooling”). In the case of test portion pooling, the samples are pooled in the initial suspension. In the case of pooling (pre)-enriched test portions, aliquots of the primary enrichments are combined into a single secondary enrichment (Fig. 1). When pooling samples it is important to consider the impact if a positive result is obtained. For example, retesting of individual samples may be required following a positive result to trace back the original contamination source and this may not be possible or provide the same result due to the low-level contamination of some samples. Therefore, ideally, only samples from the same batch/lot are pooled as a positive result for one or all samples will result in the same action.

Since legislation mandates the absence of LM in 25 g of finished product in a two-class sampling plan, detection methods are developed and validated to detect one target organism in a 25 g sample. Therefore, empirical evidence is required to assess the impact of compositing and pooling to ensure that these sample manipulations do not negatively affect the recovery of the target organism. This is of particular importance when testing for the presence of LM as it is well known that the recovery of LM can be negatively impacted by the presence of background organisms competing for growth such as *Listeria* spp. (Gnanou-Besse et al., 2010). This negative impact may have resulted from the production of inhibitors, nutritional competition, differences in growth rates as well as competition from non-*Listeria* background (Gnanou-Besse, Favret, Desreumaux, Decourseulles Brasseur, & Kalmokoff, 2016).

Several approaches are described in the literature to establish the impact of pooling matrices for pathogen detection. There are publications that assess the impact by comparing method performance characteristics such as sensitivity, specificity and efficiency (Vitas, Diez-Leturia, Tabar, & Gonzalez, 2014), limit of detection (LOD) and relative limit of detection (RLOD) (Tomas Fornes, McMahon, Moulin, & Klijn, 2017). Another approach is using a model measuring the growth during pre-enrichment to predict the impact of modifications, such as pooling, to the enrichment protocol. This was used for *Cronobacter* (Miled et al., 2011) and LM (Augustin et al., 2016) detection in pooled samples. In the international standard detailing the preparation of test samples, initial suspension and decimal dilutions for microbiology examination, ISO-6887-1:2017 (Anonymous, 2017a), a verification protocol to assess the impact of pooling on an analytical method is described.

However, only a limited number of scientific studies have been reported on the effect of test portion pooling for the detection of LM and *Listeria* spp. (Augustin et al., 2016; Becker et al., 2012; Curiale, 2000; Vitas et al., 2014). Curiale (2000) evaluated the performance of test portion pooling for the detection of LM in RTE meat and poultry products and found that there was no significant difference when samples

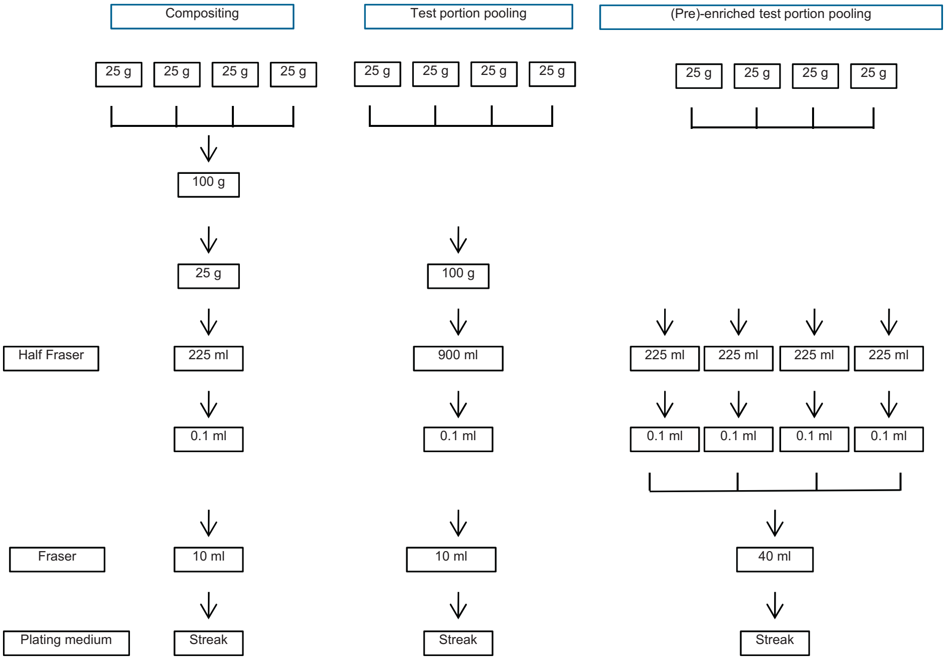

Fig. 1. Schema describing compositing, test portion (dry) pooling and (pre)-enriched test portion (wet) pooling. Test portion pooling was evaluated in this study where multiple 25 g samples were combined and processed as a single sample.

were pooled only up to 125 g. Becker et al. (2012) evaluated LM detection in frozen vegetables and reported reduced percent positives at low levels of inoculation in pooled sample sizes of 125 g and 375 g. In 2014, Vitas et al. reported that wet pooling of six portions of 25 g of smoked salmon did not have a significant impact on the analytical results of the batch. However, Augustin et al. (2016) using a modelling approach found a significant impact on LM detection due to wet pooling six portions of 25 g smoked salmon. Augustin et al. (2016) also proposed that the design recommended in ISO-6887-1:2017 Annex D cannot be applied with statistical confidence. Primarily, the authors questioned the applicability of a limited number of replicates suggested in the standard to verify the impact of pooling and recommended the use of mathematical models to assess the effect of procedures such as wet pooling on the performance of LM detection methods.

In this context, the objective of this study was to validate the principle of test portion pooling for the detection of LM and *Listeria* spp. in artificially contaminated processed dairy products using the experimental design based on the international validation standard, ISO-16140-2 (Anonymous, 2016). The increased sample size was considered as the alternative method, validated against the 25 g sample as the reference method. Relative limit of detection 50% ($RLOD_{50}$) [ratio of limit of detection 50% (LOD_{50}) pooled versus LOD_{50} 25 g sample] was calculated to determine the impact of the increased sample size on the detection of low levels of stressed organism. Two cultural methods, namely ISO-11290-1:1996/Amd 1: 2004 (hereinafter ISO-11290-1) (Anonymous, 2004) and a commercially available rapid method employing chromogenic plating media (Rapid'L.Mono) were used in this study. We decided to start the evaluations in 375 g sample portions, by pooling up to 15 samples of 25 g weight as has been previously reported for *Salmonella* detection in multiple food matrices (Tomas Fornes et al., 2017). In addition to $RLOD_{50}$, LOD_{50}, background organism level, pH of enrichment broth, temperature profile of enrichment broth and plate count of target organism after pre-enrichment were also done to study their influence on the detection of the target organism from pooled samples.

