Comparison of the ISO method and three modifications of it for the enumeration of low concentrations of *Listeria monocytogenes* in naturally contaminated foods

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Abstract

Sensitive methods for enumeration of *Listeria monocytogenes* (*L. monocytogenes*) are needed to verify compliance with microbiological criteria in ready-to-eat foods. Here, we assessed the reference EN ISO 11290-2 method and three modifications of it with lower threshold levels for enumeration in terms of specificity, false results and practical limitations for use. Two of the methods, called the EURL and the Cyprus protocols, use membrane filtration to obtain a more concentrated test suspension, and the third, called the Norway protocol, uses less diluent. This study included 18 samples of foods naturally contaminated with *L. monocytogenes* at concentrations of 0.2-80 CFU/g. All four tested methods yielded valid results with good repeatability (Fisher's test, $p<0.01$). The Norway protocol was the least laborious method and gave good results even for samples that could not be filtered.

Keywords

- Enumeration
- *Listeria monocytogenes*
- Filtration
- Microbiological methods
- Food

Acronyms and units used

- CFU/g: colony forming units per gram
- EURL: European Union reference laboratory
- ISO: reference method EN ISO 11290-2
- NRL: national reference laboratory
- RTE: ready-to-eat

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Introduction

Although its incidence is low, foodborne listeriosis causes serious symptoms and has a high fatality rate, up to 30% (Anonymous, 2000). Moreover, since 2000, the number of listeriosis cases has increased in several European countries (EFSA, 2007; EFSA 2012). For reasons not fully understood, this increase in infection appears to be linked to the increased use of ready-to-eat (RTE) products in which *L. monocytogenes* can grow during chilled storage. Several meat products and some seafood products belong to this category. Surveys of the incidence of *L. monocytogenes* in RTE foods at the retail level have been carried out during the past few decades. Across the EU, the estimated prevalence of foods with more than 100 CFU/g of *L. monocytogenes* at the end of shelf life is 1.7 and 0.43% for RTE seafood and meat products, respectively (EFSA BIOHAZ Panel, 2018). The majority of positive samples contain less than 1 000 CFU/g, but 2-4% contain 10 000 CFU/g or more at the end of shelf life (EFSA BIOHAZ Panel, 2018). Risk assessments from recent years have concluded that nearly all cases of listeriosis occur due to a very high dose of *L. monocytogenes* (1 000 to 1 000 000 CFU depending on the vulnerability of the consumer) after consumption of food that has been stored for a long time and/or at temperature abuse conditions (for review, see Buchanan *et al.*, 2016). Due to growth of the bacterium during storage, the concentration of *L. monocytogenes* in such products at the initial, processing stage may be below 10 CFU/g. Sensitive methods for the enumeration of *L. monocytogenes* in foods are therefore needed to demonstrate compliance with microbiological criteria and to limit the number of listeriosis cases.

The European and international standard method for enumeration of *L. monocytogenes* in food, EN ISO 11290-2 (ISO, 1998, 2004) is the reference method for *L. monocytogenes* in Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs. The method is characterised by a theoretical limit of enumeration of 10-100 CFU/g in solid food. Its specificity has been improved with the introduction a more specific agar, Agar *Listeria* according to Ottaviani and Agosti (ALOA) (Gnanou Besse and Colin, 2004). However, this method still lacks sufficient sensitivity to determine bacterial counts in a precise manner at the limit of 100 CFU/g and to quantify the bacterium at even lower levels. Sensitivity of the ISO standard method can be improved by using more agar plates at the lowest dilution, but the feasibility of this approach is limited due to the costs of the selective medium and available space in incubators. Therefore, other alternative ways to obtain a more concentrated sample solution have been explored. (for review, see Gnanou Besse and Colin 2004, Välimaa *et al.* 2015; Jadhav *et al.* 2012). For routine analysis, however, it is important to ensure the same specificity and detection principle as the ISO standard method specified by the microbiological criteria. Here, we assessed three modifications of the EN ISO method that have been developed by reference laboratories, called the EURL, Cyprus and Norway protocols.

