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A contribution to the improvement of *Listeria monocytogenes* enumeration in cold-smoked salmon

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Abstract

For the enumeration of *Listeria monocytogenes* in food, a sensitive enumeration method based on membrane filtration followed by transfer of the filter to a selective medium has been developed. This study was carried out with cold-smoked salmon, a product likely to be contaminated with *L. monocytogenes*. The operating protocol utilizes three filtration runs in parallel (5, 15 and 30 ml) of a 1 in 10 dilution of the salmon suspension through 0.45-μm pore-size cellulose ester membranes, and then culture of the filters on Aloa agar (AES Laboratoires, Combourg, France). The results obtained with the technique were compared with those from the reference EN ISO 11290-2 method and found to provide more precise results in the enumeration of *L. monocytogenes* from both artificially and naturally contaminated cold-smoked salmon. Moreover, for several samples contaminated at low levels, *L. monocytogenes* could be recovered only by the filtration method. The examination of increasing volumes of salmon suspension enabled readable results to be obtained for all levels of *L. monocytogenes* and competitive microflora investigated. In most cases, the optimised operating protocol enabled 5.1 g of salmon to be examined, instead of 0.01–0.1 g with the reference EN ISO 11290-2 method, thus improving the sensitivity of the method.

Keywords: *Listeria monocytogenes*; Enumeration; Filtration; Microbiological methods; Cold-smoked salmon

1. Introduction

Despite its low incidence, food-borne listeriosis can be characterised by the seriousness of symptoms and a high rate of lethality, up to 30% (Anonymous, 2000). In addition, detection of *Listeria monocytogenes* in food involves important economic consequences like withdrawal of products and decrease of sales for incriminated products.

Vacuum-packed cold-smoked fish is a ready-to-eat product processed by a method that does not inactivate *L. monocytogenes* (Anonymous, 1999). After processing, the product is packaged and stored for a

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varying time, i.e., 3–6 weeks after the date of production, at refrigeration temperatures before consumption, allowing potential growth of *L. monocytogenes* (Anonymous, 1999, 2000). These products are mainly eaten without heat treatment, with the risk of consuming products contaminated with *L. monocytogenes*. However, no outbreak of listeriosis has yet been associated with the consumption of smoked fish (Anonymous, 1999), except an outbreak of listeriosis with a low number of cases (eight cases, two deaths), which was linked to "gravad" trout and cold-smoked rainbow trout in Sweden in 1994–1995 (Ericsson et al., 1997). An association between sporadic human listeriosis and consumption of cold-smoked fish is suggested, based on a characterisation of *L. monocytogenes* strains isolated from patients and from fish products (Loncaveric et al., 1998). Several studies reveal a relatively high prevalence of *L. monocytogenes* in such products, ranging from 5% to 60%, most of the surveys indicating a 15–20% prevalence (Richard et al., 1996; Anonymous, 1998a, 1999; Cohen Maurel, 1999; Johansson et al., 1999). The contamination levels are usually low, less than 100 CFU g^{-1}, and levels rarely exceed 10^3 CFU g^{-1} at consumption (Anonymous, 1998a, 1999). Such data indicating a relatively high incidence have raised concern about *L. monocytogenes* in cold-smoked fish, and over several years, studies using predictive microbiology, epidemiology, and quantitative risk assessment have been carried out (Cortesi et al., 1997; Dalgaard and Jorgensen, 1998; Jorgensen and Huss, 1998; Johansson et al., 1999; Lindqvist and Westöö, 2000; Fonnesbech Vogel et al., 2001). However, despite this interest, an adequate enumeration method is still lacking, which is essential to provide reliable data for both research studies and routine analysis. In most cases, *L. monocytogenes* was enumerated in cold-smoked fish using most probable number (MPN) technique (Cortesi et al., 1997; Dalgaard and Jorgensen, 1998), direct enumeration by spread plating on selective agar (Jorgensen and Huss, 1998), or semiquantitative determination (Jorgensen and Huss, 1998).

