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Comparative evaluation of most probable number and direct plating methods for enumeration of *Escherichia coli* in *Ruditapes philippinarum,* and effect on classification of production and relaying areas for live bivalve molluscs

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ABSTRACT

A precise and accurate method for enumeration of *Escherichia coli* is critical to classify production and relaying areas for live bivalve molluscs. Paired comparison of most probable number and direct plating count of *E. coli* was performed on a total of 918 samples of Manila clams (*Ruditapes philippinarum*) collected from official monitoring and from a regional shellfish monitoring program conducted from 2011 to 2017 in Emilia Romagna region, Italy. The records of *E. coli* enumeration resulting from both MPN reference method and alternative TBX count method were compared with McNemar's test using three cut-off values, namely 230, 700 and 4.600 MPN/100g, based on the requirements laid down in Regulation (EU) 2019/627. The relative trueness was calculated. Significant differences were observed between the two methods for each investigated cut-off: these incongruities concerned a total of 129 (14%), 131 (14%) and 56 (6%) samples observed as negative with TBX but positive by MPN (respectively using 230, 700 and 4.600 *E. coli* as cut-off), as well as, quite the opposite, a total of 20 (2,2%), 8 (0,9%) and 5 (0,5%) samples observed negative by MPN but positive with TBX method. A negative bias is reported between the two methods. Applying the outcomes of the two different methods to a real scenario, the Veterinary Competent Authority could classify production and relaying areas in the same area of classification only for 69% of samples. This high degree of disagreement between MPN and direct plating outcomes clearly shows the different and not negligible impact of the two methods on the specific *E. coli* requirements for the classification of production and relaying areas for *R. philippinarum.* Analytical methods, either official or validated, if used officially by Veterinary Authority must lead to the same classification of production and relaying areas for live bivalve molluscs.

1. Introduction

International and national trades of live and raw bivalve molluscs require very stringent sanitary requirements. For the production of bivalve molluscs, the microbiological contamination of the water in which they grow is known as the main hazard, especially when the animals are intended to be eaten live or raw. Given the filter feeder nature of molluscs, these animals concentrate contaminants to a much higher degree than the surrounding seawater. The microbiological contamination with both viruses and bacteria in the molluscs production area is therefore critical for the end-product requirement (FAO & WHO, 2020). Worldwide shellfish sanitation programmes hinge on the application of indicator organisms for a certain contamination rather than on monitoring the presence of pathogens. Based on Section 7 of the Codex Code of Practice for Fish and Fishery Products, *Escherichia coli*/faecal coliforms or total coliforms may be globally used as an indicator for the presence of faecal contamination (FAO & WHO, 2020) in water and foods. Within EU, live bivalve molluscs are produced and marketed in compliance with Regulation (EU) 2017/625 and its implementing Regulation (EU) 2019/627. The Veterinary Competent Authorities

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classify, accordingly to article 18(6) of Regulation (EU) 2017/625, production and relaying areas and authorize the harvesting of live bivalve molluscs as Classes A, B and C areas according to the level of faecal contamination, namely the level of Most Probable Number (MPN) *E. coli* in 100 g of flesh and intravalvular liquid.

The MPN method is a standardised and well characterized statistical approach based on an estimation of the concentration of viable micro-organisms in a given sample by replicate 10-fold dilutions, assessing their presence/absence in multiple subdivisions of each dilution. The results obtained by MPN tubes reactions, and namely the number of tubes and those with growth at each dilution, is then used to derive an estimate by probability calculation of the undiluted concentration of bacteria present in the original sample (Walker et al., 2018). In short, the MPN is the number which makes the observed outcome most probable. The MPN is more advisable in case of low concentrations of organisms (usually <100/g) as well as for those foods whose particulate matter could interfere with precise colony counts (BAM, 2020).