The EURL and the Cyprus protocols are based on membrane filtration followed by transfer of the filter to ALOA (AFNOR, 2009; Baudouin *et al.*, 2010; Barre *et al.*, 2015). The EURL protocol has been validated through an inter-laboratory study (Gnanou Besse *et al.*, 2008). With the analysis of a 5 g test portion of cold-smoked salmon, an enumeration limit of 0.2 CFU/g can be reached. The method has already been successfully used to monitor the growth and initial concentrations of *L. monocytogenes* in cold-smoked salmon (Gnanou Besse *et al.*, 2006). The method was recently evaluated for other food categories (meat, sausages, vegetables and seafood; Barre *et al.*, 2015), but this method proved non-applicable for some meat products. In light of this need for other protocols for meat products, the Cyprus protocol includes filtration, but the additives to the sample suspension are different to the EURL protocol, thereby representing an alternative for some matrices. The Norway protocol does not apply filtration to concentrate the sample suspension, but uses a smaller volume of diluent before homogenisation. Skjerdal *et al.*, (2014) used a 1:1 ratio for fresh salmon and diluent leading to a five-times more concentrated sample suspension than the ISO method and the-

reby a five-times lower detection level for enumeration. The protocol was used successfully for salmon, but not tested on other food matrices.

The objective of the present study was to assess the EURL, Cyprus and Norway protocols in terms of specificity, false-positive and -negative results and practical limitations for use compared with the reference method (EN ISO 11290-2) for the enumeration of *L. monocytogenes* in food, with focus on meat products, because the protocols have been tested on seafood products previously.

Materials and methods

■ Food samples and experimental design

The study was conducted in our capacities as the European Union and National Reference Laboratories for *Listeria monocytogenes*.

Samples naturally contaminated with *L. monocytogenes* (n=35) were kindly provided by the NRLs for *Lm* from various EU Member States and private laboratories. NRLs receive many samples, and the panel used in this study is therefore considered representative. Samples were received and stored frozen at -18°C. They were thawed the night before use at 3 ± 2°C.

The same suspension of a naturally contaminated sample was analysed with four methods, each repeated five times (see Figure 1) in parallel with the modified reference EN ISO 11290-2 standard method (current version at the time of the study), the EURL, Cyprus and Norway protocols (20 analyses in total per sample).

The reference method was modified by using a tryptone salt (TS) diluent and spreading 5 ml on 15 ALOA agar plates to increase its sensitivity. A maximum of 25 colonies per assay were confirmed.