2. Materials and methods

2.1. Food matrices, bacterial strains and culture conditions

The food matrices, LM and *Listeria* spp. strains used in the experiments and their sources if known are listed in Table 1. Six food matrices were selected to represent the diversity of the food types within the dairy products category. An aerobic plate count was determined from all of the uninoculated matrices to estimate the background microbial load. Additionally, a 25 g sample was screened for the presence of *Listeria* spp. utilizing ISO-11290-1 and Rapid'L.Mono (RLM) (Biorad, Hercules, CA, USA) methods prior to artificial inoculation.

Isolates listed in Table 1 were obtained from the Silliker Food Science Center Culture Collection (FSC-CC) and used for the inoculation of the test matrices. Biochemical confirmations were performed on each *Listeria* spp. and LM strain before the start of the experiments. Each strain was separately transferred from frozen stock culture to 10 mL tryptic soy broth plus 0.6% yeast extract (TSB-YE) (Product Numbers BD211823 and BD211929, Becton, Dickinson and Company, Sparks, MD, USA) and incubated at 35 °C ± 1 °C for 18–24 h prior to the inoculation of food matrices.

2.2. Preliminary study

A preliminary study was done to determine the feasibility of pooling test portions and the sample size to be included in the main study as literature indicated that matrix type could have a major impact on the recovery of *Listeria* spp. and LM in pooled samples (Becker et al., 2012; Curiale, 2000). Samples were inoculated only with unstressed LM, as the goal was to assess the matrix impact on the recovery of the target organism from the pooled samples. Briefly, frozen stock culture (−80 °C) of the strain was transferred to 10 mL of TSB-YE broth using a sterile loop and incubated at 35 °C ± 1 °C for 18–24 h. After incubation, the culture was refrigerated at 4 °C while the levels were determined by plating serial dilutions on tryptic soy agar with yeast extract (TSA-YE) plates (Product number BD211046, Becton, Dickinson and Company, Sparks, MD, USA). No heat stress was applied to the culture prior to inoculation of the matrices. Though it would have been optimal to include *Listeria* spp. in the preliminary trials, it was not practical to cover the diversity within *Listeria* spp. and therefore the results obtained from LM experiments were used to guide the design of *Listeria* spp. inoculation studies.

2.2.1. Preparation of inoculated products and pre-enrichment

During the preliminary trials, five replicate samples from each product were inoculated with approximately 5 CFU of LM per test portion size. Each test portion was 375 g. All five samples were required to be positive for 375 g portion size to be included in the full validation study. If the test portion size affected method sensitivity, then a reduced test portion (e.g. 250 g or 125 g) was evaluated.

A 1:10 dilution of the sample was prepared using pre-warmed (30 °C) half (demi) Fraser broth (Product number BD211766, Becton, Dickinson and Company, Sparks, MD, USA) in a sterile bag (Catalog number 01-815-397, Fisher Scientific, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and homogenized in a JumboMix 3500 VP (Interscience, Woburn, MA, USA) for 2 min. Each homogenate was inoculated with approx. 5 CFU of non-stressed LM. Following inoculation, samples were incubated at 30 °C ± 1 °C for 26 h. After 26 h of incubation, aliquots were removed and analysed as described below.

2.2.2. Microbiological analyses

Samples were tested by following ISO-11290-1 and Rapid'L.Mono methods for the detection of LM.

2.3. Validation study

A schematic representation of the study protocol is outlined in Fig. 2. Briefly, separate validation studies were done with *Listeria* spp. and LM for all matrices listed in Table 1. Organisms were stressed using the procedure described below (2.3.1) to simulate the real-life stress conditions encountered by the bacteria during their presence in the food matrices.

Table 1
Food matrices and bacterial strains used in the validation study.

No.	Matrix	*Listeria* spp. & source	*L. monocytogenes* & source
1	Pudding	*L. seeligeri* ATCC 35967 (first trial); soil *L. seeligeri* ATCC 51335 (second trial); not known	*L. monocytogenes* ATCC 51775; dairy products
2	Yogurt	*L. ivanovii* ATCC 49954; food	*L. monocytogenes* FSC-CC 1791; yogurt flavour
3	Brie cheese	*L. innocua* ATCC 33090; cow brain	*L. monocytogenes* ATCC 51780; dairy products
4	Milk	*L. seeligeri* ATCC 51335; not known	*L. monocytogenes* ATCC 51414; raw milk
5	Ice cream	*L. ivanovii* ATCC BAA-139; Washing water	*L. monocytogenes* ATCC 51777; dairy products
6	Infant formula	*L. innocua* ATCC 51742; cabbage	*L. monocytogenes* ATCC 51779; dairy products