The European and International Standard method for enumeration of *Listeria* EN ISO 11290-2 (Anonymous, 1998b) is characterised by a limit of enumeration of 10–100 CFU g^{-1}, and the method does not seem to be well adapted to the examination of cold-smoked fish products and, more generally, of food products which are usually contaminated at low levels, <100 CFU g^{-1} (Anonymous, 1998a). In several countries and for specific products, the level of contamination accepted at consumption is <100 CFU g^{-1} (Anonymous, 2000).

Several attempts to enumerate *L. monocytogenes* with the MPN technique (Yu and Fung, 1993; Blysick-McKennal and Schaffner, 1994; Yu et al., 1995; Cortesi et al., 1997; Dalgaard and Jorgensen, 1998) or with methods based on molecular biology (Wang and Hong, 1999; Nogva et al., 2000) have been reported. These methods do not always provide accurate results (Wang and Hong, 1999; Nogva et al., 2000), and they may be difficult to use for routine analysis.

Another approach to improve the sensitivity of the enumeration method would be to use a concentration technique, such as membrane filtration. This method is already widely used for microbiological analysis of water and liquids. Several studies have already dealt with the improvement of food suspension filterability, in order to adapt this technique to the analysis of solid products (Sharpe et al., 1979, 2000; Peterkin and Sharpe, 1980; Peterkin et al., 1982). Detection of target strains can then be achieved through culture of the membrane on selective agar or through various rapid techniques, such as, for example, direct epifluorescence technique (DEFT) or immunoassays. The hydrophobic grid membrane filtration (HGMF) technique, including a filtration stage, has been widely tested and is already validated by AOAC International for several bacterial species in food (Entis, 1986, 1989, 1990; Entis and Lerner, 2000). Recently, membrane filtration was applied to the detection and enumeration of *L. monocytogenes* (Hale et al., 1990; Jehanno et al., 1999; Carroll et al., 2000; Entis and Lerner, 2000; Gnanou Besse and Lafarge, 2001).

We have developed an enumeration method involving membrane filtration and incubation on agar medium for *L. monocytogenes* in cold-smoked fish product. Firstly, filterability of cold-smoked salmon has been optimised with respect to filtration temperature, prefiltration, dilution rate, type of diluent, rinsing of the filter, and addition of enzymes and surfactant, and secondly, the optimal culture conditions after membrane filtration (selective media, incubation temperature, etc.) have been evaluated with artificially and naturally *L. monocytogenes* contami-

nated samples. A resuscitation step on solid nonselective agar was also investigated, but results were unsatisfactory, suggesting poor performance of the resuscitation step, or presence of limited stressed *Listeria* population in such products. Examination of cold-smoked salmon with the standard method with resuscitation both in liquid or solid media yielded similar results.

The objective of this work was to evaluate the standardised operating protocol and to compare it to the ISO 11290-2 reference method.

2. Materials and methods

2.1. Strains and samples

2.1.1. Bacterial strains and preparation of inocula

Experiments were carried out with three different strains of *L. monocytogenes* serovar 1/2a (strains 42 LM, 46 LM, and 47 LM, Afssa LERHQA collection), isolated from cold-smoked salmon and characterised in our laboratory. A stock culture was maintained at −80 °C in cryobank (AES Laboratoires, Combourg, France). Appropriate dilutions of *L. monocytogenes* cultures grown in Brain Heart Infusion (BHI) broth (CM 225, Oxoid, Dardilly, France) for 24 h as two successive cultures of 6 and 18 h at 37 °C served as inocula for tests. The final BHI culture usually contained around 1×10^9 CFU ml^{-1}. All decimal dilutions were prepared in Tryptone Salt (TS, 42 076 bioMérieux, Marcy l'Etoile, France).