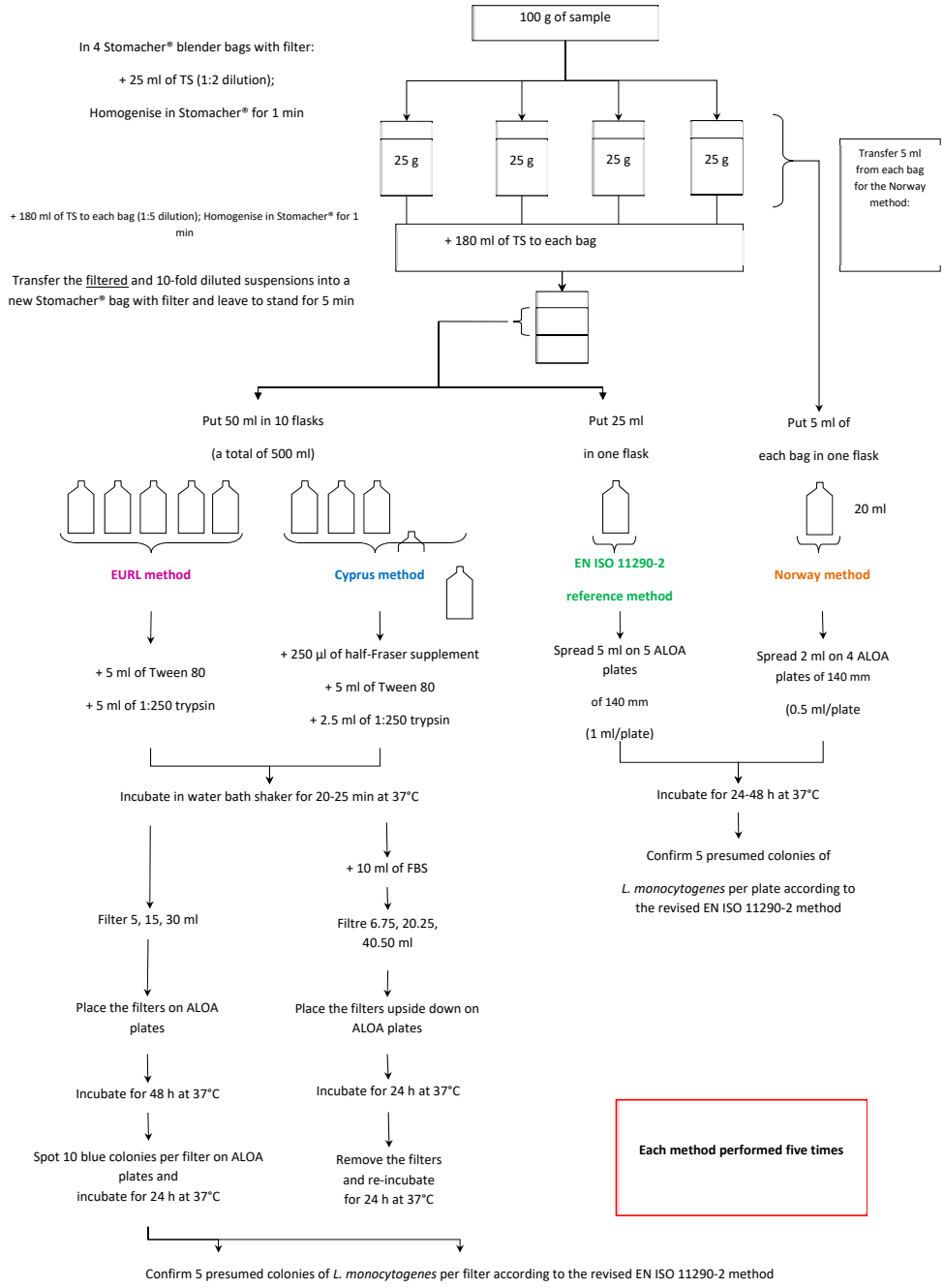
■ Samples and sample preparation

The flow chart for sample preparation and all protocols tested are shown in Figure 1. Samples of 100 g were aliquoted into four 25 g portions to which tryptone salt (TS) diluent (25 ml) was added in a Stomacher bag fitted with a filter, then homogenised for 1 min using a blender (either a Smasher™, Biomérieux, France, or a Stomacher 400, Seward, West Sussex, UK) at normal speed. The homogenate (1:2 dilution) was transferred to flasks. Five ml from each flask was transferred to a new flask and used for the Norway protocol. The remaining homogenates (45 g per bag, sample and diluent included) were added another 180 ml of TS to obtain a 1:5 dilution, which together with the first dilution gives a 1:10 dilution. The contents in the flasks were combined and used as test suspension for the EURL protocol, the Cyprus protocol and the modified EN ISO 11290-2 protocol. Five sample preparations were carried out on each product.

■ EURL protocol for enumeration of *L. monocytogenes* using a membrane filtration method

The filtration method was carried out according to the protocol described by Gnanou Besse *et al.* (2008) with the same media and chemicals. Briefly, filtration was carried out using a standard commercial Pyrex apparatus, and a vacuum pump with a maximum vacuum power of 630-635 mm Hg (around 80-85 kPa) and an airflow rate of around 34 l/min. A 4.7 cm diameter and 0.45 µm pore-size membrane, composed of mixed cellulose esters and single-use filtration units with an effective 12.25 cm2 filtration area were used.