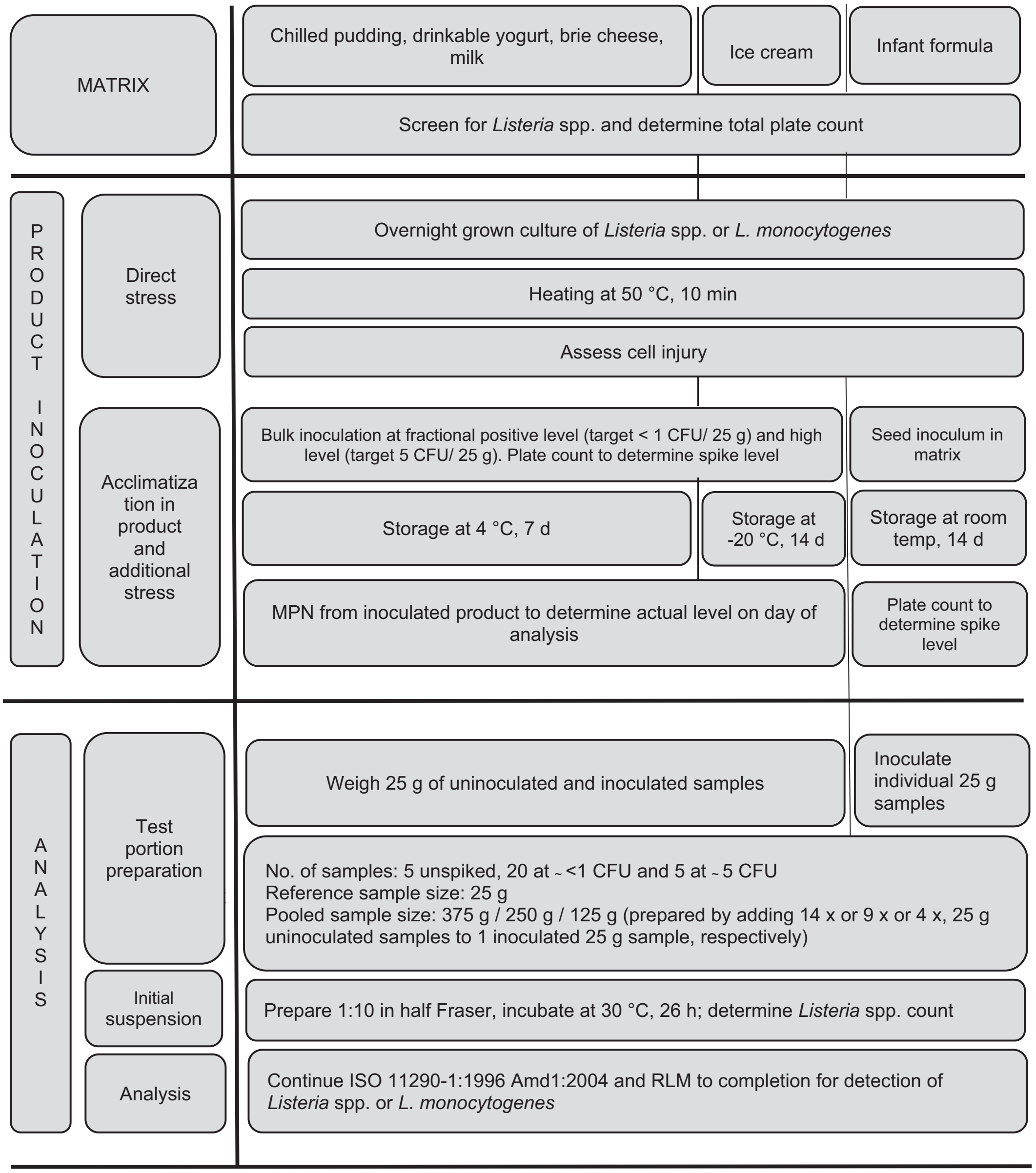

Fig. 2. Schematic diagram of the study protocol.

2.3.1. *Stress protocol*

Each strain was separately transferred from frozen stock culture using a sterile loop to 10 mL of TSB-YE in 16 × 150 mm disposable tubes, (Catalog number 14961-31, Fisher Scientific, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and incubated at 35 °C ± 1 °C for 18–24 h. Following incubation, each 10 mL of growth were heat-stressed by incubating at 50 °C ± 1 °C for 10 min in a water bath (Model Number 2335, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The timing started once a blank 10 mL control sample reached 50 °C ± 1 °C. The degree of injury was estimated by plating an aliquot of diluted culture onto modified oxford agar (MOX) (Product number BD222510 Becton, Dickinson and Company, Sparks, MD, USA) and TSA-YE (Product number BD211046, Becton, Dickinson and Company, Sparks, MD, USA). The plates were incubated at 35 °C ± 1 °C for 24 h and the colonies were counted.

The degree of injury was estimated by

$$\left(1-\frac{n_{select}}{n_{nonselect}}\right)\times 100$$

where n_{select} = number of colony forming units (CFU) on selective agar and $n_{nonselect}$ = number of CFU on nonselective agar.

2.3.2. *Preparation of inoculated products*

Matrices were inoculated to achieve a low and high target level of approximately 1 CFU/25 g and 5 CFU/25 g, respectively. The aim of low-level inoculation was to obtain fractional positive results, whereas all of the high-level inoculated samples were expected to test positive following the analysis.

To inoculate the different products, three separate protocols were used:

1) Refrigerated products (pudding, milk, yogurt, brie cheese): Matrices were bulk inoculated using heat stressed culture described in Section 2.3.1. After inoculation, samples were mixed thoroughly for approximately 15 min and stored at 4 °C for 48 h to adapt the microorganism to the matrix.
   Also, a second strain of *L. seelgeri* (ATCC 51335) was evaluated for detection in pudding in 125 g test portions after laboratory investigations determined that the growth of the first strain selected for the trial (*L. seeligeri*, ATCC 35967) was insufficient regardless of

the inoculated matrix (data not shown).

2) Frozen product (ice cream): Ice cream was brought to room temperature and bulk inoculated using heat stressed culture as described in 2.3.1. After inoculation, samples were mixed thoroughly for approximately 15 min and stored at −20 °C for 2 weeks to adapt the microorganism to the matrix.
3) Dry product (infant formula): In order to prepare the inoculum for infant formula, after overnight incubation in TSB-YE, the culture was centrifuged, supernatant discarded and the bacterial pellet was washed two times in Butterfields Phosphate Buffer (BPB) (Catalog number P285500 Fisher Scientific, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and re-suspended in 10 mL BPB. Following the application of heat stress as described in Section 2.3.1 the culture was again centrifuged to remove the supernatant and the pellet was dried in a desiccator for 48 h at room temperature. After drying, the culture was ground to a fine powder using a mortar and pestle and mixed separately with 500 g of infant formula to make seed inoculum. The seed inoculum was stored at 25 °C for 14 d to adapt the microorganism to the product. Samples (10 g) were taken during the 14-day period for enumeration, and the seed inoculum level was determined by plating serial dilutions on TSA (Product number BD211046, Becton, Dickinson and Company, Sparks, MD, USA) with a MOX agar overlay. The plates were incubated at 35 °C ± 1 °C for 46–50 h prior to enumeration. The seed inoculum at levels of < 10% of the test portion weight was then used to inoculate individually 25 g samples to achieve the desired inoculation level.