The MPN method is used internationally for products intended for human consumption and animal feeding of as well as for environmental samples in the area of food production and food handling. MPN method is also used by official food safety criteria monitoring for molluscs placed on the market during their shelf-life (Regulation 2073/2005). Regulation (EU) 2019/627 - Annex IV, and previously Regulation 854/2005 - Annex I, actually no longer in force, indicates the MPN technique specified in ISO 16649-3 as the reference testing method for analysis of *E. coli* in live bivalve molluscs, but clearly specifies that alternative methods may be used if they are validated against this reference method in accordance with the criteria in ISO 16140. ISO 16140-3:2021, and previous, is the protocol for reference methods verification and validation of alternative methods. Besides, the Competent Authority has to authorize the use of these alternative analytical methods. Over the years, the most suitable and used alternative methods for faecal coliforms and/or *E. coli* counts in molluscs, resulted the colony count and impedance methods, that have been validated and considered satisfactory by the EURL. Indeed, besides MPN test, the direct plating method for determining *E. coli* levels in bivalves is used in official control laboratory by the Netherlands, whereas impedance test is not used widely across the EU, with the exception of some laboratories located in France and Italy (Walker et al., 2018). Pour plate method, initially on Mac Conkey agar and later on Tryptone Bile X-glucuronide (TBX) agar, has been validated for the enumeration of *E. coli* in bivalve molluscs at a level of 200-18.000 CFU/100g with MicroVal certificates changing over the years. The last revision was performed in 2021 following EN ISO 16140 revision, and therefore the previous validation study was renewed according to EN ISO 16140-2:2016 (Pol-Hofstad & Jacobs-Reitsma, 2021) and new experiments using oysters, mussels, cockles and ensis were carried out. Equivalent results were observed for TBX and MPN methods, approving this alternative method for counting *E. coli* bacteria in shellfish (Pol-Hofstad & Jacobs-Reitsma, 2021).

Considering that the official Veterinary Authorities perform a continuous microbiological monitoring of the shellfish harvesting areas, as laid down by the EU regulations, but the bivalve mollusc species Manila clam (*Ruditapes philippinarum*) was not considered in the validation studies with TBX counts as alternative method, this study conducts a thorough comparison of MPN and direct plating *E. coli* methods.

2. Material and methods

2.1. Manila clam sampling and sample preparation

A total of 918 samples of Manila clams were collected during the official monitoring activities performed by the regional Veterinary Authority and from a shellfish monitoring program from 2011 to 2017 in the province of Ferrara, Emilia Romagna region, Italy. All the samplings were performed in class B area in the Adriatic Sea, near a wide delta (31 Km^2 and an average depth of 1,5 m) of the Po river which flows through the Po Valley, that is a densly populated area with high number of intensive farms. All samples were transported between 0 and 10 °C to the laboratory, then stored at 4 °C ± 2 °C, and processed within 24 h after collection. Any material adhering to molluscs was removed prior to opening by rinsing/scrubbing under cold, running tap water of potable quality. Each mollusc sample comprised a number of individuals (commercial size or adult product) sufficient to obtain at least 100 g of clam homogenate comprehensive of flesh and fluid. As far as possible, the same sample was used for *E. coli* enumeration with both MPN and colony-count method in parallel.

2.2. Microbiological analysis

All samples were analyzed for the *E. coli* enumeration at the Experimental Institute for Zooprophylaxis of Lombardy and Emilia Romagna in Ferrara. ISO/TS 16649-3, 2005 and ISO 16649-2:2001, 2001 were respectively used for the reference MPN method and the colony-count technique. A revision of the reference MPN method occurred during the study, but the differences between ISO 16649-3:2015 Cor. 2016-12-15, 2015 (Cor. 2016-12-15) and previous ISO/TS 16649-3:2005 version are minor, and therefore the experimental design of the study was not affected.

The 100 g of clam homogenate were homogenized with 100 ml peptone salt solution to obtain a 1:1 primary dilution which was used directly for the alternative method (ISO 16649-2:2001), whereas for the reference MPN method (ISO/TS 16649-3, 2005) a total of 80 ml of peptone salt solution were added to 20 ml of the primary dilution.

Based on ISO 16649-3, *E. coli* was enumerated using a two-stage, five tube by three dilutions MPN method: briefly, the first stage is a resuscitation step, where primary dilution and two dilutions are inoculated into 5 Mineral Modified Glutamate Broth (MMGB) tubes and incubated at 37 ± 1 °C for 24 ± 2 h. *E. coli* is subsequently confirmed by subculturing tubes, showing acid production and turning from purple to yellow in case of growth, onto TBX and detecting β-glucuronidase activity by the presence of blue or blue-green colonies after incubation at 37 ± 1 °C for 24 ± 2 h and other 18 h ± 3 h at 44 °C ± 1 °C. The observed number of positive tubes was reconducted to a concentration/100 g *R. philippinarum* using the 5-fold MPN table in EN ISO 7218: 2007/A1:2014–04.