FIGURE 1 / Flow scheme of the protocols

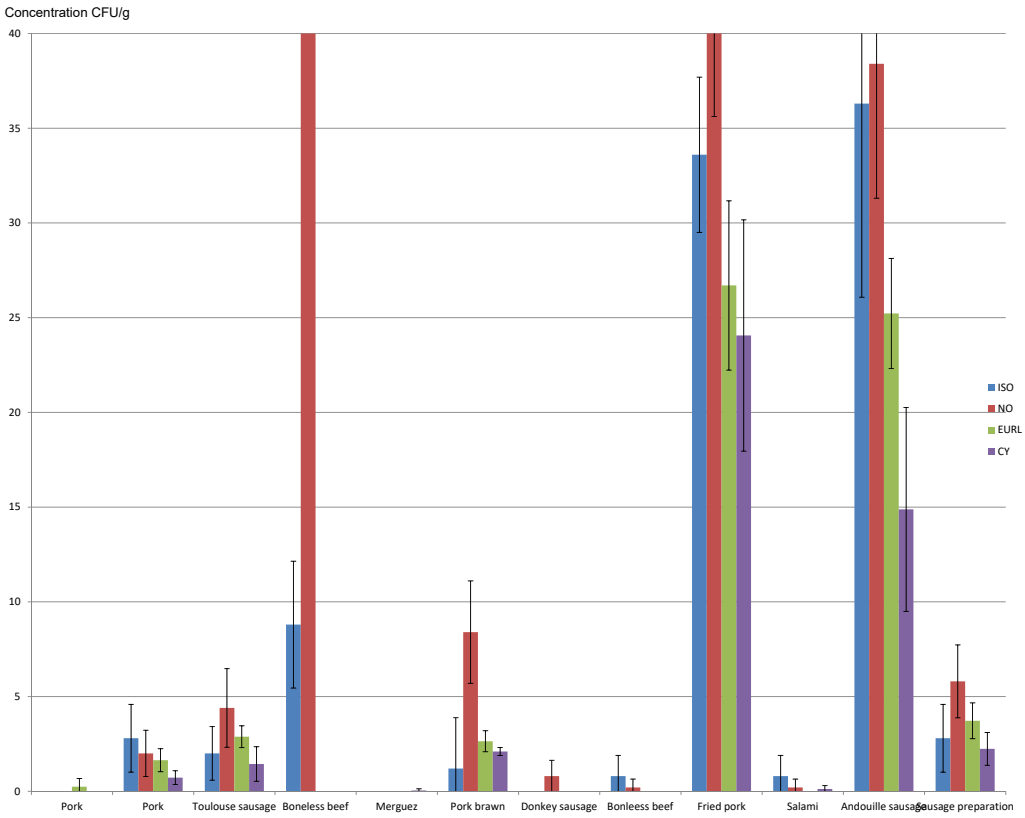


Three different volumes of the 1:10 diluted suspension (5, 15 and 30 ml) were immediately treated for a minimum of 20 and a maximum of 25 min at 37°C in a water bath shaker with 0.83% Tween 80 and 0.83% trypsin (addition of 1 ml of each reagent per 10 ml suspension to filter), and filtered. The procedure was repeated five times for each treatment. Every week, 10% trypsin from 1:250 stock solutions were prepared in phosphate buffer pH 7.5 containing 20 g dipotassium phosphate per litre of deionised water and stored at 4°C. The theoretical threshold of detection was 0.2 CFU/g.

Maximal filtration duration was set to 3-5 min. After this time, volumes of suspension which were not entirely filtered were considered as unfilterable. The filters were laid on ALOA plates. The plates were incubated upside down for 48 h at 37°C, and read after 24 and 48 h.

All *L. monocytogenes* colonies obtained on readable filters were counted. The volume analysed (corresponding to the selected filters) was recorded. *L. monocytogenes* colonies were blue without a halo, due to trypsin remaining on the filter. Consequently, in the present study, five typical blue colonies per filter were spot-inoculated on an ALOA plate, and incubated for approximately 6 to 18 h at 37°C to read the halo formation. Then, the typical *L. monocytogenes* colonies were confirmed according to the ALOA confirmation method.

FIGURE 2 / Comparison of enumeration results for *Listeria monocytogenes* obtained using the alternative methods (EURL, Cyprus (CY) and Norway (NO)) and the modified reference method (ISO) on naturally contaminated meat products (mean and standard deviation).



Cyprus protocol for enumeration of *L. monocytogenes*

The Cyprus protocol was similar to the EURL protocol with modifications intended to increase selectivity of the method. To reduce background microflora growth on the filter, half Fraser selective agents (Life Technologies, 10106169) were added to the suspension (0.5% final concentration). To favour better development of colonies and halo formation on ALOA agar without the need of subsequent inoculation, trypsin concentration was diluted with two parts of foetal bovine serum (FBS) that was added to neutralise trypsin activity before filtration (20% final concentration) (FBS, Oxoid, ref SR0166E) in order to obtain halo formation on ALOA agar. The filters were laid upside down on the selective agar and removed after 24h incubation at 37°C.