2.3.3. Sample preparation

Twenty random 25 g reference test portions at fractional positive level (approx. 1 CFU/25 g sample) were prepared from the inoculated products (Section 2.3.2). Similarly, 20 random pooled test portions were prepared at fractional positive level (approx. 1 CFU/pooled test portion). This was achieved by adding 14, 9 or 4, 25 g test portions to one inoculated 25 g test portion to make 375 g, 250 g or 125 g pooled sample, respectively.

In addition to the above samples, 5 replicates at high level (5 CFU/25 g or pooled test portion) of inoculation and 5 uninoculated controls were prepared and analysed for the 25 g and pooled test portions, respectively for all matrices. Two levels of contamination (fractional positive and high level) and a set of negative samples were used for the RLOD study following the procedure outlined in ISO-16140-2 (Anonymous, 2016). Reduced pooled test portion sizes namely, 250 g or 125 g were evaluated only if satisfactory results were not obtained with 375 g or 250 g test portions, respectively.

2.3.4. Estimation of contamination level prior to analysis

Most Probable Number (MPN) as recommended by AOAC International (Feldsine et al., 2002) was used to quantify the contamination levels. MPN was performed on each matrix (except for infant formula where plating method was used on the seed inoculum to determine the spike level as samples were individually spiked), by following the same enrichment, isolation and confirmation protocols as described in ISO-11290-1 cultural reference method to determine the actual inoculation levels. Test portion sizes were 3 × 100 g, 3 × 10 g and 3 × 1 g. Dilutions (1:10) from the 3 × 1 g test portions were prepared for determination at 0.1 g (3 replicates) following the established AOAC protocol. MPN analysis was done on the same day as the comparative analyses.

2.3.5. Microbiological analyses

A 1:10 dilution of each test portion was prepared using pre-warmed (30 °C) half Fraser broth (Product number BD211766, Becton, Dickinson and Company, Sparks, MD, USA) and incubated at 30 °C ± 1 °C for 26 h (maximum incubation time prescribed in ISO-11290-1) and analyses was continued as described below.

ISO-11290-1: After 26 h of incubation, 0.1 mL of half Fraser was transferred to 10 mL of Fraser broth and incubated at 37 °C ± 1 °C for 46–50 h. In addition, a loopful was streaked from the primary enrichment to Agar *Listeria* according to Ottaviani and Agosti (ALOA) (Product number AEB520079, bioMerieux, Inc., Hazelwood, MO, USA) and PALCAM (Product number OXCM0877B, Oxoid, Hampshire, UK) agars and incubated at 37 °C ± 1 °C for 46–50 h. Following the completion of Fraser broth incubation, a loopful was streaked to ALOA and PALCAM agar and incubated at 37 °C ± 1 °C for 46–50 h.

Following incubation of the plates, they were examined for the presence of LM and *Listeria* spp. colonies. Typical colonies were streaked to TSA-YE and incubated at 35 °C ± 1 °C for 18–24 h for purity. Confirmations of isolated colonies were completed following the procedure described in ISO-11290-1. Biochemical confirmations were done using the VITEK 2 ((Product number 21342, bioMerieux, Inc., Hazelwood, MO, USA) and or API *Listeria* system (Product number 10300, bioMerieux, Inc., Hazelwood, MO, USA).

Rapid'L.Mono (RLM): After 26 h incubation of the primary enrichments, 0.1 mL was streaked for isolation onto RLM plate and incubated at 37 °C ± 1 °C for 22–24 h for LM. Typical colonies of LM were confirmed by inoculating three isolated colonies as spots onto a dish of ALOA agar incubated for 22–26 h at 37 °C ± 1 °C (up to 12 spots per ALOA plate). RLM plates were re-incubated for additional 22–26 h for *Listeria* spp. Typical colonies were streaked to TSA-YE and incubated at 35 °C ± 1 °C for 18–24 h for purity. Following incubation, confirmations of isolated colonies were completed following the procedure described in ISO-11290-1 followed by biochemical confirmation using the VITEK 2 and or API *Listeria* system.

2.3.6. Monitoring of temperature profile

Throughout the study, a programmable data tracer was included in one negative control enrichment for each sample weight at 25 g and pooled sample (375 g, 250 g or 125 g) to monitor the temperature profile during incubation. The enrichment was placed besides other control samples on the top shelf of the incubator.

2.3.7. Pre- and post-enrichment pH

The pH of each sample enrichment was measured before the start of incubation and after the end of enrichment period in half Fraser broth using Fisherbrand™ Accumet™ AB15 Basic pH meter (Catalog number 13-636-AB15, Fisher Scientific, Thermo Fisher Scientific, Inc., Waltham, MA, USA).

2.3.8. Enumeration of Listeria spp. and LM in half Fraser enrichment

After 26 h of pre-enrichment, *Listeria* spp. or LM count was determined on TSA-YE with MOX overlay to assess the impact of the matrix on the recovery and growth of the target organism. The plates were incubated at 35 °C ± 1 °C for 46–50 h prior to enumeration.

2.4. Data analyses

$RLOD_{50}$ was used as the primary criteria to evaluate the performance of a method on pooled test portions. $RLOD_{50}$ by matrix, method and organism (*Listeria* spp. or LM) was calculated as the ratio between LOD_{50} of pooled test portion versus LOD_{50} of 25 g inoculated test portion. The 50% limit of Detection (LOD_{50}) was calculated according to Wilrich and Wilrich (2009). As the 25 g and pooled test portions followed separate pre-enrichment steps, the acceptability limit of $RLOD_{50}$ for pooling was set at 2.5, as recommended in ISO-16140-2 for evaluating results obtained from unpaired samples. In addition to $RLOD_{50}$, LOD_{50} was also considered to assess the impact of pooling on method performance. In this study, a LOD_{50} value of 1 MPN or CFU/test portion was determined to be an acceptable guiding value to evaluate the performance of a method on pooled test portions as theoretically a method should be capable of detecting even a single pathogen that can be present in a sample.