Based on the validation of the TBX pour plate method (ISO 16649-2) for the enumeration of *E. coli* in live bivalve molluscs, 2 ml of the primary dilution were inoculated in each of the 5 Petri dishes of selective TBX agar. After an incubation for 24 ± 2 h at 37 ± 1 °C and other 18 h ± 3 h at 44 °C ± 1 °C, all the typical blue green colonies confirming the presence of *E. coli* were counted.

2.3. Statistical analysis

For the expression of the results by MPN, MPN values per 100 g of sample as well as lower and upper 95% confidence limits were used. For the expression of the results by TBX count, given the EURL protocol (issue 3) "Enumeration of *Escherichia coli* in bivalve molluscan shellfish by the colony-count technique (Microval, 2014), the sum of all colonies was multiplied by the dilution factor to obtain the total number of colonies in 100 g of shellfish meat and fluid using the following equation:

$$N = (\sum \text{colonies}/V) * tv$$

where $\sum$colonies is the sum of the blue/green colonies counted on all 5 dishes.

V is the total volume of the inoculum in ml on 5 TBX dishes (10 ml)
tv is the total volume of the sample (x g shellfish flesh + x ml of peptone salt solution)

For the statistical analysis, the *E. coli* enumeration in *R. philippinarum*

resulting from both MPN reference method and alternative TBX count method were dichotomized into different groups considering three different cut-off values of *E. coli*/100 g, namely 230, 700 and 4.600. Theses cut-offs were arbitrarily identified based on requirements for the classification of production and relaying areas for live bivalve molluscs laid down in Regulation (EU) 2017/625 and its implementing Regulation (EU) 2019/627 for which molluscs' samples may: i) contain between 230 and 700 *E. coli*/100 g in 20% of flesh and intravalvular liquid, while the remaining 80% must be < 230 MPN/100g for class A areas; ii) not exceed, in 90% of the samples, 4.600 *E. coli* per 100 g of flesh and intravalvular liquid (the remaining 10% of samples shall not exceed 46.000 *E. coli*) for class B areas. The records of *E. coli* enumeration resulting from both MPN reference method and alternative TBX count method were compared with McNemar's test in order to determine whether the two methods disagree with each other in classification by these cut-off values.

For the calculation of relative trueness study, all the MPN numbers were log transformed. As described in the RIVM report, results above or below the detection limit recorded for both methods were assigned values 1 $\log_{10}$ unit higher or lower. Samples with only positive tubes in the reference method (MPN code 555 > 16.000 MPN CFU/100 g) leading to $\log_{10}$ > 4,3/100 g were assigned values of $\log_{10}$ 5,3/100 g. All the data were analyzed using the scatter plot, with the reference method results plotted against the alternative method results (Pol-Hofstad & Jacobs-Reitsma, 2021).

3. Results

Out of the 918 *R. philippinarum* samples analyzed by both the MPN and direct plating methods, 287 yielded no colonies by direct plating (below LOD) and, among those, 79 were also zero positive MPN tubes. A total of 20 samples resulted or were estimated positive by direct plating (≥1 colonies) and negative by MPN. In relation to MPN results, a total of 98 samples (10.6%) resulted below the detection limit of the method, 385 samples (41,9%) showed a contamination between the detection limit of the method and 230 MPN/100 gr and respectively 153 (16,7%), 215 (23,4%) and 66 (7,1%) were comprised between 230 and 700, >700 and ≤ 4.600 and >4.600 MPN/100 gr. In relation to TBX results, in a total of 287 (31.2%) samples no colonies were observed at all, and 305 (33,2%), 167 (18,1%), 143 (15,6) and 16 (1,7%) samples showed an estimated contamination between the LOD and the limit of 230, 230 and 700, >700 and ≤ 4.600 and >4.600 CFU/100gr respectively. The arrangement of the results obtained with the two methods is reported in Table 1.