2.1.2. Cold-smoked salmon samples

For experiments using artificial contamination, cold-smoked salmon were purchased locally, divided into 25-g portions and stored at 3 ± 2 °C, or if necessary at −18 °C, in Stomacher® bags with a mesh screen liner with 280-μm pore size (Bagpage Plus 400, Intersciences, Saint-Nom La Bretèche, France) which removed any large salmon particles after the peristaltic homogeniser treatment. When frozen, the samples were thawed a night before use at 3 ± 2 °C. Absence of *L. monocytogenes* in each purchased cold-smoked salmon was previously checked according to the ISO 11290-1 reference method (Anonymous, 1996). Results showed that no product used in these studies was contaminated by *Listeria*. For artificial contamination, 25-g samples were spiked with a low volume (around 1 ml) of appropriate TS dilution of *L. monocytogenes* culture prepared as described below, gently mixed, and left for 1 night at 3 ± 2 °C before use. Rates of contamination ranged from 0.3 to 30 CFU g^{-1}.

Most of the naturally contaminated samples were received from Afssa LERAC laboratory (Afssa Laboratoire d'Etudes et de Recherches pour l'Alimentation Collective), which studied the prevalence of *L. monocytogenes* in vacuum-packed cold-smoked salmon sold at retail in France. Thirty five naturally contaminated samples were collected from six different cold-smoked salmon French processors for 1 year. They were analysed at the "best before" date or a few days later. If possible, samples were analysed without having been frozen at the laboratory. When it was necessary, they were stored and frozen as previously described. Approximately 20% of the samples were kept for 1–3 weeks at −18 °C.

2.2. L. monocytogenes enumeration using filtration method

2.2.1. Filtration apparatus

Filtration was carried out using standard commercial Pyrex apparatus, and a vacuum pump with a maximum vacuum power of 630–635 mm Hg (around 80–85 kPa) (XX 55 220 50 Millipore, Saint Quentin en Yvelines, France). A 4.7-cm diameter and 0.45-μm pore-size membrane, composed of mixed cellulose esters (Millipore HAWP 047 00) and single-use filtration units with an effective 12.25-cm^2 filtration area (MicroFunnel™ Filter Funnels, 4800, Pall Gelman Sciences, Champs sur Marne, France), were used.

2.2.2. Reagents

A 10% sample of Tween 80 (P 4780, Sigma-Aldrich, Saint Quentin Fallavier, France) or trypsin 1/250 (Difco 0152-13-1) stock solutions were prepared everyday in phosphate buffer pH 7.5 containing 20 g dipotassium phosphate (Merck 10 5104) per litre of deionized water and stored at 4 °C. Unfiltered Tween or trypsin stock solutions contained no detectable level of *Listeria* and, consequently, could also be used without sterilisation.

2.2.3. Protocol

The protocol of the enumeration method is shown in Fig. 1. Samples were homogenised in TS diluent (1 in 10 dilution) for 1 min at normal speed using a Stomacher® apparatus (Stomacher 400, Seward, London, UK). Cold-smoked salmon suspensions of 5, 15, and 30 ml were immediately treated for 20–25 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 filters were laid on Aloa agar (AEB 520080, AES Laboratoires) containing (per liter) 18 g meat peptone, 6 g trypton, 10 g yeast extracts, 2 g sodium pyruvate, 2 g glucose, 1 g magnesium glycerophosphate, 0.5 g magnesium sulfate, 5 g sodium chloride, 10 g lithium chloride, 2.5 g anhydric disodium hydrogen phosphate, 0.05 g 5-bromo-4-chloro-3-indolyl-D-glucopyranoside, 0.02 g nalidixic acid sodium salt, 0.02 g ceftazidim, 76700 IU polymixin B sulfate, 0.05 g cycloheximide, 0.01 g amphotericin B, 2 g L-phosphatidylinositol (Sigma P 6636), and 12–18 g agar, pH 7.2. The plates were incubated upside down for 48 h at 37 °C. The standard ISO 11290-2 method should be conducted in parallel.

For enumeration, colonies on filters containing less than 100 colonies were counted. All *L. monocytogenes* colonies obtained on readable filters were counted. The volume analysed (corresponding to the selected filters) was recorded in Results. *L. monocytogenes* colonies were blue without a halo, due to trypsin remaining on the filter. Consequently, all typical blue colonies had to be spot inoculated on an Aloa plate and incubated for approximately 4 h at 37 °C to read the halo formation. Then, five typical *L. monocytogenes* colonies were confirmed according to the EN ISO 11290-2 method.

