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Validation by an interlaboratory collaborative trial of EN ISO 21528 - microbiology of the food chain - horizontal methods for the detection and enumeration of *Enterobacteriaceae*

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ABSTRACT

The methods for the detection and enumeration of *Enterobacteriaceae*, described in EN ISO 21528, parts 1 and 2, were validated by order of the European Commission under the mandate M/381. Fourteen laboratories from seven European countries participated in the collaborative trials, organized by The Netherlands Food and Consumer Product Safety Authority (Wageningen/Utrecht, The Netherlands).

Five different matrices from different food categories were selected to be tested in the collaborative trials, in order to validate the method horizontally, according to ISO 16140. The matrices included meat, tiramisu, infant formula, liquid egg, smoked salmon (detection method only) and animal feed (enumeration method only). The raw meat and liquid egg were naturally contaminated and the other matrices were artificially contaminated with a cocktail of four different *Enterobacteriaceae* strains.

The samples used in the trial were tested for homogeneity and stability before distribution. The method for detection of *Enterobacteriaceae* showed a specificity and sensitivity above 95% for all matrices. The method for the enumeration had a repeatability limit r of 0.37 (expressed as a difference between $\log_{10}$-transformed test results) and a reproducibility limit R of 0.87 (expressed as a difference between $\log_{10}$-transformed test results).

The validation data were incorporated in the newly published ISO standards EN ISO 21528:2017- Microbiology of the food chain - Horizontal methods for the detection and enumeration of *Enterobacteriaceae* - Part 1: Detection method, and Part 2: Colony-count technique.

1. Introduction

Within the framework of the European food hygiene legislation, the Commission Regulation No (EC) 2073/2005 on microbiological criteria for foodstuffs contains provisions requiring the use of analytical methods in the food chain (Anonymous, 2005a). In this Regulation a reference method has been established for each microbiological criterion. EN or EN ISO methods that apply to the testing of samples taken across the entire food chain (i.e. horizontal), when available, have received the status of reference methods in the Regulation. The establishment of standardised reference methods is of importance to safeguard a uniform application and control of Community legislation in all Member States. Standardised and validated reference methods are an indispensable element to guarantee a high level of food safety, and are the references against which other methods can be validated.

In the field of food hygiene, including production environments, there is a lack of standardised methods. The validation (i.e. the process of gathering evidence that a method is fit for purpose) of European standards is important since it will lead to improved recognition of the standards by the international community, better substantiation of the microbiological criteria and therefore enhanced consumer protection. It will also result in better recognition of analytical results by courts, the European Food Safety Agency, authorities and laboratory inspections at International level. Finally quality of test results produced by (accredited) laboratories are improved due to the verification and implementation of validation data of the standard methods as part of their quality system based in ISO/IEC 17025 (Anonymous, 2005b).

The European Union, represented by the European Commission, decided to award a grant for the standardization of 15 published European Validated Standards (EN) in the field of food hygiene legislation under mandate SA/CEN/ENTR/381/2010-06 Microbiology of Food and animals feeding stuffs (Mandate M/381) (Anonymous, 2010).

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Table 1
Number of *Enterobacteriaceae*-positive confirmed results of the participants for detection of *Enterobacteriaceae* for eight biological replicates per matrix and per contamination level.

Matrix	Inoculum level	laboratory code													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Meat	Blank[a]	0	0	0	0	0	1	0	0	0	0	0	0	1	0
	Low[b]	8	8	8	8	8	8	8	8	8	8	8	8	7	8
	High[c]	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Tiramisu	Blank[a]	0	0	0	0	0	0	0	0	0	0	0	1	0	0
	Low[b]	8	8	8	8	8	8	8	8	8	8	8	8	8	8
	High[b]	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Smoked salmon	Blank[a]	0	0	0	0	0	0	0	0	0	0	0	2	0	3
	Low[b]	8	8	7	8	8	8	8	8	8	8	8	8	8	8
	High[c]	8	8	8	8	8	8	8	8	8	8	8	7	8	8
Infant formula	Blank[a]	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	Low[b]	8	8	8	8	8	8	8	8	8	7	8	8	7	8
	High[c]	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Egg	Blank[a]	0	0	1	0	0	8	0	0	0	0	0	0	0	0
	Low[b]	8	8	8	8	8	8	8	8	8	8	8	8	8	8
	High[c]	8	8	8	8	8	8	8	8	8	8	8	8	8	8

[a] The samples meat and egg product were gamma sterilized to obtain blank samples. The samples tiramisu, smoked salmon and infant formula were tested and found negative for *Enterobacteriaceae* (not detected in 10 g of sample).
[b] Contamination level was 6 cfu/10 g for raw meat, 12 cfu/10 g for egg product and 2 cfu/10 g for tiramisu, smoked salmon and infant formula, respectively.
[c] Contamination level was 61 cfu/10 g for raw meat, 25 cfu/10 g for egg product and 6 cfu/10 g for tiramisu, smoked salmon and infant formula, respectively.