Table 2 shows positive and negative findings hypothetically assigned by the use of reference and alternative methods considering three different cut-off values of *E. coli* per 100 g of flesh and intravalvular liquid. Significant differences (p=<0,05) were observed by McNemar's test between the two methods for each investigated cut-off.

Fig. 1 shows the scatter plot for all the 918 samples tested, including samples with results below or above the detection limit. Considering the overall samples, the several observed deviating data points indicate the differences between TBX and MPN methods: the average difference is −0.5896 $\log_{10}$ (CI95% −0,6396 to −0,5395), meaning that a negative bias is to be reported between the two methods, and namely that lower *E. coli* levels are detected by TBX counts than MPN. Excluding all the samples with a contamination level <0.3 $\log_{10}$ and >5.3 $\log_{10}$ for both the two methods, the average difference calculated in 598 *R. philippinarum* sample maintains a negative bias but with lower average difference: −0,34 $\log_{10}$ (CI95% −0.38 to −0,30 $\log_{10}$).

4. Discussion

In the present study, paired data of MPN and direct plating from a significant number of naturally contaminated *R. philippinarum* samples collected for a long period (2011-2017) during veterinary official controls were compared. This study has the merit to be based on a primary production real scenario achieving the reliability and power of statistical analysis. In fact our data consider: i) a mollusc species not included in previous studies (family *Veneridae* versus *Cardiidae* and *Pharidae*); ii) only naturally contaminated samples with background flora and unknown (from absent and/or low to high) levels of *E. coli*; iii) a large amount of mollusc samples, of which several very close to the in-force requirements for primary production.

Based on the results obtained with the application of the two different methods to a real scenario, the Veterinary Competent Authority might classify production and relaying areas: i) within the same area for 69, 76 and 37% of samples considering respectively the MPN value, the lower and the upper 95% confidence limits (white boxes in Table 1); ii) in a lower area of classification than TBX method in 3, 15 and 1% of samples, considering respectively the MPN value, the lower and the upper 95% confidence limits (light grey boxes in Table 1); iii) in an higher area of classification than the TBX method in 28, 9 and 62% samples, considering respectively the MPN value, the lower and the upper 95% confidence limits (dark grey boxes in Table 1).

Table 1
Distributions of the level of *E. coli* per 100 g of flesh and intravalvular liquid in all the investigated Manila clam samples by plate count and MPN methods, using the MPN value (a), the lower (b) and the upper (c) 95% confidence limits.

a)

Escherichia coli		Most probable number method (MPN/100 gr)				
		<230	230-| 700	701-| 4600	>4600	Total (%)
Plate count method (CFU/100gr)	**<230**	463	85	40	4	**592 (64,4)**
	230-| 700	16	64	79	8	**167 (18,1)**
	700-| 4600	4	4	91	44	**143 (15,6)**
	>4600	0	0	5	11	**16 (1,7)**
	Total (%)	**483 (52,6)**	**153 (16,7)**	**215 (23,4)**	**67 (7,1)**	**918**

b)

Escherichia coli		Most probable number method (MPN/100 gr)				
		<230	230-| 700	701-| 4600	>4600	Total (%)
Plate count method (CFU/100gr)	**<230**	548	35	7	2	**592 (64,4)**
	230-| 700	80	56	30	1	**167 (18,1)**
	700-| 4600	8	42	81	12	**143 (15,6)**
	>4600	0	0	7	9	**16 (1,7)**
	Total (%)	**636 (69,2)**	**133 (14,4)**	**125 (13,6)**	**24 (2,6)**	**918**

c)

Escherichia coli		Most probable number method (MPN/100 gr)				
		<230	230-| 700	701-| 4600	>4600	Total (%)
Plate count method (CFU/100gr)	**<230**	263	207	114	8	**592 (64,4)**
	230-| 700	2	15	119	31	**167 (18,1)**
	700-| 4600	2	2	48	91	**143 (15,6)**
	>4600	0	0	0	16	**16 (1,7)**
	Total (%)	**267 (29)**	**224 (24,4)**	**281 (30,6)**	**146 (15,9)**	**918**

Table 2
Observed *E. coli* positive and negative samples tested by reference (MPN) and alternative (TBX) methods using the cut-off values of 230, 700 and 4.600 *E. coli* per 100 g of flesh and intravalvular liquid as cut-off and results of statistical analysis using McNemar's test.

