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Comparative evaluation of direct plating and most probable number for enumeration of low levels of *Listeria monocytogenes* in naturally contaminated ice cream products

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ABSTRACT

A precise and accurate method for enumeration of low level of *Listeria monocytogenes* in foods is critical to a variety of studies. In this study, paired comparison of most probable number (MPN) and direct plating enumeration of *L. monocytogenes* was conducted on a total of 1730 outbreak-associated ice cream samples that were naturally contaminated with low level of *L. monocytogenes*. MPN was performed on all 1730 samples. Direct plating was performed on all samples using the RAPID'*L.mono* (RLM) agar (1600 samples) and agar *Listeria* Ottaviani and Agosti (ALOA; 130 samples). Probabilistic analysis with Bayesian inference model was used to compare paired direct plating and MPN estimates of *L. monocytogenes* in ice cream samples because assumptions implicit in ordinary least squares (OLS) linear regression analyses were not met for such a comparison. The probabilistic analysis revealed good agreement between the MPN and direct plating estimates, and this agreement showed that the MPN schemes and direct plating schemes using ALOA or RLM evaluated in the present study were suitable for enumerating low levels of *L. monocytogenes* in these ice cream samples. The statistical analysis further revealed that OLS linear regression analyses of direct plating and MPN data did introduce bias that incorrectly characterized systematic differences between estimates from the two methods.

1. Introduction

Listeria monocytogenes has been involved in numerous foodborne outbreaks linked to a variety of contaminated food commodities (Cartwright et al., 2013). In the United States (U.S.), several major listeriosis outbreaks linked to stone fruit (Chen et al., 2016b; Jackson et al., 2015), caramel apple (CDC, 2015b), ice cream (CDC, 2015a), cantaloupe (McCollum et al., 2013) and cheese (CDC, 2014) have been reported in recent years. In the European Union (EU), the number of reported cases of human listeriosis increased substantially during the period of 2008–2012 (EFSA, 2014). Reliable quantitation of *L. monocytogenes* in artificially inoculated or naturally contaminated foods is critical to obtain highly reliable research data to address various issues related to predictive microbiology, epidemiology, risk assessment, regulatory testing, etc. (Auvolat and Besse, 2016). Most probable number (MPN) and direct plating are the primary methods for the enumeration of foodborne bacterial pathogens. There are advantages and disadvantages for each method. The sensitivity of direct plating is limited to the spreadable volume on each plate. Colony counting can be challenging if high levels of interfering background flora grow on the agar plates (Buchanan et al., 1989). The accuracy can be compromised if some of the viable, but stressed cells fail to develop colonies on selective agars (Lavieri et al., 2014). In contrast, MPN can detect much lower levels of bacteria and the enrichment schemes employed by MPN can help reduce the level of background (Capita and Alonso-Calleja, 2003). However, MPN is more labor intensive and time consuming, especially when narrow confidence intervals are desired, although the use of 96-well plates (Macarisin et al., 2013) allows the increase of the number of tubes without significant amount of additional labor and time. MPN performance depends on the ability of its enrichment scheme to recover stressed cells and inhibit competing flora. In some occasions, the

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presence of non-pathogenic *Listeria* spp. can outgrow low levels of *L. monocytogenes* during enrichment, which resulted in underestimation by MPN (Besse et al., 2010). MPN could also underestimate the true levels when bacterial cell clumping occurs in food commodities (Auvolat and Besse, 2016; Jongenburger et al., 2011).

The standard method for the enumeration of *L. monocytogenes* described in EN ISO 11290–2 had a theoretical lower limit of detection (LOD) of 10–100 CFU/g or ml when analyzing 1:10 diluted foods (Auvolat and Besse, 2016). The uncertainty of measurement of this method is high when enumerating *L. monocytogenes* near the lower LOD (Auvolat and Besse, 2016; Baudouin et al., 2010; ISO, 2013; Loncarevic et al., 2008). The *L. monocytogenes* chapter of *Bacteriological Analytical Manual* (BAM) by U.S. Food and Drug Administration (FDA) recommends a combination of MPN and direct plating for *L. monocytogenes* enumeration in foods; and for MPN, FDA allows the increase in the number of dilution tubes in situations that would require narrow confidence intervals (Hitchins et al., 2016). Lack of precision in the quantitative assessment of low levels of bacterial cells could compromise the reliability of subsequent statistical analysis (Duffy et al., 1994). Therefore, in order to obtain precise enumeration data for low levels of bacteria, the number of MPN tubes or agar plates and the number of sample replicates need to be increased.