2.3. Evaluation of L. monocytogenes recovery using artificially contaminated samples

Cold-smoked salmon samples spiked with varying concentrations of an equal cocktail of *L. monocytogenes* strains 42 LM, 46 LM, and 47 LM were enumerated according to the EN ISO 11290-2 refer-

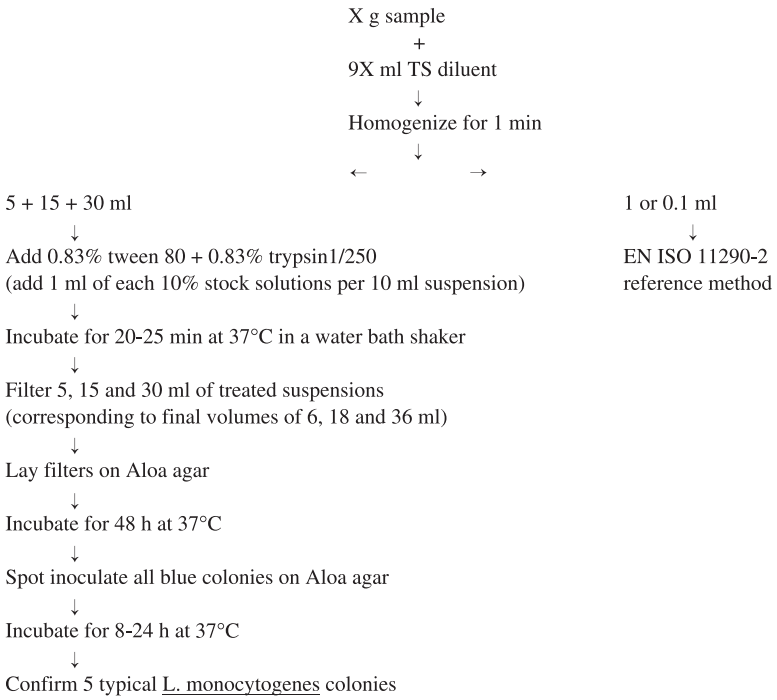

Fig. 1. Filtration method.

ence method and using the membrane filtration protocol. The standard method was modified by using TS diluent and by spreading 5 ml of the suspension on 15 plates in order to decrease the limit of enumeration and to approach the sensitivity of the filtration protocol. Previous studies with artificially and naturally contaminated samples had shown no differences in enumeration result, using TS or buffered peptone water diluent (unpublished data). For the filtration protocol, only 30-ml filtrations were carried out.

2.4. Evaluation of *L. monocytogenes* recovery using naturally contaminated cold-smoked salmon

2.4.1. First evaluation of the filtration method

Naturally contaminated cold-smoked salmon samples were enumerated according to the EN ISO 11290-2 reference method modified as described previously and using a filtration protocol. For each sample, the aerobic mesophilic microflora was enumerated using aerobic plate count Petrifilm™ (06400, 3M Laboratoires, Cergy-Pontoise, France) incubated for 72 h at 30 °C.

The aim of this study was to evaluate performances of the filtration method through the analysis of numerous naturally contaminated samples with various background microflora levels and potentially stressed *L. monocytogenes* populations compared to the reference method. This study was also useful to indicate any potential toxicity of the combined Tween and trypsin treatment towards stressed *L. monocytogenes*.

2.4.2. Comparison of the filtration method with the standard method

The same 10-fold dilution of naturally contaminated cold-smoked salmon was analysed five times in parallel both with the modified reference method and the filtration protocol. Results were converted in $\log_{10}$ for statistical analysis, which was performed according to the NF V03-110 AFNOR standard (Anonymous, 1998c): stability of precision was assessed with a Cochran test, repeatability variances were compared with a Fisher test, and relative accuracy of the methods was evaluated by comparison of means.