		MPN										
		>230	≤230	Sum		>700	≤700	Sum		>4600	≤4600	Sum
TBX	**>230**	306	20	326	**>700**	151	8	159	**>4600**	11	5	16
	≤230	129	463	592	**≤700**	131	628	592	**≤4600**	56	846	902
	Sum	435	483	918		282	636	918		67	851	918

McNemar's chi-squared for 230, 700 and 4.600 *E. coli* cut-offs = 79.74, 107.08, 42.64; d.f. = 1, all p-values <0,001.

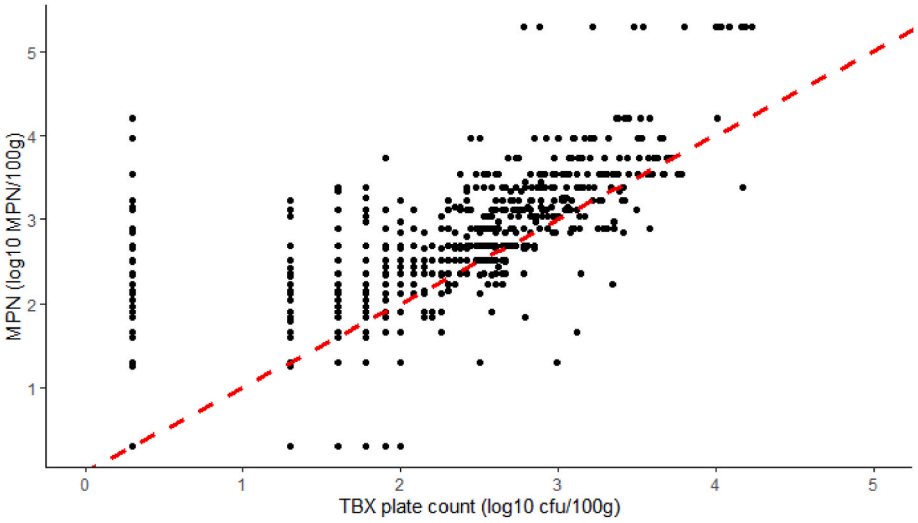

Fig. 1. Scatter plot of the reference method versus alternative method of the overall 918 *E. coli* levels in *R. philippinarum* samples observed in this study.

In addition, the significant differences observed by McNemar's test between the two methods, for each of the three investigated cut-offs, clearly underline that the frequency of discordant findings is not by chance but it is systematic, and therefore, the use of either the reference or the alternative method don't lead to the same result. In fact, these incongruities concern a total of 129 (14%), 131 (14%) and 56 (6%) samples observed as negative with TBX but positive by MPN (respectively using 230, 700 and 4.600 *E. coli* as cut-off), as well as, quite the opposite, a total of 20 (2,2%), 8 (0,9%) and 5 (0,5%) samples observed negative by MPN but positive with TBX method (Table 2, grey boxes). About two-third of the discordant data refer to the contamination interval between 230 and 700 *E. coli* per 100 g of flesh and intravalvular liquid of molluscs. This aspect has an important impact considering the classification of molluscs' harvesting areas into three different quality levels. In fact, bivalve mollusc from class A (80% of samples ≤230 MPN/100 g and 20% of samples maximum ≤700 MPN/100 g) can be placed on the market with no post-harvesting treatment required. Molluscs from class B (90% of samples ≤4.600 MPN/100 g and 10% of samples maximum ≤46.000 MPN/100 g) before they can be supplied for human consumption must be submitted to one of three approved processes: purification, relaying or cooking. Whereas molluscs originating from class C (≤46.000 MPN/100 g) must undergo to resuspension for a long time in a class A area or must be submitted to an approved heat treatment process (Regulations 853/2004 and 2019/627). Therefore, the discrepancy observed using the two methods might not only affect the classification of mollusc areas in primary production but for molluscs belonging to class A area, also their potential safety control. Obviously, no food safety considerations could be gathered from these data considering the stage into the bivalve molluscs chain where the criterion applies, but it should be reminded that the adequate analytical method could influence the ways to both control the final products and to perform an adequate risk-based monitoring approach. Indeed, all the tested samples were collected from mollusc areas B, where a post-harvesting treatment is foreseen to protect the consumers, but in case of an initial misclassification of the area, inappropriate application of post-harvesting treatment might have represented a potential threat for human health.