Due to the above mentioned technical limitations associated with enumerating low levels of bacteria, the studies on the growth kinetics of *L. monocytogenes* in artificially inoculated food commodities often used 10^2 to 10^4 CFU/g or higher levels of inoculum (Huang et al., 2015; Luo et al., 2015; Panagou and Nychas, 2008; Schvartzman et al., 2014; Xanthiakos et al., 2006). However, these inoculum levels do not reflect the low levels of *L. monocytogenes* often times sparsely distributed in food (EFSA, 2012). Several studies have found that the lag phase durations during recovery and growth of foodborne pathogens were longer when the initial levels of inoculum were lower (Augustin et al., 2000; Baranyi, 1998; Gay et al., 1996). For example, Chen et al. (2016a) demonstrated that the average lag phase duration associated with *L. monocytogenes* growth in milkshakes containing ≤3 CFU/g of *L. monocytogenes* was longer ($P < 0.01$) than that in milkshakes containing >3 CFU/g of cells. Currently, European Community Regulation 2073/2005 on microbiological criteria for food stuffs establishes a quantitative threshold of 100 CFU/g for *L. monocytogenes* in certain categories of ready-to-eat food products and manufacturers must demonstrate that *L. monocytogenes* does not exceed 100 CFU/g in their product throughout the shelf-life (European Commission, 2005). As a result, challenge tests, recovery and growth studies under realistic conditions are preferred and low levels of initial experimental contamination are desired (Auvolat and Besse, 2016). This requires the development and validation of the methods for precise quantitation of low levels of *L. monocytogenes* in foods.

The accuracy of a direct plating scheme depends on the agar's selectivity for target bacteria as well as its efficiency in recovering stressed cells. Jantzen et al. (2006) demonstrated that *Listeria* Ottaviani and Agosti (ALOA) agar recovered most of the injured *L. monocytogenes* in artificially inoculated ground chicken exposed to high pressure treatment, because samples initially containing injured *L. monocytogenes* yielded similar plate counts on ALOA before and after a resuscitation step. Many studies evaluated the efficiency of an agar to recover stressed cells by comparing direct plating counts on selective agars and non-selective agars of pure culture of *L. monocytogenes* or *L. monocytogenes* artificially inoculated in foods, exposed or not exposed to sublethal treatment (Back et al., 2012; Lachica, 1990; Lavieri et al., 2014; Lin et al., 2006; Yan et al., 2006). However, this approach may not work well with foods containing competing flora that can interfere with plate counting on non-selective agars (Lin et al., 2006). Comparison of selective agars for direct plating enumeration of *L. monocytogenes* was performed with artificially inoculated foods in a few studies, but direct plating using selective agars was not compared to MPN or direct plating using non-selective agars, and thus, the efficiency of selective agars in recovering stressed cells was not fully evaluated (Beumer and Hazeleger, 2007; Loncarevic et al., 2008). Thus, quantitative comparison between MPN and direct plating using naturally contaminated samples that contain background flora and target bacteria in realistic physiological status could provide an excellent evaluation of the selective agar for the purpose of direct plating enumeration.

Comparative evaluation of MPN and direct plating has been performed for *L. monocytogenes*. Several factors, such as the levels of contamination, the presence of background flora and the physiological status of *Listeria* cells, could affect the performance of enumeration methods (Jasson et al., 2010). However, comparison using paired data of MPN and direct plating obtained from sufficient number of samples naturally contaminated with low levels (e.g., ≤100 CFU/g) of *L. monocytogenes* has rarely been reported. Several studies analyzed paired data from food samples that were artificially inoculated with high levels of *L. monocytogenes* (e.g., >100 log CFU/g) (Martin et al., 2004; Yu and Fung, 1993) and revealed a general agreement between MPN and direct plating. Other studies investigated naturally contaminated samples; however, either the analyses were not focused on low levels of *L. monocytogenes*; the direct plating schemes could not enumerate very low levels of cells; or direct plating agars did not allow clear distinction of *L. monocytogenes* from background flora, and thus not sufficient number of paired data of low levels of bacteria were obtained (Buchanan et al., 1989; Capita and Alonso-Calleja, 2003; Martin et al., 2004; Yu and Fung, 1993). Many comparative evaluations of MPN and direct plating for other bacteria had similar study designs, which combined data points from a wide range of cell levels (e.g., 2 log CFU/g to 7 log CFU/g) (Berry and Wells, 2008; Chenu et al., 2013; Gooch et al., 2001; Line et al., 2001; Line et al., 2011; Stephens et al., 2007). MPN and direct plating data of low levels of *Cronobacter* in naturally contaminated infant formula were reported (Jongenburger et al., 2011). We found that MPN and direct plating in that report produced comparable results because for samples determined to be negative by direct plating (below LOD), MPN values were also less than LOD of direct plating.