Norway protocol for enumeration of *L. monocytogenes*

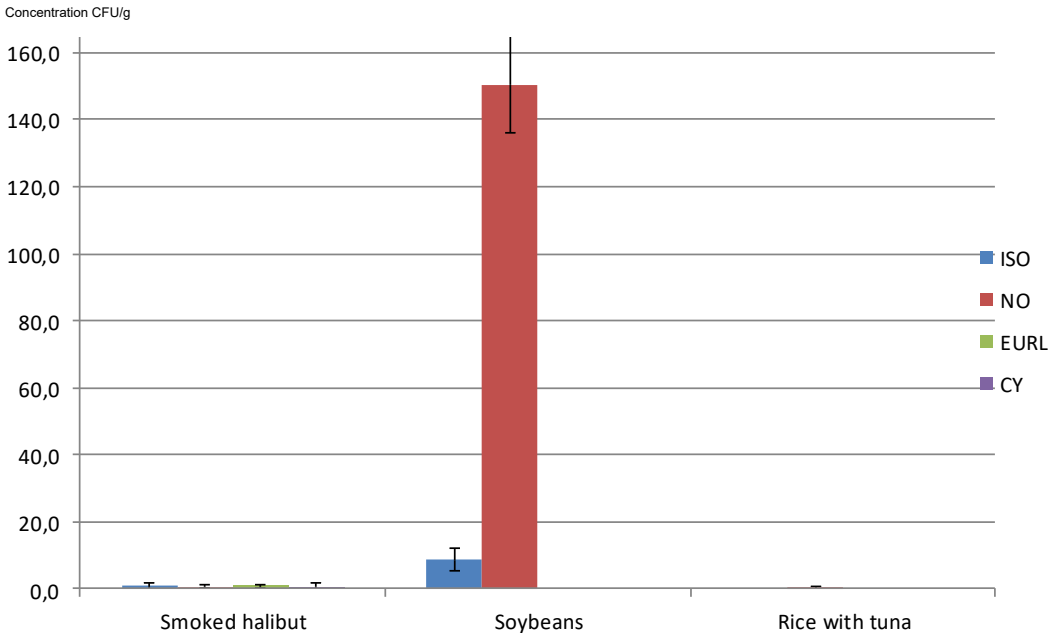
The reference method was modified by using TS diluent and spreading 2 ml of a 1:2 food suspension on four ALOA plates of 140 mm (0.5 ml/plate) or 2 ml on six ALOA plates of 90 mm (0.3 ml, 0.3 ml, 0.4 ml/plate) to obtain a limit of enumeration at approximately 1 CFU/g. Confirmation of presumptive colonies were carried out according to EN ISO 11290.

Statistical analysis

Samples with either counts higher than 100 CFU/g or no colonies were excluded from the statistical analysis. The statistical analysis was performed according to the AFNOR NF V03-110 standard (AFNOR, 1998), which describes an intra-laboratory validation procedure for an alternative method compared with a reference method.

The Cochran test was used to check that the sample variances did not differ statistically and that the precision was stable over the scope of the method. The repeatability variances of the alternative and reference methods were compared using Fisher's test. The relative trueness of the alternative methods against the reference method was assessed by comparing the means with an error risk (α) of 1%.

FIGURE 3 / Comparison of enumeration results for *L. monocytogenes* obtained by the alternative methods and the reference method with other naturally contaminated products (mean and standard deviation)



Results and Discussion

Applicability of the methods

Results are given in Table 1. Samples with *L. monocytogenes* concentrations greater than 1 CFU/g gave results in the same range for all methods. In most cases, the techniques made it possible to examine a larger quantity of food, thus greatly improving the sensitivity of the enumeration of *L. monocytogenes* in foods.

TABLE 1 / Result of the mean concentration (CFU/g) of *Listeria monocytogenes* obtained in each food sample, with the modified reference method analysing 5 mL and the alternative methods.