3. Results and discussion

3.1. Preliminary study results

Results of uninoculated products tested for the presence of *Listeria* spp. (in 25 g test portion size) were negative by both ISO-11290-1 and RLM methods. Total plate counts of the matrices tested were generally low for all matrices (1–2.5 log$_{10}$ CFU/g) except for brie cheese, where it was between 4.7 and 7.2 log$_{10}$ CFU/g. A preliminary trial was performed to decide on the sample size to be included in the validation experiments. In the first preliminary trial, LM was detected in all five replicates of 375 g test portion size for yogurt, brie cheese, 2% milk, ice cream and infant formula. In the case of pudding, LM was detected in four out of five replicates of 375 g test portion size and only in 250 g test portion size were all five replicates positive for LM. Based on these results, the full validation study started with the evaluation of 375 g sample size for the detection of *Listeria* spp. and LM for all matrices except for pudding, where 250 g was evaluated.

3.2. Pooling validation

Stressed cells of LM and *Listeria* spp. were used for artificially inoculating the food matrices. The degree of injury inflicted upon the organisms due to heat stress ranged from 39% to 94% and was determined to be an acceptable level of injury for matrix inoculation.

Acceptable RLOD$_{50}$ and LOD$_{50}$ values were obtained for 5/5 matrices (pudding was not tested at 375 g) with both ISO-11290-1 and RLM methods for LM detection, whereas equivalent results were obtained with only 3/5 matrices for *Listeria* spp. detection (Table 2). (Refer to the tables in supplementary materials for information on inoculation levels and the number of positives obtained). In this case, yogurt and ice cream were the two matrices that provided unacceptable RLOD$_{50}$ and LOD$_{50}$ values when samples were pooled up to 375 g. Though a reduced 250 g sample size was successful for yogurt, it was not the case with ice cream, where acceptable results were obtained only when sample size was further reduced to 125 g. In addition, for pudding, acceptable values were obtained only with 125 g samples for both LM and *Listeria* spp. using both methods (Table 2). LOD$_{50}$ values obtained for pooled test portions were higher or equivalent to 25 g samples, except for LM brie cheese and *Listeria* spp. in milk and yogurt (250 g) with both methods. Better performance of the methods in pooled test portions is difficult to explain and is most likely is due to a random effect.

The results obtained from LM experiments would suggest that samples could be pooled to > 125 g (except pudding) for LM detection. However, this is not recommended as equivalent results were not obtained for *Listeria* spp. detection in pooled samples. Equal weightage was given to the results obtained from both LM and *Listeria* spp. experiments as it can be envisaged that testing and reacting to the presence of *Listeria* spp. as if LM were to be detected can be practiced in a proactive food safety approach, given that *Listeria* spp. is often used as an environmental hygiene indicator of LM (Anonymous, 2017b). Therefore, it was decided to do two separate sets of experiments with LM and *Listeria* spp. and the results obtained were interpreted together to decide on the impact of pooling. In addition, the LM pooling study did not include any competition from other *Listeria* spp., which is known to influence its recovery. The reason co-inoculation was not performed is because the co-occurrence ratio of *Listeria* spp. and LM have not been studied across different matrices and would have made it difficult to determine the relative contribution of increased sample size versus the role of competitor organisms on the recovery of LM. This is supported by the fact that co-inoculation of *Listeria* spp. with LM is not required in RLOD studies following ISO 16140-2 guidelines. Based on the arguments presented in this paper, the sample size of 125 g was considered acceptable for test portion pooling. The results obtained from 125 g samples correlates well with the findings of Curiale (2000), where it has been shown that pooling of ready-to-eat (RTE) meat and poultry products (frankfurters, deli turkey and deli ham) was acceptable only up to 125 g sample and not 375 g.

3.3. Effect of background microorganisms

It has been previously reported that the growth and consequently the detection of *Listeria* spp. and LM is influenced by several factors like

Table 2
RLOD$_{50}$ and LOD$_{50}$ in pooled test portions.

	Pooled sample weight (g)	*L. monocytogenes* study results				Pooled sample weight (g)	*Listeria* spp. study results			
Matrix		LOD$_{50}$ pooled sample (MPN/test portion)		RLOD$_{50}$ (based on MPN)			LOD$_{50}$ pooled sample (MPN/test portion)		RLOD$_{50}$ (based on MPN)	
		ISO-11290-1	RLM	ISO-11290-1	RLM		ISO-11290-1	RLM	ISO-11290-1	RLM
Pudding	375	ND	ND	ND	ND	375	ND	ND	ND	ND
	250	0.75	1.023	3.575	4.878	250	ND	ND	ND	ND
	125	*0.989*	*1.145*	*1.429*	*1.655*	*125*	*0.515*	*0.572*	*1.705*	*1.894*
Yogurt	*375*	*1.025*	*1.025*	*1.426*	*1.426*	375	0.786	1.426	1.849	3.36
	250	ND	ND	ND	ND	*250*	*1.413*	*1.413*	*0.797*	*0.797*
	125	ND	ND	ND	ND	125	ND	ND	ND	ND
Brie cheese	*375*	*1.154*	*1.154*	*0.719*	*0.719*	*375*	*0.957*	*0.957*	*1.79*	*1.79*
	250	ND	ND	ND	ND	250	ND	ND	ND	ND
	125	ND	ND	ND	ND	125	ND	ND	ND	ND
Milk	*375*	*0.762*	*0.864*	*1*	*1.135*	*375*	*0.439*	*0.439*	*0.568*	*0.568*
	250	ND	ND	ND	ND	250	ND	ND	ND	ND
	125	ND	ND	ND	ND	125	ND	ND	ND	ND
Ice cream	*375*	*0.425*	*0.425*	*1*	*1*	375	2.981	2.981	4.436	4.436
	250	ND	ND	ND	ND	250	2.639	3.436	1.356	1.765
	125	ND	ND	ND	ND	*125*	*0.698*	*0.901*	*1.886*	*2.435*
Infant formula	*375*	*1.636*[a]	*2.123*[a]	*1.284*[a]	*1.666*[a]	*375*	*0.599*[a]	*0.912*[a]	*0.874*[a]	*1.157*[a]
	250	ND	ND	ND	ND	250	ND	ND	ND	ND
	125	ND	ND	ND	ND	125	ND	ND	ND	ND

RLOD$_{50}$ = LOD$_{50}$ [pooled test portion] / LOD$_{50}$ [25 g test portion].
ND: Not done.
Acceptable RLOD$_{50}$, LOD$_{50}$ (*in italics*).