In addition to the inefficient microbiological methods employed and a small number of paired data points collected for low levels of bacteria, statistical comparison of MPN and direct plating estimates of low level of contamination may be inaccurate if the statistical method is not appropriately chosen (GroneWold and Wolpert, 2008). Ordinary least squares (OLS) linear regression has certain underlying assumptions. The independent variable x should be fixed values (i.e. non-random) (Matthews, 2005; Weisberg, 2005), and thus, OLS is suitable to study the correlation between MPN/direct plating estimates and fixed levels of bacteria artificially inoculated in food samples (Berry and Wells, 2008); it could introduce bias for studying correlation between MPN and direct plating estimates for naturally contaminated samples because both estimates are known with uncertainty. Linear regression and comparison of means using analysis of variance have been commonly used and showed a general agreement between MPN and direct plating when analyzing a wide range of cell levels (e.g., 2 log CFU/g to 7 log CFU/g) (Berry and Wells, 2008; Chenu et al., 2013; Gooch et al., 2001; Line et al., 2001; Line et al., 2011; Martin et al., 2004; Stephens et al., 2007; Yu and Fung, 1993). However, poor correlation between MPN and direct plating was reported for a set of naturally contaminated samples (Berry and Wells, 2008), and poor agreement between MPN and direct plating was observed when data points of only low levels of (≤100 CFU/g) bacteria were analyzed (Stephens et al., 2007). Direct plating and MPN estimates are both discrete, rather than continuous, values, which impair their use as a dependent variable in an OLS method (GroneWold and Wolpert, 2008). Additionally, the hypothesis of homoscedasticity (i.e. homogeneity of variance around the regression line) for MPN and direct plating would be invalid on the arithmetic scale. A log transformation is typically used to stabilize the variance (Cochran, 1950; Dalgaard et al., 1994). However, the presence of a large number of zeroes from the direct plating estimates (leading to non-finite log)

must then be resolved. Removing these zeroes from the analysis (non-random censorship) could bias the regression results because it would preferentially remove results that were low in direct plating. Gronewold and Wolpert (2008) thus proposed a probabilistic method for the improved modeling of the correlation between MPN and plating, and demonstrated that the often large observed differences between MPN and direct plating estimates for the same samples were within the ranges predicted by the probabilistic model.

In 2015, a listeriosis outbreak in the United States was linked to the consumption of milkshakes prepared from contaminated ice cream scoops manufactured on a production line (CDC, 2015a). We subsequently obtained individually packaged ice cream portions (scoops) produced on that production line between November 2014 and March 2015. These naturally contaminated samples provided an opportunity to 1) conduct a rigorous comparison of MPN and direct plating data from samples naturally contaminated with low levels of *L. monocytogenes*, and 2) evaluate appropriate statistical methods for comparing paired enumeration data collected from these samples.

2. Materials and methods

2.1. Ice cream samples and microbiological methods

A total of 1730 ice cream samples from seven lots were analyzed. These ice cream products were manufactured on the production line implicated as the source of a recent listeriosis outbreak (CDC, 2015a). The seven lots were produced in November 2014, December 2014, January 2015 and March 2015. Each lot represented a day of production. Frozen ice cream samples were allowed to melt at room temperature and thoroughly homogenized; and each individual sample was enumerated by both MPN and direct plating, resulting in 1730 paired data points. Without knowing the range of *L. monocytogenes* levels in these samples during the initial enumeration, we tried four different MPN schemes (I, 3 tubes × 10 g ice cream, 3 × 1 g, 3 × 0.1 g; II, 3 × 10 g, 16 × 1 g, 3 × 0.1 g; III, 3 × 10 g, 5 × 1 g, 5 × 0.1 g; and IV, 3 × 10 g, 5 × 1 g, 5 × 0.1 g, 5 × 0.01 g) for 190 samples. This led to the development of the final MPN scheme (3 × 10 g, 5 × 1 g, 8 × 0.1 g, 8 × 0.01 g), designated as the 3-5-8-8 scheme, with a LOD of 0.03 MPN/g. This scheme was best suited for the levels of *L. monocytogenes* in these samples and provided a balance between labor intensity and confidence interval. This 3-5-8-8 scheme was used for the remaining 1540 samples. MPN sample dilution, enrichment and calculation were performed according to Chapter 10 of FDA BAM (Hitchins et al., 2016) and confidence intervals were derived from the method from Fisher (1921) as reported by Hurley and Roscoe (1983). An additional portion of each sample was directly plated on ALOA (Cat. No. AEB150072, bioMerieux-USA, St Louis, MO) or Rapid' *L. mono* (RLM) (Cat. No. 3564293, Bio-Rad Laboratories, Hercules, CA). Preliminary testing of some ice cream samples on ALOA agar revealed blue background flora colonies without halo, and thus, accurate counting required careful visual examination for the presence of a halo, which was difficult due to the presence of the fat content in these samples. Therefore, RLM agar, which does not rely on white halo for the identification of *L. monocytogenes*, was chosen for direct plating enumeration of the majority of the samples. During the initial testing of 50 samples, ice cream (density, 0.9 g/ml) samples were first diluted with equal weight of Bufferfield's Phosphate Buffer (BPB), and 200 μl of diluted ice cream was plated onto ten RLM plates (0.95 g of ice cream total, LOD, 1.1 CFU/g) for 10 samples and onto sixteen RLM plates (1.52 g of ice cream total, LOD, 0.66 CFU/g) for 40 samples. Recognizing low levels of contamination, undiluted ice cream was plated directly onto RLM for the remaining samples, as follows: 400 μl of ice cream was plated onto four RLM plates (1.44 g total) for 185 samples (LOD, 0.7 CFU/g); 200 μl was plated onto five RLM plates (0.9 g total) for 85 samples (LOD, 1.1 CFU/g), and 400 μl was plated onto two RLM plates (0.72 g total) for 1280 samples (LOD, 1.4 CFU/g). We also enumerated 130 samples using ALOA agar due to a temporary manufacturer backorder of RLM agar in the course of the study. For this, 400 μl of undiluted ice cream was plated onto two ALOA agar plates (0.72 g total, LOD, 1.4 CFU/g). The plating was performed by easySpiral® automatic spiral plater (Interscience, Inc., France) set to constant volume plating. Plates were left to dry before incubation. For direct plating, confidence intervals were derived from the exact 95% confidence interval for a Poisson distribution. A subset of representative colonies from MPN and direct plating were confirmed according to Chapter 10 of FDA BAM (Hitchins et al., 2016).