Samples	Method				
	ISO 11290-2, modified	EURL protocol	Cyprus protocol	Norway protocol	Filtration difficulties (filtration of 15 ml > 3 min)
	CFU/g (standard deviation)				
Pork	0	0.2 (0.4)	0	0	No
Pork	2.8 (1.8)	1.6 (0.6)	0.7 (0.4)	2.0 (1.2)	No
Toulouse sausage	2.0 (1.4)	2.9 (0.6)	1.4 (0.9)	4.4 (2.1)	No
Boneless beef	8.0 (3.3)	unreadable	unreadable	151.0 (14.7)	No
Boneless beef	0.8 (1.1)	unreadable	unreadable	0.2 (0.4)	No
Merguez	0	0	0.04 (0.1)	0	No
Pork brawn	1.2 (2.7)	2.6 (0.6)	2.1 (0.2)	8.4 (2.7)	No
Donkey sausage	0	unreadable	unreadable	0.8 (0.8)	Yes*
Fried pork	33.6 (4.1)	26.7 (4.5)	24 (6.1)	44.2 (8.6)	No
Smoked halibut	0.8 (1.1)	1 (0.5)	0.8 (0.9)	0.6 (0.5)	No
Salami	0.8 (1.1)	0	0.1 (0.2)	0.2 (0.4)	No
Soybeans	60.8 (12.0)	unreadable	unreadable	80.1 (18.9)	Yes*
Andouille (tripe) sausage	36.3 (10.2)	25.2 (2.9)	15 (5.4)	38.4 (7.1)	No
Sausage meat	2.8 (1.8)	3.7 (0.9)	2.2(0.9)	5.8 (1.9)	No
Rice with tuna	0	0	0	0.5 (0.4)	0.5 (0.4)
Sushi maki	0.1 (0.2)	0	Not tested	0	Yes*
Beef and mutton sausage	0.4 (0.4)	unreadable	Not tested	unreadable	No
Veal milanese	0.1 (0.2)	2.3 (3.2)	Not tested	1.8 (1.6)	Yes*

* Filtration difficulties indicate clogging of the filters in the EURL and/or the Cyprus protocols. Not relevant for the Norway and modified reference ISO protocol.

At *L. monocytogenes* concentrations below 1 CFU/g, the bacterium was detected with only some of the methods, and there was no clear pattern for any given method. The number of *L. monocytogenes* in the samples was likely too low to be homogenously present in samples for all protocols, and therefore it is not possible to determine which method was the most sensitive. In addition, four samples yielded unreadable results for both filter methods (EURL and Cyprus), but readable with the two others (reference and Norway). This discrepancy was due to either accumulation of the food matrix on the filters, or presence of bacteria that were able to grow on ALOA medium and cover the *L. monocytogenes* colonies (Baudouin *et al.*

2010). Given that the filter methods lead to a higher concentration of suspension than the other methods, the effect of background flora was more visible for these two methods. The samples with most overgrowth of background bacteria were generally products that included more ingredients. For the Norway protocol, in which the suspension was less concentrated than the filter methods, but more concentrated than in the reference method, only one sample gave unreadable results due to overgrowth (Table 1). The challenges observed with overgrowth will be the same for any method based on identifying characteristic colonies on ALOA agar, e.g. the pour-plate method recently described by Hunt *et al.* (2017). Our observations also illustrate the importance of testing new methods on naturally contaminated samples with a realistic concentration of background flora.

Despite the caveats described above, the sensitive methods hold promise for the majority of the tested food samples. Practical limitations were mainly due to low filterability of the sample. Food matrices with high fat content or containing moisture-absorbing compounds such as flour, soybean and rice, were the ones with lowest filterability.

Seven of the samples used in this study were meat products typical of a specific region and/or prepared with several different ingredients, often cut in large pieces and pressed or fermented together. Such non-homogeneous matrices represent different niches, which in turn make it possible for more kinds of microbes to survive than in homogenous products. The likelihood of interfering background bacteria is therefore high. Analytical methods that are suitable for these kinds of product are likely to be suitable for more homogenous products as well, which indicates that the analytical methods studied here are suitable for more products than the ones tested.

■ Selection of data for statistical analysis

Thirty-five samples were analysed and we obtained 18 interpretable results (Table 1), 15 of which were analysed using all four methods. Data from three samples were analysed with three methods. These data were not included in the statistical analysis, but are given in Table 1 to illustrate the challenges and benefits of methods and food matrices studied. The non-interpretable results were either due to a concentration of *Listeria* too low to detect with any of the methods (64%) or overgrowth of background flora (36%).

Among the 18 results given in Table 1, 50% were interpretable for all the methods. About 40% of the samples were interpretable only with the plating methods (Norway and modified reference protocol) because (i) the concentration of background microbiota or of *Listeria* spp. was too high to enumerate characteristic colonies, particularly on the filter (see above), or (ii) the sample was not filterable. One sample was only interpretable for the modified reference protocol. About 10% of the samples were interpretable only using the filtration methods (EURL and Cyprus), because the concentration of *Listeria* was too low and no colonies were found with either direct plating method.