[a] Inoculation level determined by plating method; LOD$_{50}$ is expressed as CFU/enrichment and RLOD$_{50}$ is based on plate count.

the competition from background flora, growth rate of co-existing *Listeria* spp. and interaction with the food matrix (Gnanou-Besse et al., 2010). Interestingly, in this study the presence of background organisms at levels that ranged from $< 1 \log_{10}$ CFU/g to $6.7 \log_{10}$ CFU/g, did not seem to have an adverse effect as even the matrix with the highest background organisms (brie, $4.7 \log_{10}$ and $6.7 \log_{10}$ CFU/g in *Listeria* spp. and LM inoculation experiments) could be pooled to a higher sample weight.

In the study by Becker et al. (2012) background organisms present in raw materials from plant origin such as broccoli, tomato, onion and potato, though not reported, could have also influenced the ability to detect LM in pooled test portions of frozen vegetables inoculated with low levels of LM. This speculation on the role of the background organisms is also supported by the fact that testing of 25 g portion size of raw materials has been later excluded from the scope of an Association Française de Normalisation (AFNOR) validation for VIDAS LMO2 assay (when Fraser broth is incubated at 30 °C) (Anonymous, 2014), as there is a possibility to obtain false negative results due to the presence of a higher number of background microorganisms and or other competing *Listeria* spp. that may co-exist. It was also for this same reason that we decided to exclude raw materials and environmental samples from the scope of this validation study as it may be difficult to simulate the diversity and the levels of the background organisms existing in those samples. Consequently, the results obtained in this study pertain only to processed dairy products, i.e. foods that have been subjected to some form of thermal treatment and not for raw materials or environmental samples.

In general, the background organisms by themselves did not seem to negatively affect the detection of LM in pooled test portions as the RLOD_{50} obtained was < 2.5. Similarly, the background microorganisms did not adversely influence the detection of *Listeria* spp. in brie, milk and infant formula, where pooling was found to be satisfactory in 375 g samples. This can also be concluded from the fact that LM was successfully detected in 375 g yogurt and ice cream samples, whereas it was not the case with *Listeria* spp. though the levels of the background organisms itself was similar in both the trials. However, it is not possible to rule out the adverse impact of the background organisms on *Listeria* spp. detection in these matrices. It is to be noted that *Listeria* spp. was not naturally present during the pre-screening of the samples and a lack of competition from non-monocytogenes *Listeria* spp. could have aided the recovery of LM from the spiked samples. Metagenomics may provide an interesting insight into the dynamics between species during pre-enrichment as described by Ottesen et al. (2016) especially when other organisms are present that are known to compete.

3.4. Growth of target organism

Though LM provided consistently acceptable results in pooled test portions, it was not the case in *Listeria* spp. inoculation studies. In order to understand the impact of pooling on the detection of *Listeria* spp. and LM, enumeration of the target organism following pre-enrichment in half Fraser broths were performed. Counts in samples that tested positive by at least one method ranged from 1.9 to $5.1 \log_{10}$ CFU/mL. In all except one evaluation (brie cheese, LM 375 g), selective plate counts were higher in 25 g test portions when compared to the pooled test portions. The counts obtained from 25 g test portions were higher by 0.6–1.6 log CFU/mL for LM and 0.3–1.8 log CFU/mL for *Listeria* spp., respectively (Fig. 3a and b).

In addition, counts in smaller pooled test portions (e.g., 125 g pudding for LM, 250 g yogurt for *Listeria* spp. or 125 g ice cream for *Listeria* spp.), were higher in comparison to bigger test portion sizes of the same matrix/organism combination. The reduced counts noticed in pooled samples could have been also due to the increased dilution of the target organism in the pre-enrichment broths. Thus, it is evident that the growth of *Listeria* spp. including that of LM is affected by the test portion size though the number of positives obtained itself was not significantly impacted when the pooled sample size was at 125 g.

3.5. Methods used for detection of target organisms

Culture based methods using half Fraser as primary enrichment were evaluated in this study. RLM is a rapid alternate method, which uses a proprietary chromogenic plating media and has been previously shown to be equivalent to the ISO-11290-1 standard for the detection of *Listeria* spp. and LM (Anonymous, 2015). Overall, a slightly increased LOD_{50} and RLOD_{50} values were observed for both LM and *Listeria* spp. detection with the RLM method. This could be because it is an abbreviated method with no secondary enrichment in Fraser broth, unlike ISO-11290-1. However, LOD_{50} and RLOD_{50} results obtained with RLM were comparable to ISO-11290-1 for all matrices except 375 g yogurt samples inoculated with *Listeria* spp. Thus, no major differences were noted between the methods evaluated in this study as both of them used the same end-point detection principle, i.e. plating on selective solid media. However, different conclusions may have been reached by using other end-point techniques like PCR and Enzyme Linked Immuno-Sorbent Assay (ELISA). As discussed before, the results obtained by Curiale (2000) using cultural, ELISA and PCR methods showed that pooling is acceptable only up to 125 g sample size with culture method and PCR, but not with ELISA. The study by Becker et al. (2012) reported highest positive detection using the cultural method though it was not significantly different from PCR or ELFA. Results obtained from both these studies highlight the fact that methodology plays a critical role in determining the outcome of testing pooled samples. Thus, it is important that the method intended to be used by a laboratory should be validated for sample pooling.