2.2. Statistical analytical methods

As stated in the introduction, OLS methods could introduce bias when comparing MPN and direct plating data because the underlying assumptions regarding value certainty and continuity are not met. In order to illustrate this bias, we applied an OLS method to the data and compared it with an appropriate probabilistic method. A linear regression of the logarithm of the MPN results (M, in MPN/g) and the logarithm of the direct plating results (C, in CFU/g) was tested, i.e. $\log_{10}(C) = \alpha + \beta \log_{10}(M) + \varepsilon$ with ε as the error variable (Weisberg, 2005).

We then adapted a probabilistic method from GroneWold and Wolpert (2008) to evaluate the agreement between direct plating and MPN. The direct plating process could be considered as a random sampling from a Poisson($v_j \times c_{1j}$) distribution where c_{1j} was the (latent) concentration of bacteria in sample j and v_j was the volume of product plated. The dilution assay process, from which the MPN was evaluated, could be considered as a random sampling from a binomial(n_{ij}, $1 - \exp(-v_{ij}\, c_{2j})$) distribution, where n_{ij} and v_{ij} were the number of tubes and the volume, respectively, at each dilution i for sample j, and c_{2j} was the (latent) concentration of bacteria in sample j. The direct plating and the MPN results would be considered in agreement if the concentrations evaluated through direct plating c_1 and the concentrations evaluated through MPN c_2 were in agreement. Using this theoretical probabilistic model, we compared the direct plating and MPN results by evaluating the probability to observe the number of colonies in direct plating, given the observed MPN outcome. If this probability was lower than 0.025 (overall $\alpha = 5\%$ for this bilateral test), the result of direct plating was deemed higher than expected (when the direct plating result was higher than the MPN) or lower than expected (when the direct plating result was lower than the MPN). We finally developed a Bayesian model (model #1) to evaluate the relationship

$$\log_{10}(c_1) = a_0 \times \log_{10}(c_2) + b_0$$

between c_1 and c_2 where a_0 and b_0 are parameters. In this framework, we also tested if the type of direct plating method (ALOA or RLM) was significant or not, by comparing model #1 to a model #2

$$\log_{10}(c_1) = (a_0 + a_1 \times ALOA) \times \log_{10}(c_2) + (b_0 + b_1 \times ALOA)$$

where *ALOA* is an indicator variable with value 1 if the results was obtained using an ALOA method and 0 if it was a RLM method and a_1 and b_1 are parameters. See the appendix for additional details on the methods.

3. Results

Out of the 1730 samples analyzed by both the MPN and direct plating, 301 yielded no colonies by direct plating (below LOD) and, among those, nine were also negative in any MPN tubes. Seven samples were at the maximum of the MPN schemes (all MPN tubes positive); one of these samples was enumerated by the 3-5-8-8 scheme (the six others being observed with the Schemes I to IV). There were no samples that were positive by direct plating and negative by MPN.