The proportion of discrepancies suggests a high extent of disagreement between the MPN and the direct plating outcome, differently to the studies performed by RIVM 2021-0127 and previous reports. In addition, based on RIVM report 2021-0127, it should be noted that for the plating count method, the results between 4 and 10 colonies per 5 plates (overall 114 in our study) into TBX were considered an estimation rather than a true count. Indeed, the count is intended so when a minimum of 11 colonies can be counted, which corresponds approximately to 220 CFU/100 gr, value that is definitely in close proximity to the limit of 230 *E. coli* per 100 g of flesh and intravalvular liquid (Regulation 2019/627). As observed by Walker et al. (2018), the lack of sensitivity of TBX count compared to the MPN method (practical LOD <18 versus 200 CFU/100 g) may limit its application in clean environments, such as class A sites or end product testing. This is in line with the assumption that dates back to the earliest days of microbiology theorizing that an MPN procedure is used when a low number of bacteria is expected. In contrast, the pour plate method is useful where high *E. coli* levels are expected, whereas in samples with high microbial load, the MPN determinations are less precise and often report higher values than those obtained by pour plate colony count techniques (Seafish, 2021). This could raise some concerns about the choice of an alternative method to the MPN count, even if validated, to be applied in shellfish producing areas when these are classified as A. Within the investigated area of the Goro lagoon, more than one third of the surface is exploited for clam farming, with an annual production that reached a maximum of 87.000 t y^{-1} in 2011. This area faces the Po Valley that is highly populated with abundant large animals' intensive farms where mussels, Manila clams and oysters

are farmed.

As expected, our findings confirm that where low results are expected, low results are normally observed with the MPN method and therefore it suggests that this tool is more advisable for class A harvesting mollusc areas in order to opt for the better preventive approach and measure. A precise and accurate method for enumeration of low levels of *E. coli* in foods is critical to class A areas that produce molluscs for direct sale, for which the plate count method feels weak. However, considering that plate colony count technique is less time-consuming and less labor-intensive than MPN, which is particularly relevant when public health interventions are required (Seafish, 2021), its use could be an interesting and well-effective tool for harvesting areas not very clean as well as not highly contaminated by *E. coli* and in which post-harvesting treatments were performed. The well-known characteristics of an analytical method, namely higher precision for MPN at low levels compared to direct plating, and lower precision for MPN at high levels, have to be considered not only for the result but also in consideration to the assessment of a proper risk-based monitoring plan.

Although a real scenario was used for the proposed comparison, some sources of uncertainty may be identified in this study. The main one is the fact that even though the MPN method is prescribed as reference method actually in force to count *E. coli* in bivalves, it is not validated and a high variability is reported in the literature. In this regard, the uncertainty measurement performed by comparison of food microbiology methods by an EU Commission working group (2003) showed that the MPN method has a variability of 0,6 $\log_{10}$ *E. coli* for four replicates in molluscs, similarly to other food matrices (Walker et al., 2018). In the last RIVM report 2021-0127 the calculated values of relative trueness per food-type and for the category of molluscs as a whole showed an overall slight negative bias (-0,0920) with some differences between the oysters (-0,0981), mussels (-0,1264) and cockles and ensis (0,0042). Another reported factor of variability is linked to subsampling for which a perfectly uniform and homogeneous distribution of *E. coli* in the material is not achievable (Walker et al., 2018). Our bias (media -0.34 $\log_{10}$ and IC95% -0.38 to -0.30 $\log_{10}$) are higher, at least the triple of the above mentioned results.

Also the species accuracy of MPN EU reference method used for detection of *E. coli* in marine bivalves was examined by Grevskott et al. (2016). Considering that the β-glucuronidase enzyme is possessed by other members of the Enterobacteriaceae, false-positive blue-green colonies on the TBX agar could be assumed/suspected: indeed API 20E and MALDI-TOF MS did not confirm the identification as *E. coli* in 10% of the presumptive colonies, leding to an overestimation of the *E. coli* level of contamination. However, it should be mentioned that, in this study, only one single colony was isolated and cultivated for further examination whereas by reference method (EN ISO 16649-3), all the positive tubes with colour change in MMGB have to be spread into TBX plates.