2.4.3. Statistical analysis

Different statistical tests, according to the AFNOR Standard NF V 03-110, were used.

The Cochran test was used to check that all sample variances did not differ statistically and that the precision was stable all over the scope of the method. The ratio C_{obs} is calculated as follows:

$$C_{\text{obs}} = \frac{s^2_{\max}(x)}{\sum_{i=1}^{p} s_i^2(x)},$$

where $s^2_{\max}(x)$ is the highest variance, and p is the number of samples. This value is compared to the critical value of Cochran test, with an error risk α of 1%.

The repeatability variances of the filtration and reference methods were compared, using a Fisher test. The ratio q is calculated as follows:

$$q = \frac{s^2_{\text{r}}(x)}{s^2_{\text{r}}(z)},$$

where $s^2_{\text{r}}(x)$ is the largest repeatability variance of the method (either filtration or reference), and $s^2_{\text{r}}(z)$ is the repeatability variance of the other method.

This value is compared to the critical value of the Fisher variable $F(N(x)-p,\ N(z)-p,\ 0.99)$, with a confidence level of 99%, $N(x)-p$ and $N(z)-p$ degrees of freedom.

To assess the relative accuracy of the filtration method against the reference method, the means were compared. The w ratio is calculated as follows:

$$w = \frac{|\bar{d}|}{s_d},$$

where $\bar{d} = (\sum_i d_i)/p$ (d_i being the difference between the means of the filtration and the reference method for a given sample i), and $s(d) = \sqrt{(\sum_i (d_i - \bar{d})^2)/(p-1)}$ calculated for each sample. The ratio w is compared to the critical value 3.0, with an error risk α of 1%.

3. Results

3.1. Recovery of *L. monocytogenes* from artificially contaminated cold-smoked salmon

A close correlation between the known inoculum levels of *L. monocytogenes* and the levels enumerated using the new method was observed (Fig. 2). Regres-

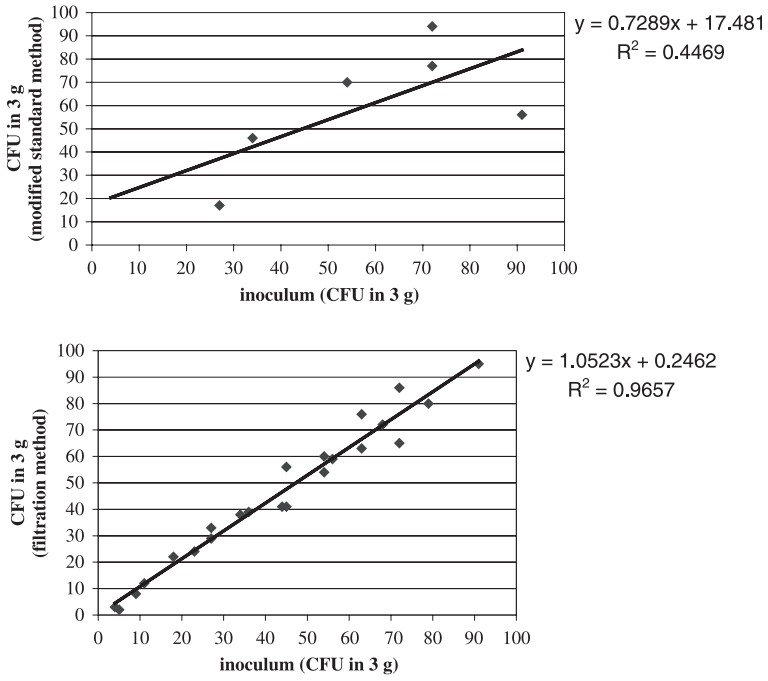

Fig. 2. Linearity study using artificially contaminated cold-smoked salmon: comparison of the enumeration results obtained by the modified standard method or the filtration method (30-ml filtrations), and the inoculum size.

sion analysis showed that there was a linear relationship between results obtained using filtration and inoculum concentration up to around 100 CFU g^{-1} (equation of the linear relationship: CFU g^{-1} filtered = 1.0523 CFU g^{-1} inoculum concentration + 0.2462, $r^2 = 0.9657$). Fig. 2 shows that the test method yielded more accurate results (nearer to the true value) than the modified standard method, whose linearity was poor (equation of the linear relationship: CFU g^{-1} = 0.7289 CFU g^{-1} inoculum concentration + 17.481, $r^2 = 0.4469$).