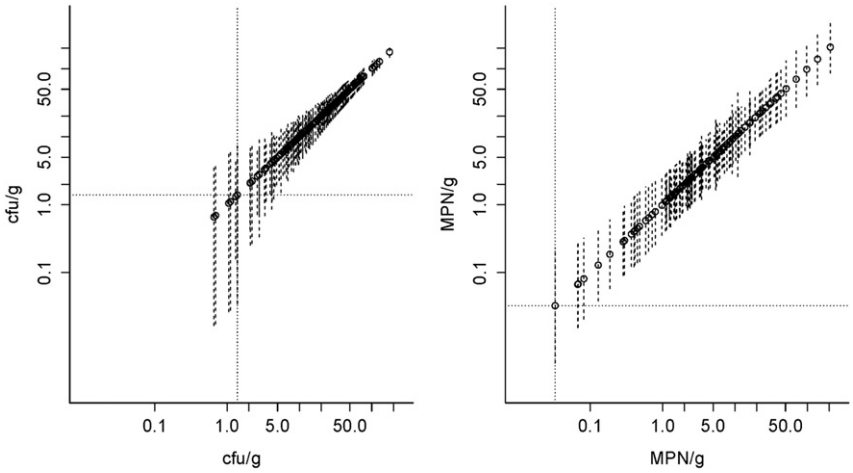

Fig. 1. Outcomes (open circle) and confidence intervals (dashed vertical bars) for the direct plating (left) and the MPN (right) estimates of *L. monocytogenes* levels in ice cream samples obtained in this study. Dashed line represents the limit of detection (LOD) for the schemes as applied for the majority of samples, 0.72 g of products plated in direct plating (LOD, 1.4 CFU/g) and the 3-5-8-8 MPN scheme (3 × 10 g, 5 × 1 g, 8 × 0.1 g, 8 × 0.01 g) (LOD, 0.03 MPN/g). Values below these limits were obtained using other schemes.

Fig. 1 illustrates the range and the confidence intervals of the various direct plating and MPN outcomes obtained in this study. This graph shows the LOD (0.03 MPN/g for the 3-5-8-8 MPN scheme vs. 1.4 CFU/g for directly plating 0.72 g of the sample), the discrete nature of the values, the higher precision (narrower confidence intervals) for MPN at low levels compared to direct plating, and the lower precision (wider confidence intervals) for MPN at high levels compared to direct plating. The scatter plot of MPN results vs. direct plating results (Fig. 2) illustrates the level of agreement between the estimates provided by these two methods and the relatively high variability of results around the $x = y$ line (MPN and direct plating values being equal). An OLS linear regression of the logarithm of the MPN results (M, in MPN/g) and the logarithm of the direct plating results (C, in CFU/g) provided an equation of $\log_{10}(C) = 0.70\ (\pm 0.016) \times \log_{10}(M) + 0.27\ (\pm 0.014)$. The intercept and slope derived from this analysis were not significantly different when comparing MPN and ALOA direct plating results and when comparing MPN and RLM direct plating results.

With the exception of the seven MPN outcomes for which all tubes were positive, we estimated the likelihood to observe the direct plating outcome (number of colony forming unit from v grams of ice cream, including zero) given the MPN outcome (number of positive tubes, from n_i tube of v_i grams) obtained for the same sample. Out of the 1723 samples for which this likelihood could be estimated, 14 (0.8%) samples showed a direct plating result that was lower than expected ($p < 0.025$), and 54 (3.1%) showed a direct plating result that was higher than expected ($p < 0.025$). The overall proportion of unexpected direct plating results was thus 3.9%, which is lower than the 5% that would be expected to be found by chance. These 68 data pairs are listed in Supplemental Table 1. Out of the 54 samples in which the direct plating result was higher than expected, 49 of them had *L. monocytogenes* < 10 MPN/g, and 39 of them had *L. monocytogenes* < 20 CFU/g. The 14 samples in which the direct plating result was lower than expected had *L. monocytogenes* between 11 MPN/g and 98.2 MPN/g (Supplemental Table 1). Fig. 3 also illustrates those results. Two out of 129 (1.6%) of