■ Comparison of the alternative methods with the reference method

For each interpretable sample, the mean of five *L. monocytogenes* counts is shown with standard deviations for all four protocols in Figures 2 and 3. The mean contamination levels of *L. monocytogenes* ranged from 0.04 to 150 CFU/g. According to the statistical analysis of data, both filtration methods and the Norway method yielded true results, according to the criteria set up in the statistical analyses, compared with the reference ISO standard method. The precision (repeatability) of all methods was as good as for the reference method (Fisher's test, $p<0.01$), and seemed to be better for the filtration methods: s_r^2 were respectively 3.7, 1.7 and 2.6 for the Norway, EURL and Cyprus protocols.

Assessment of each method

Both filtration methods showed good performance in terms of trueness and precision. The EURL method is sensitive, relatively rapid, easy to implement and cheap: to achieve the same sensitivity (e.g. the analysis of 50 ml of a 1:10 sample suspension) without filtration, up to 150 Petri dishes of selective agar would be necessary for each sample, spreading 1 ml on three 90-mm plates. Practicability is important to consider when choosing an analytical method. It includes ease of use, speed and cost. New methods including multiple steps, unusual materials and costly reagents may be difficult to implement in routine analysis. The filtration method is quite simple to use, but requires a specific apparatus and is more laborious than the reference method. However, the filtration part of the method is performed in a single step (no pre-filtration needed). Nonetheless, according to our experience in organising an inter-laboratory study, a very detailed protocol generally requires a training period before being able to use the method satisfactorily, particularly to overcome technical difficulties, such as filtering issues. The EURL method does not appear to be applicable to some food products, due to background microbiota that hinder colony reading on plates. Similarly, a previous study with naturally contaminated samples showed that the EURL method is not adapted for various meat products (Barre *et al.*, 2015).

The Cyprus protocol shares the same advantages/disadvantages as the EURL method, but was developed to obtain a clearer halo on ALOA agar. In the present study, no halo formation was observed below the filters with the EURL protocol, and re-plating to another ALOA plate was needed to obtain typical, visible blue colonies. Among the 12 samples that could be interpreted using the Cyprus protocol, halo formation occurred only in two samples, indicating that the protocol needs to be developed further. Addition of FBS in the ALOA medium and/or the washing of the membrane filter before plating with FBS are some options. In some cases, the overgrowth of the filters by background microflora was a disadvantage observed for both EURL and Cyprus methods, due to the large volume of the filtrate and the small diameter of the filters, making them inappropriate for some food matrices. The use of filters with a larger diameter or a lower volume of filtration, are possible alternatives to overcome this problem. Both filter protocols apply a 0.45 µm pore-size membrane, which allows some bacteria to pass. However, no sign of underestimation of *L. monocytogenes* was observed for these two filter protocols, and a smaller pore size would further reduce the filterability of the matrix.

The Norway protocol is rapid and easy; it requires fewer plates than the reference method to obtain the same limit of enumeration and it can be used for routine analysis regardless of the product. It also represents a good alternative to filtration protocols for some unfilterable food categories (Table 1). The enumeration level for the Norway protocol, 1-2 CFU/g depending on the number of plates used, is higher than for the filter protocols, but an improvement compared with the 10 CFU/g in the standard methods currently applied. A drawback of the method is its tendency for overestimation, even though the difference with other protocols was not significant in our study. Other studies in our laboratory (results not shown) with artificially contaminated salmon (N=20) and naturally contaminated heat-treated chicken meat (N=53) indicated a systematic overestimation of up to 50% with the 1:2 dilution compared with the 1:10 dilution. The difference is likely to be due to that the amount of dry, inert material in the suspension is five times higher in 1:2 dilutions and, as a result, the concentration of bacteria in the liquid fraction of the suspension higher. This bias can be corrected for by subtracting the dry weight of the sample in the calculations.

Conclusions

Despite limitations related to low filterability of samples containing fat or moisture absorbing compounds like rice and flour, the filter methods were better than the reference ISO standard method for the enumeration of low levels of *L. monocytogenes* in samples of other types of food products. Overgrowth of background bacteria was observed for all three alternative methods, but less frequently for the Norway protocol, which requires less diluent than the filter protocols. All methods showed satisfactory sensitivity, which is essential for implementation the European regulatory limit of 100 CFU/g as well as to conduct shelf-life studies and surveys for risk assessments at realistic conditions. The Norway protocol using less diluent to obtain a more concentrated sample was the least laborious one, and gave results as precise as the filter methods.

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