3.2. Recovery of L. monocytogenes from naturally contaminated cold-smoked salmon

Aerobic plates counts ranged from 10^4 to 10^8–10^9 CFU g^{-1} (results not shown). With respect to the presence of background microflora on the membranes, results were very similar within samples belonging to a same lot number. In most cases, no or few background microflora colonies were observed on the filters; however, in a limited number of cold-smoked salmon lots (4:26), unreadable results were obtained for the largest volumes examined because of overgrowth. However, most cold-smoked salmon at retail level is likely to contain lower levels of aerobic microflora than our samples, which were examined at the end of their shelf-life.

During this study, 42 examinations of naturally contaminated cold-smoked salmon (35 different samples) have been performed both with the modified standard enumeration method and the filtration meth-

Table 1
Comparison of enumeration results obtained by the modified standard method and filtration method with naturally contaminated samples: results of five counts obtained by each method on the same sample

Method	Sample code			
	1	2	3	4
Standard method (CFU g^{-1})	10, 20, 8, 34, 10	18, 68, 20, 42, 28	4, 4, 6, 6, 2	2, 2, 2, 1, 1
Filtration method (CFU g^{-1})	18, 16, 16, 17, 17	48, 45, 45, 43, 46	8, 8, 9, 7, 10	2, 2, 1, 1, 1

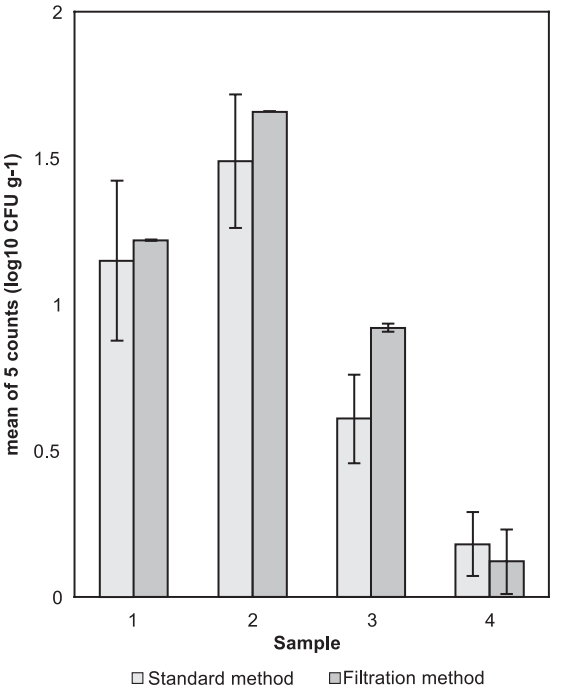

Fig. 3. Comparison of enumeration results obtained by the modified standard method and filtration method with naturally contaminated samples: mean of five counts obtained by each method on the same sample and standard deviation.

od. *L. monocytogenes* was recovered by both methods on 64.3% of occasions. On one occasion (2.4%), *L. monocytogenes* was recovered only by the modified standard method, and on 14 occasions (33.3%) *L. monocytogenes* was recovered only by the filtration method. When *L. monocytogenes* contamination levels were sufficient to compare methods, filtration method and standard method gave similar results.

3.3. Comparison of the filtration method with the standard method

Results are shown in Table 1 and Fig. 3. According to the statistical analysis of $\log_{10}$ data in Table 1, the precision was stable for all samples tested and for the reference method (Cochran test, $C_{obs} < C_{crit}$, $p < 0.01$) but not for the filtration method, due to the very low variances for three samples (0.000, 0.000, 0.003), the fourth having a relatively higher variance (0.027). The precision of filtration method ($s_r^2 = 0.006$) was significantly better (Fisher test, $p < 0.01$) than the precision of the standard method ($s_r^2 = 0.038$). Filtration method yielded accurate results when compared to the standard method (at $\alpha = 1\%$, where $w = 0.8$, $w_{crit} = 3.0$).