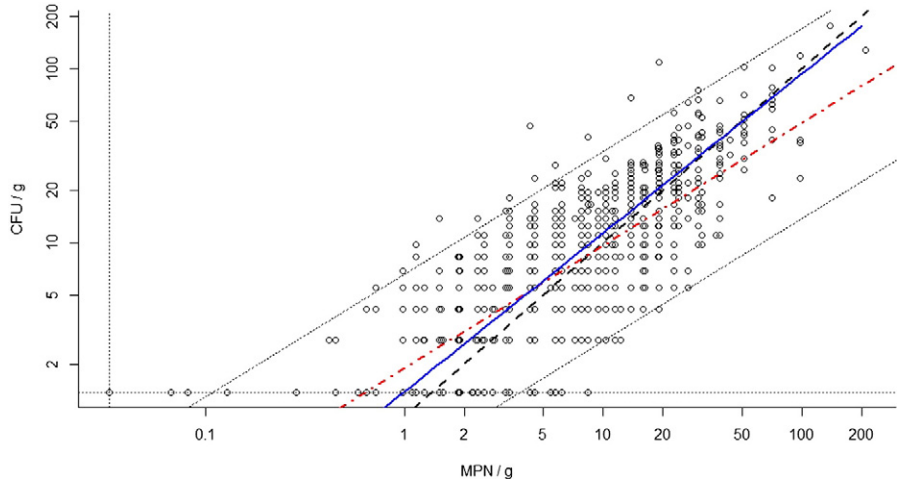

Fig. 2. Correlation of direct plating (C) vs. MPN (M) estimates of *L. monocytogenes* levels in ice cream samples analyzed in this study. The dashed line is the $x = y$ (M and C being equal) line. The dot-dashed, red line is the result from the ordinary least squares regression analysis (OLS), i.e. $\log_{10}(C) = 0.70\ (\pm 0.016) \times \log_{10}(M) + 0.27\ (\pm 0.014)$. The dotted lines are the 95% prediction interval from the regression. The plain, blue line is the results of the Bayesian model analysis, i.e. $\log_{10}(C) = 0.91\ (\pm 0.014) \times \log_{10}(M) + 0.15\ (\pm 0.015)$. The horizontal dashed line represents the limit of detection (LOD) for the direct plating method (0.72 g of products plated, LOD, 1.4 CFU/g) applied for the majority of samples; the vertical dashed line represents the LOD for the 3-5-8-8 MPN scheme (3 × 10 g, 5 × 1 g, 8 × 0.1 g, 8 × 0.01 g, LOD, 0.03 MPN/g) applied for the majority of samples.

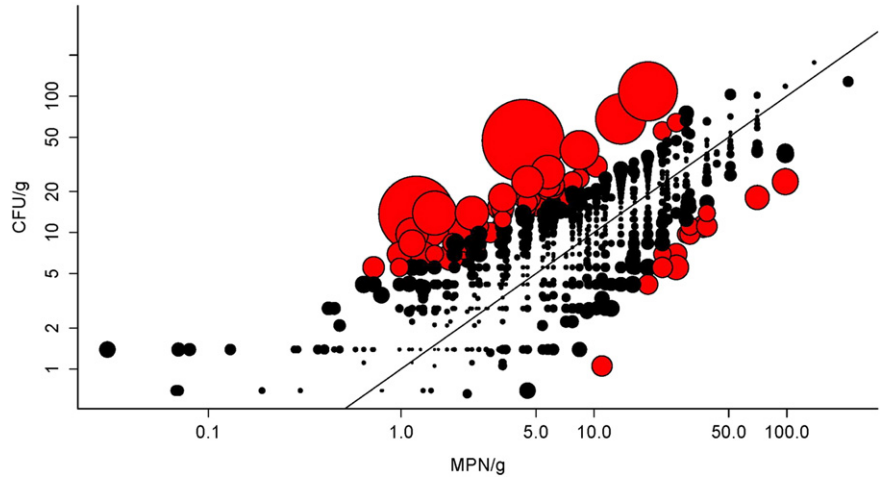

Fig. 3. The probabilistic analysis of the agreement of direct plating vs. MPN estimates of *L. monocytogenes* levels in ice cream samples analyzed in this study. The radii of the circles are proportional to the inverse of the log of the probability of occurrence of the direct plating given the MPN outcome (the larger the circle, the lower the probability). Red circles indicate significant departure (<0.025). The line is the $x = y$ line (MPN and direct plating values being equal).

ALOA direct plating and 66 out of 1594 RLM direct plating results (4.1%) were unexpected; these proportions were not statistically different (Fisher-exact test, $p = 0.23$), suggesting that the accuracy of ALOA direct plating and RLM direct plating estimates were comparable for these samples. The Bayesian inference model results indicated that the impact of the direct plating agar (ALOA vs. RLM) was not significant (the credible intervals for parameters a_0 and b_0 include 0 in model #2, see appendix for details and Table 1). The final model was thus model #1 with $\log_{10}(C) = 0.91\ (\pm 0.014) \times \log_{10}(M) + 0.15\ (\pm 0.015)$. This result indicated that the direct plating method estimated a higher concentration than MPN, when the *L. monocytogenes* concentration in the sample was low (lower than 46 CFU/g or MPN/g) and a lower value than the MPN method when the concentration was higher (>47 CFU/g or MPN/g), at least in the tested domain (0 to 208 MPN/g).

4. Discussion

In the present study, paired data of MPN and direct plating from a large number of naturally contaminated ice cream samples involved in a listeriosis outbreak were compared. This study is novel for the following reasons: 1) we were able to obtain a relatively large number of paired data points from samples containing low levels of *L. monocytogenes*, which improved the reliability and power of statistical analysis; 2) the samples were naturally contaminated with background flora and low levels of *L. monocytogenes* that suffered certain degree of injury (as discussed below), which were needed for proper evaluation of agars for direct plating enumeration purpose; 3) a probabilistic model was used to evaluate the relationship between MPN and the direct plating, which did not introduce bias, unlike OLS linear regression.

Bacterial cell clustering or clumping in food commodities could result in an underestimate of true levels by MPN (Auvolat and Besse, 2016). Jongenburger et al. (2011) investigated powdered infant formula contaminated with low levels of *Cronobacter* spp. and found very heterogeneous contamination levels, with presence of cell clusters; and this resulted in MPN estimates 2 log units lower than the direct plating estimates for one bag of samples. In the present study, no evidence of cell clustering was observed. Either there were no cell clusters in these samples, or the clusters were disrupted during the sample homogenization. The ice cream samples were much easier to homogenize than solid foods, e.g., infant formula, and this greatly increased the accuracy of enumeration.