3.6. Time to reach incubation temperature

Temperatures of enrichment media were monitored to determine its impact on the recovery of the target organism. Even though pre-warmed enrichment broths were used to prepare the initial suspension, the time to reach the actual incubation temperature was directly dependent on the sample size and its initial temperature that varied from 24 °C–25 °C for ice cream, milk and yogurt after adding pre-warmed media compared to 26 °C–27 °C for infant formula, pudding, and brie cheese. The time to reach the required incubation temperature ranged between 0.6 and 1.3 h for 25 g versus 3.3–14.9 h for pooled test portions in LM validation study. In *Listeria* spp. validation study, 25 g test portions reached 29 °C after 0.2–7.0 h, whereas it required between 1.0 and 9.8 h for pooled test portions to reach the same temperature. The differences seen in the come up time between *Listeria* spp. and LM validation study is most probably due to the day to day variations in the experimental conditions itself and cannot be attributed to any other specific factor. Thus, pooled samples required a longer time in comparison to 25 g samples to reach the desired incubation temperature. This delay to reach the set incubation temperature could have potentially contributed towards the lower counts of target organism obtained following the completion of pre-enrichment step. However, it is of interest to note that the lower counts itself did not adversely impact the total number of positives obtained in pooled test portion sizes of 125 g which were determined to be acceptable, though it will be interesting to determine whether a shorter delay or an extended incubation period can result in increased count and thus the detection of the target organism.

In order to minimize the impact of come up time on the growth of LM and *Listeria* spp. in a pooled sample it is recommended to use pre-warmed enrichment medium for the preparation of the initial suspension. Performance of incubators in individual laboratories can also affect the recovery of the target organism from the pooled samples. Therefore, it is critically important for the laboratories to assess and verify the pooling performance not only considering the specificity of their matrices but also taking into account lab specific conditions such

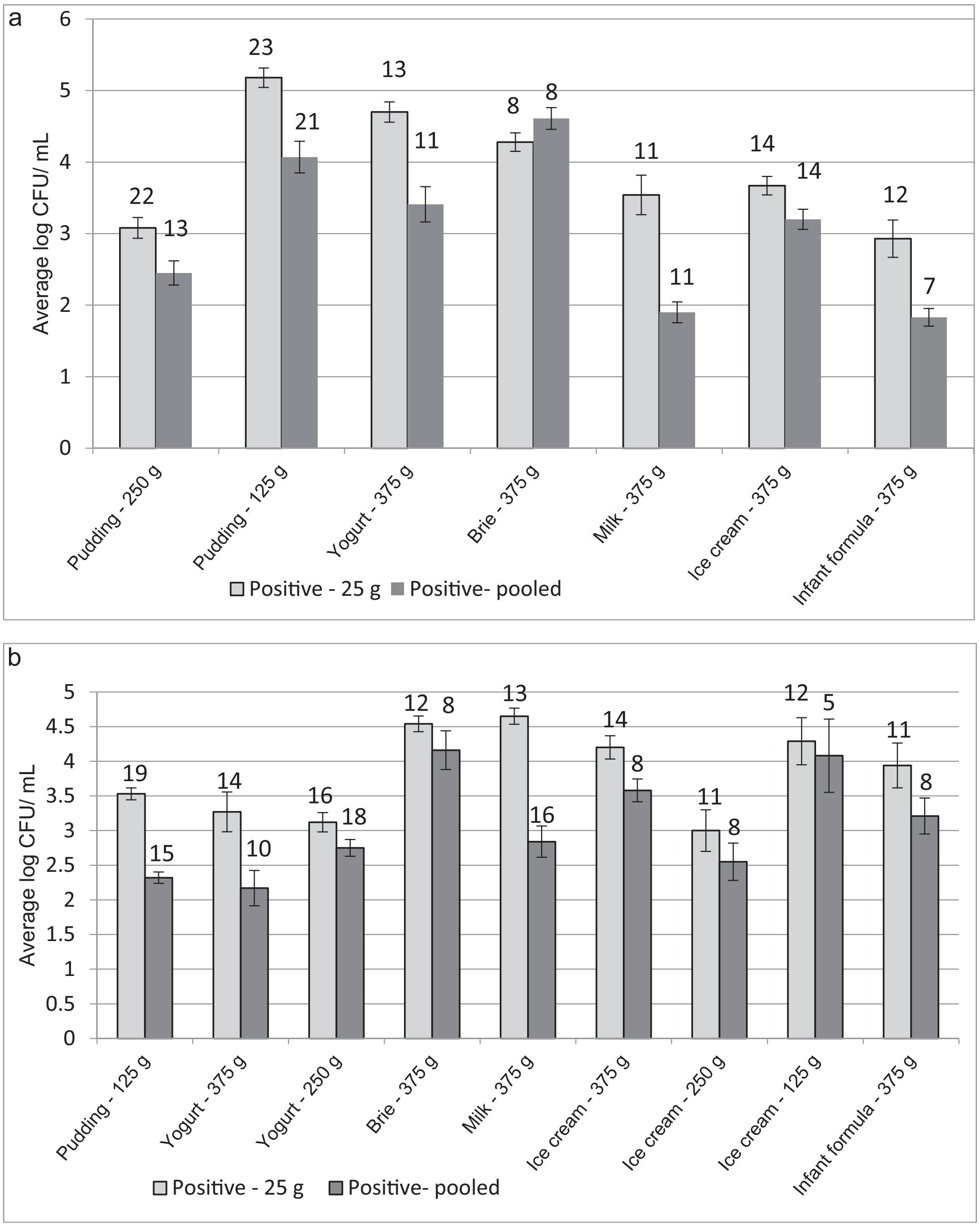

Fig. 3. Average LM/*Listeria* spp. counts obtained from pre-enrichment broths for samples that tested positive by ISO-11290-1. a: LM counts; b: *Listeria* spp. counts. Numbers on top of each bar represent the number of spiked samples that tested positive.
Samples which tested positive, but whose counts were < 1 log CFU/mL were excluded.

as incubators.

3.7. pH of enrichment media

pH of the enrichment broths were monitored during the study to determine its impact on the recovery of the target organism. Average pH of half Fraser broth post enrichment in pooled and 25 g negative and positive samples are presented in Fig. 4a and b. While significant pH differences (*t*-test, p value < 0.05) were noticed in 13 comparisons between samples that tested positive and negative, these did not correlate to the pooling outcome. All pH values stayed in a narrow range (6.8–7.3) and no significant pH differences were noticed for other samples (p value > 0.05). In addition, the pH values observed in this study are well above the reported growth limit of pH 4.3 for LM.