3.4. L. monocytogenes contamination levels of cold-smoked salmon

Of 35 different samples (some of them belonging to the same lot), 91.4% contained < 100 CFU g^{-1}, and 88.2% contained < 50 CFU g^{-1} (Table 2). The percentage of samples containing less than 0.2 CFU g^{-1} remains unknown: in some cases, the enumeration method did not allow to recover *L. monocytogenes* from samples which have not been tested with the detection protocol, but belonged to the same lot number than positive samples.

4. Discussion

The method that we have developed is relatively rapid (same duration as the standard method), easy to implement, and cheap; to achieve the same sensitivity (analysis of 50 ml of 1:10 fish suspension) without filtration, up to 150 petri dishes of selective agar would be necessary per sample (spreading 1 ml on three plates). Our protocol permits filtration of 5 g cold-smoked salmon diluted to 1:10, thereby decreasing the enumeration limit (10–100 CFU g^{-1}) of the reference method to 0.2 CFU g^{-1}. Moreover, it improves the precision, especially at low levels of contamination. The sensitivity of the method could possibly be increased still further if more filtrations were performed in parallel or by using filters of wider diameter.

The filtration method involves the use of a specific apparatus and is more laborious than the reference

Table 2
L. monocytogenes contamination in naturally contaminated cold-smoked salmon samples

Contamination level (CFU g^{-1})	0.2–1	1–25	25–50	50–75	75–100	>100	>1000
Samples number	11 (31.4%)	13 (37.1%)	6 (17.1%)	0 (0%)	2 (5.7%)	3 (8.6%)	0 (0%)

method. For one analysis, all filtrations usually take less than 5 min. However, some salmon samples may have a composition (high fat content) that could extend the filtration time. In those cases, gently mixing suspensions with a sterile plastic loop during filtration or transferring the cold-smoked salmon suspension into another Stomacher® bag with a mesh screen liner may be useful to limit filter silting and to accelerate filtration. The use of the same type of materials than the ones described here (Stomacher® apparatus, etc.) is also important to avoid filtration difficulties.

The method developed is also based on the use of Aloa agar. Special attention must be paid to some bacteria producing bluish colonies with halo (some *Bacillus cereus* strains and *Listeria ivanovii*) or bluish colonies (nonpathogenic *Listeria* and some enterococci and bacilli), which could induce false-positive presumptive results or, in the case of the filtration method, decrease the detection ratio (Vlaemynck et al., 2000). However, in our study, no or few background microflora colonies were observed on the filters, and most bluish colonies were *Listeria* colonies. The good performances of this media have been demonstrated in terms of productivity ratio, selectivity and detection ratio (Vlaemynck et al., 2000), and it has been adopted in 2002 by the International Organization for Standardization as the official media for *L. monocytogenes* standard detection and enumeration methods.

The protocol that we have developed improves the specificity and the reproducibility and repeatability of the method, as compared to the current reference method, since the selected isolation medium, Aloa, permits differentiation of the different *Listeria* species. Of the naturally contaminated cold-smoked salmon of various origin examined in this study, only one shown to be contaminated by *L. monocytogenes* contained other *Listeria* species. It was the only one examined several days before the "best before" date. The lack of diversity of *Listeria* species in our samples could be explained by their long storage duration, which may have allowed a competition between *Listeria* strains (Kalmokoff et al., 1999), and thus, one species or one strain to dominate. Further studies are necessary to assess this phenomenon.

Low contamination levels found in this study at the "best before" date confirm previous studies indicating generally very low contamination levels of cold-smoked salmon (Anonymous, 1998a, 1999). These results highlight the need for a sensitive enumeration method, such as a filtration method, for examining this type of products.

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