In this study, a probabilistic method was used to compare the estimates provided by MPN and direct plating enumeration. When we estimated the likelihood to observe the direct plating outcome given the MPN outcome that was obtained for the same sample, we found that only 0.8% samples showed a direct plating result that was lower than expected, and only 3.1% showed a direct plating result that was higher than expected. The relative likelihood of each reported MPN outcome was evaluated on the basis of the rarity index as defined by Blodgett (2010). None of the unexpected results were associated with MPN patterns with a low rarity index (<0.01), suggesting that the MPN dilution procedure was adequate. Very low likelihood (probability < 1.0E-03) was observed in 10 samples, all yielding higher direct plating estimates than MPN estimates, which could then be explained by possible insufficient homogenization prior to analyses. Overall, this low proportion of

Table 1
Results from the Bayesian models.

	Model #1[a]				Model #2[b]			
	Mean	Standard deviation	Quantile 2.5%	Quantile 97.5%	Mean	Standard deviation	Quantile 2.5%	Quantile 97.5%
a_0	0.91	0.014	0.89	0.94	0.91	0.016	0.88	0.95
a_1					1.82×10^{-5}	0.047	−0.091	0.094
b_0	0.15	0.015	0.11	0.17	0.14	0.016	0.11	0.18
b_1					−0.0093	0.050	−0.11	0.089

[a] $\text{Log}_{10}(c_1) = a_0 \times \log_{10}(c_2) + b_0$ where c_1 is the concentration of bacteria measured by the direct plating method and c_2 is the concentration of bacteria measured by the MPN method.
[b] $\text{Log}_{10}(c_1) = (a_0 + a_1 \times ALOA) \times \log_{10}(c_2) + (b_0 + b_1 \times ALOA)$ where *ALOA* is an indicator variable with value 1 if the result was obtained using agar *Listeria* Ottaviani and Agosti (ALOA) and 0 if it was obtained using RAPID'*L.mono* (RLM) agar.

discrepancies (<5%) suggests a high degree of agreement between the MPN outcome and the direct plating outcome. Contrary to the OLS method, this probabilistic method is able to take into account most samples, including the ones with a direct plating result of 0 or no positive tube in any MPN dilutions. Additionally, this method is an exact test that does not use any other assumption than those used to calculate the direct plating result and the MPN result (i.e. a Poisson distribution of the bacteria at different dilutions and a certain growth of each bacterium in the culture media).

The Bayesian model describing the relationship between the direct plating and MPN estimates indicated also that the two methods were in good correlation. This model considered all samples. While not providing identical results, the analysis indicated only small statistical differences in the estimates from the two methods; e.g., slope of linear inference model is 0.91 (±0.014) as compared with 1.00. In the concentration range for which both methods can provide estimates, the Bayesian model predicted that, on average, the direct plating result would be 1.4 CFU/g for a MPN result of 1.0 MPN/g and, on average, the direct plating result would be 175 CFU/g for a MPN result of 200 MPN/g. Such small differences could only be identified in a study with such a large number of data points that had a very high statistical power of the analysis, but may not be of practical importance. In contrast, the OLS linear regression analysis indicated that the agreement between estimates from the two methods was much worse (e.g., slope of regression line was 0.70 (±0.016)). The OLS linear regression model predicted that, on average, the direct plating result would be 1.4 CFU/g for a MPN result of 0.7 MPN/g and, on average, the direct plating result would be 76 CFU/g for a MPN result of 200 MPN/g. As stated in the introduction and method sections, a number of assumptions necessary for the OLS linear regression analysis were not fulfilled and non-randomly censored data cannot be included; together, these limitations introduced bias into the analysis. As a result, this type of analysis is not appropriate when comparing estimates from direct plating and MPN, notably at low levels of contamination; and our comparison of OLS linear regression and probabilistic based model using the same dataset provides insight to this.

Even though direct plating had lower precision relative to MPN at low levels, the agreement between MPN and direct plating schemes used in the present study suggest that direct plating can be explored for enumerating low levels of *L. monocytogenes* for practical reasons. The ability to homogenize ice cream samples with no dilutions improved the sensitivity and precision of direct plating. In addition, ice cream samples were viscous and could stay out of the edge of the agar plates. Therefore, as much as 400 μl undiluted ice cream could be evenly spread onto each plate with the help of an automatic spiral plater. Recently, plating as much as 2 g of undiluted milkshakes every hour allowed relative precise determination of the lag phase durations of *L. monocytogenes* in milkshakes, 76.7% of which contained initial contamination levels ≤5 CFU/g (Chen et al., 2016a).