In literature, studies that have compared MPN and direct plating results highlighted a clear overestimation of MPN method for *E. coli* counts in river waters (Hamilton et al., 2005) and for *Enterobacter aerogenes* from water samples (Wohlsen et al., 2006). Prats et al. (2007) compared the MPN microplate enumeration method of *E. coli* in tropical and temperate freshwaters with counts on several differential agar media: the counts by the MPN method were statistically higher than those obtained using the plate count methods and, for the accuracy (reproducibility) evaluation, the coefficient of variation calculated in five replicates was higher for the MPN (34%) than the other plating methods (from 20% to 25%). Cho et al. (2010) enumerated fecal indicator bacteria from the same water body using MPN and CFU estimates: significant differences were found with the enumerated *E. coli* in MPN greater than that in CFU (with a concentration in MPN one order of magnitude greater than that in CFU) and, contrarily, with enterococci bacteria in MPN lower than those in CFU. Gronewold and Wolpert (2008) modelled the relationship between MPN and CFU estimates of fecal coliform concentration in water samples, revealing that the observed differences (MPN estimates were higher) were within the ranges predicted by the probabilistic model and therefore that this variability is a simple consequence of the probabilistic basis for calculating the MPN. Also Chen et al. (2017), exploiting the probabilistic model of Gronewold and Wolpert (2008), conducted a paired comparison of MPN and direct plating enumeration of *Listeria monocytogenes* on 1.730 samples from an outbreak linked to ice-cream contaminated with low level of *L. monocytogenes*.

Authors reported that the use of ordinary least squares linear regression to study the correlation between MPN/direct plating estimates could introduce bias and provide an inaccurate description of the relationship between the investigated methods because it incorrectly characterized systematic differences between estimates from the two methods. On the contrary a probabilistic analysis with Bayesian inference model revealed good agreement between MPN and direct plating estimates. Briefly, on average, predicted values of 1,4, 175 and 76 CFU/g with direct plating were observed respectively by Bayesian model (versus 1 and 200 MPN/g) and linear regression model (versus 0,7 and 200 MPN/g). Interestingly, the aforementioned differences were observed only for low levels of *L. monocytogenes*, considering that a higher concentration by direct plating method was estimated by the final model given a concentration of *L. monocytogenes* $<$ 46 CFU/g or MPN/g, as well as a lower value than the MPN method in case of *L. monocytogenes* $>$47 CFU/g or MPN/g. In addition, differently from molluscs preparation neither sample preparation nor homogenization were performed on ice creams before direct plating, eventually improving the sensitivity and precision of the method.

In conclusion, although our results may have been biased by some factors, the comparison of MPN and plate count methods for enumeration of *E. coli* in *R. philippinarum* evaluated in this study reflects the current activities performed in molluscs with a real and unknown *E. coli* contamination level by regional Veterinary Authority and Experimental Institute for Zooprophylaxis in an attempt to reproduce real-life scenarios. Our findings clearly showed that the use of reference versus alternative methods, as proposed (without any correction factor) and in this specific environment, has a different and not negligible impact on the specific *E. coli* requirements for the classification of production and relaying areas for *R. philippinarum* in Emilia Romagna and, consequently, also in the complex itinerary towards placing the products on the market. In order to guarantee the free circulation of food within the European Community, the analytical method used to determine the level of fecal contamination of the investigated area is a tool that must lead Veterinary Authority to the same classification.

CRediT authorship contribution statement

Silva Rubini: Conceptualization, Resources, Investigation. **Giorgio Galletti:** Data curation, Formal analysis, Writing – original draft. **Erica Bolognesi:** Investigation. **Paolo Bonilauri:** Supervision. **Marco Tamba:** Formal analysis. **Federica Savini:** Data curation, Writing – review & editing. **Andrea Serraino:** Supervision. **Federica Giacometti:** Conceptualization, Data curation, Writing – original draft.

Declaration of competing interest

All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

Data will be made available on request.

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