4. Conclusion

This study demonstrates that infant formula, chilled and frozen dairy products can be pooled up to 125 g for the detection of LM and *Listeria* spp. LOD_{50} and RLOD_{50} values obtained for both ISO-11290-1 and RLM methods had to be below 1 CFU or MPN/sample and 2.5, respectively for pooling sample size to be acceptable. To our knowledge, this is the first detailed validation study to investigate the effect of test portion pooling on the detection of LM and *Listeria* spp. in processed dairy foods. Though it will be of interest to subject the data obtained in our study through a mathematical model as previously reported by Augustin et al. (2016) to corroborate the outcome, we believe it is not necessary to do so as we followed the statistically valid experimental procedure outlined in ISO-16140-2 to assess the impact of pooling, which is a widely accepted standard for the validation of alternate rapid methods for the detection of microorganisms in food matrices. Additionally, the RLOD approach has been used previously in a *Salmonella* pooling study (Tomas Fornes et al.,

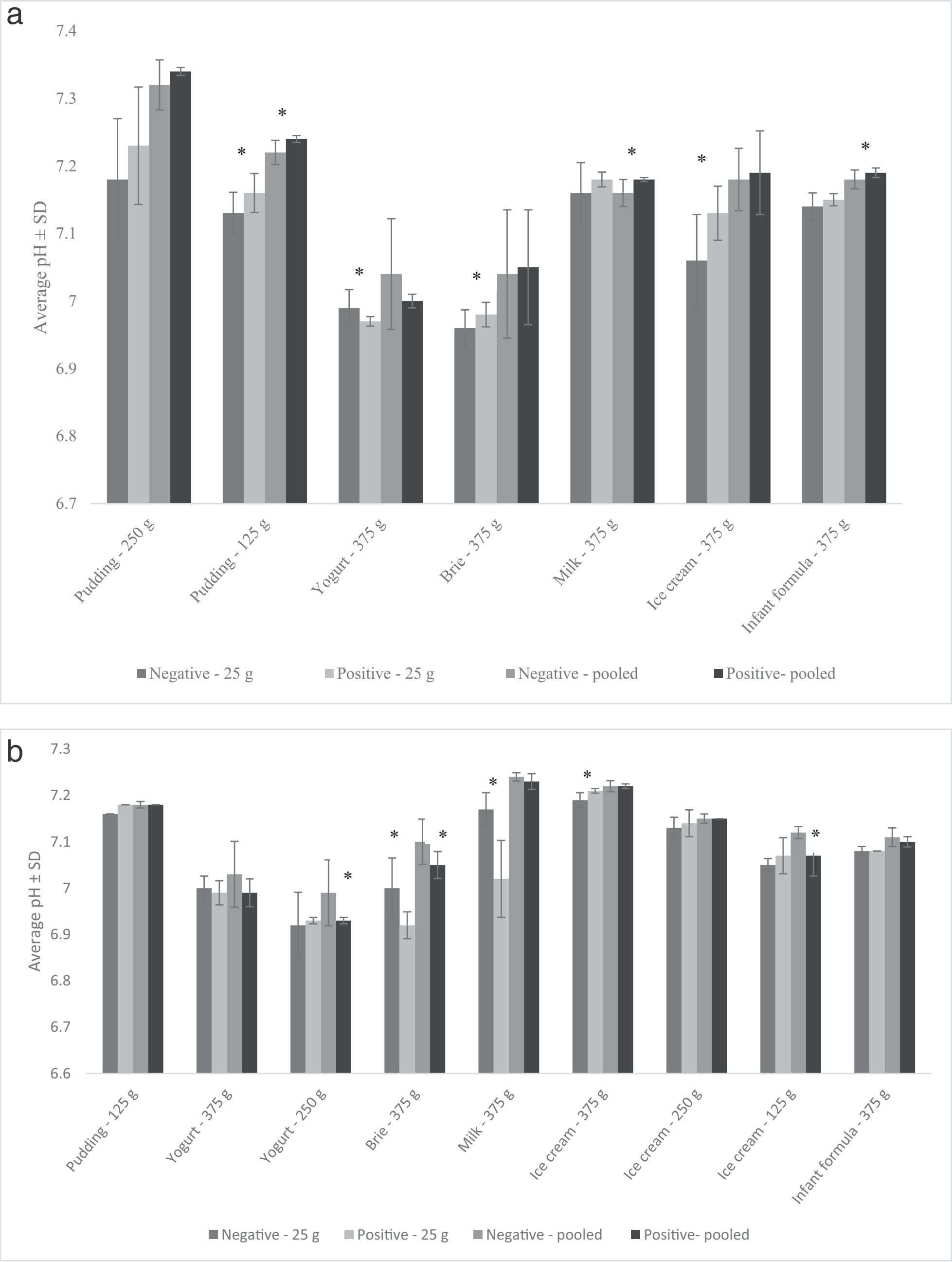

Fig. 4. Average post-enrichment pH results of positive and negative 25 g and pooled samples by both ISO-11290-1 and RLM. a: LM validation study b: *Listeria* spp. validation study.
* Significant difference between samples that tested positive and negative ($p < 0.05$).

2017) where the authors have successfully validated pooling up to 375 g for several food categories.

In summary, although acceptable $RLOD_{50}$ and LOD_{50} were obtained with some matrix combinations, the criteria were consistently met only when test portions were pooled to a maximum of 125 g. Thus pooling of up to 125 g for chilled dairy products, frozen dairy products and infant formula using ISO-11290-1 and Rapid'L.Mono methods is validated. During sample pooling it is critical that half-Fraser must be pre-warmed (30 °C) prior to the preparation of the initial suspension to reach the desired incubation temperature rapidly. Though we did not evaluate the effect of minimum enrichment period on pooled samples, the recommendation to follow maximum enrichment time (26 h) can be supported by the findings of Augustin et al. (2016) who have predicted a reduced sensitivity when the incubation period was reduced by 2 h for ISO-11290-1 method. The results obtained also indicated that the outcome of pooling is impacted by a combination of several factors such as the food matrix, time to reach the desired incubation temperature, sample size, target organism and the method used. Therefore, for laboratories intending to do pooling, it is highly recommended to do a verification for LM and *Listeria* spp. detection per food item to assess the recovery of the target strain in the presence of background organisms specific to the matrix.

Note

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Declaration of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.08.017.

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