The comparison of MPN and direct plating also provided an evaluation of the agars we used for direct plating. Even though many agars are designed to be very sensitive and specific, background flora and sublethally injured *L. monocytogenes* in many food samples pose a special challenge for direct plating, because there is no selective enrichment step that helps to recover injured cells and to increase the target to background ratio. Previous studies (Johansson, 1998; Martin et al., 2004) demonstrated that esculin based *Listeria* agars were not suitable for direct plating of food samples that had high level of background flora. In the present study, we initially explored the use of esculin based agars such as PALCAM and modified oxford agar. Even though the interference of background flora was less than what we observed when analyzing stone fruits (Chen et al., 2016b), cantaloupe rinds and certain cheese products (unpublished data), it still made the plate counting very difficult. Competing flora were also observed on ALOA and RLM plates, but they did not interfere with the plate counting. The agreement between MPN and direct plating showed that the RLM and ALOA agars provided satisfactory selectivity. The long lag phase (9 h) of *L. monocytogenes* in milkshakes prepared from these ice cream products and stored at 22.5 °C (Chen et al., 2016a) suggested that *L. monocytogenes* in these samples suffered certain degree of injury. In the present study, we showed that any possible cell injury did not cause the RLM or ALOA agars to underestimate the levels of *L. monocytogenes*.

In summary, paired enumeration data from MPN and direct plating, primarily using RLM agar, and some using ALOA agar, were obtained from ice cream samples naturally contaminated with background flora and low levels of *L. monocytogenes*. Probabilistic analyses demonstrated that our MPN schemes and direct plating schemes using RLM or ALOA provided similar estimates of *L. monocytogenes* levels in these samples where both methods were applicable. OLS linear regression analyses, which have been used by others to examine this comparison, were shown to introduce bias and provide an inaccurate description of the relationship between MPN and direct plating estimates. The applicability and accuracy of enumeration methods to a set of samples depend on the level of *L. monocytogenes* in the samples, the physiological status of the bacteria, the selectivity of the agar, the level and variety of background flora, and the direct plating and MPN schemes. Different food matrices may require different sample dilution and plating schemes, thereby affecting the LOD, accuracy and precision of direct plating enumeration. In general, MPN is preferred if the level of *L. monocytogenes* in the sample is expected to be low, as the LOD is generally lower and precision is usually higher. This held true in the present study, where MPN was more precise than direct plating for the majority of samples for which the levels of *L. monocytogenes* were low. However, the agreement between estimates by both methods in the applicable concentration range indicates that sensitive direct plating schemes can also be explored for samples containing low levels of *L. monocytogenes*. When data from both methods are available, more robust estimates of the cell levels may be obtained by including both sets of data in the analysis.

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Disclaimer

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Appendix A *Probability to observe the direct plating outcome given the MPN outcome*

For a given sample, we evaluate the probability to observe y or fewer colony forming units from g grams of product, given that we observed $x = \{x_i\}$ positive tube out of $n = \{n_i\}$ serial dilution analysis from i tubes of volume $v = \{v_i\}$. We have $f(y|x) = \int_0^\infty f(y|c)f(c|x)dc$, where c is the actual (unknown) concentration of bacteria in the sample. The Poisson distributed plate count observation has conditional probability distribution $f(y|c) = \frac{(cg)^y e^{-cg}}{y!}$. The distribution of the true bacterial distribution for an observed pattern x using Jeffreys' scale

invariant prior distribution is $f(c|x) \propto f(x|c) f(c) \propto c^{-1/2} \prod_{i=1}^{m} (e^{-cv_i})^{(n_i - x_i)} (1 - e^{-cv_i})^{x_i}$ (GroneWold and Wolpert, 2008).

We evaluated $p(Y|x) = \sum_{0}^{y=Y} f(y|x)$, if the direct plating result was lower than the MPN or $p(Y|x) = \sum_{y=Y}^{\infty} f(y|x)$ when the direct plating was higher than the MPN. If $p(Y|x) < 0.025$, the outcome of the plate count was deemed improbable given the MPN outcome.

Bayesian analysis

In order to test the relationship between c_1 and c_2, we evaluated a model (model #1) $\log_{10}(c_1) = a_0 \times \log_{10}(c_2) + b_0$ in a Bayesian framework. The model uses uninformative priors. The whole model is written: $c_{2j} \sim LogNormal(0,\ .1^{-2})$; $y_j \sim Poisson(v_j c_{1j})$; $x_{ij} \sim Binomial(n_{ij}, 1 - \exp(-v_{ij}\, c_{2j}))$; $a_0 \sim Normal(0, .1^{-2})$; $b_0 \sim Normal(0, .1^{-2})$

A second model (model #2), $\log_{10}(c_1) = (a_0 + a_1 \times ALOA) \times \log_{10}(c_2) + (b_0 + b_1 \times ALOA)$, where *ALOA* is an indicator variable with value 1 if the results was obtained using an ALOA method and 0 if it was a RLM method was tested to evaluate the impact of the microbiological method on the results. For this specific model $a_1 \sim Normal(0, .1^{-2})$; $b_1 \sim Normal(0, .1^{-2})$, were used as additional priors.

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