The agreements were concluded by the European Commission and the European Committee for Standardization (CEN). The 'Deutches Institut für Normung' (DIN) and the 'Association française de Normalisation (AFNOR)' participated as members of CEN in this specific grant agreement. The elaboration of the standards was performed by subcontractors under the mandate M/381, in which The Netherlands Food and Consumer Product Safety Authority (Wageningen/Utrecht, The Netherlands) was the appointed subcontractor to perform validation work on the standard for detection and enumeration of *Enterobacteriaceae* described in ISO 21528-1 and ISO 21528-2 (Anonymous, 2004a, 2004b; Anonymous, 2017a, 2017b). The subcontractors prepared protocols and samples for the collaborative trials and collected and evaluated the results from the collaborative trials. Based on these results, amended European Standards were also prepared by the subcontractors.

Over the past decades *Enterobacteriaceae* have gained importance as hygiene indicators for process verification in food production. Regulation (EC) 2073/2005 (Anonymous, 2005a) on microbiological criteria for foodstuffs uses the family of *Enterobacteriaceae* as a parameter in process hygiene criteria for various food products, such as dried infant formulae, dried follow-on formulae, pasteurized milk (products), carcasses (cattle and pigs), milk powder and egg products. For testing compliance to these criteria the standards ISO 21528-1 (detection) (Anonymous, 2004a) and ISO 21528-2 (enumeration) (Anonymous, 2004b) are indicated as mandatory analytical methods for this purpose.

The International Standard ISO 21528-1 (Anonymous, 2004a) is a detection (presence/absence) method which should be used when the microorganisms sought are expected to need resuscitation by pre-enrichment and when the number is expected to be below 100 per ml or per g of the test sample. Pre-enrichment is achieved in a non-selective medium (buffered peptone water), enrichment in a selective liquid medium (EE broth), isolation on a selective solid medium (violet red bile glucose (VRBG) agar) and suspect colonies are confirmed by testing for oxidase and glucose fermentation. Some isolates of *Enterobacteriaceae*, including some *Cronobacter* spp. strains, do however not grow well or even die in EE broth, which could lead to false negative results (Joosten et al., 2008). Therefore, it was decided to revise ISO 21528-1:2004 (Anonymous, 2004a) by omitting the enrichment step in EE broth. The revision of this standard also includes replacement of glucose agar by OF Medium in the confirmation step to improve the confirmation of slow-acidifying *Enterobacteriaceae* strains. The MPN method was moved to an informative Annex.

The International Standard ISO 21528-2 (Anonymous, 2004b), is an enumeration method without resuscitation which should be used when the expected number of the microorganisms sought is > 100 per ml or per g of the test sample. The method includes the preparation of an initial suspension of the sample and decimal dilutions thereof, followed by plating on a selective solid medium (VRBG agar), and confirmation of suspect colonies (oxidase, glucose fermentation). In the revised version of this standard glucose agar is replaced by OF Medium in the confirmation step, in accordance with the revised standard ISO 21528:1 (Anonymous, 2017a).

The aim of the study described in this manuscript was the elaboration of validated EN standard methods from the standards ISO 21528-1:2004 (Anonymous, 2004a) and ISO 21528-2:2004 (Anonymous, 2004b). In this manuscript we report the results of the collaborative studies performed in March/April 2013 (part 1, detection) and November/December 2013 (part 2, enumeration) and the results of the statistical analysis to determine the precision characteristics of the qualitative method and the precision data in terms of repeatability and reproducibility of the quantitative method.

2. Materials and methods

2.1. Design of the trials

The design of the trials was according to the description of the interlaboratory study for alternative methods in ISO 16140:2003 (Anonymous, 2003). In order to validate the qualitative method horizontally, five different matrices were selected for the collaborative trials, all from different food categories (Anonymous, 2003). A horizontal method applies to the testing of samples taken across the entire food chain. The matrices included meat, tiramisu, infant formula, liquid egg, smoked salmon (detection method only) and animal feed (enumeration method only). The raw meat and liquid egg were naturally contaminated and the other matrices were artificially contaminated with a cocktail of four different *Enterobacteriaceae* strains; *Escherichia coli* (NCCB 100297), *Salmonella* Enteritidis (NCCB 100284), *Klebsiella oxytoca* (ATCC 8724) and *Yersinia enterocolitica* (CCUG 8233). For qualitative methods three levels of contamination were needed; blank (samples were gamma sterilized), low (level depends on matrix, see Table 1) and high (level depends

on matrix, see Table 1), with eight replicates per level, resulting in 120 samples to test per laboratory.

For quantitative methods three levels of contamination were needed; low (at or slightly above the detection limit of the method), middle (between low and high contamination level) and high (5–10 times above the detection limit), with two replicates per level, resulting in 40 samples to be tested per laboratory.

According to ISO 16140:2003 (Anonymous, 2003) a minimum of 10 valid data sets (80 results per level and per matrix) are needed for qualitative studies, but > 10 participating laboratories were preferred in order to compensate for any unforeseen exclusions of data. A minimum of 8 valid data sets are needed for quantitative studies. All test materials during the study were coded randomly in order to prevent biased analysis based on expected outcomes.

The members of the working group TAG 16, *Enterobacteriaceae*, of CEN/TC275/WG6 "Microbiology of the food chain" were invited to participate in the study and also other laboratories were contacted based on the TAG 16 network. The participants were informed about the time schedule and procedure of the validation process prior to the start of the study. Every participating laboratory was randomly assigned a unique participant code and was provided with the standard operating procedure for the method, a procedure for artificially inoculating the samples prior to investigation, a form to report the condition of the samples upon reception and a form to report the materials used, incubation conditions and times, and final results. The test materials were frozen and then shipped on dry ice in polystyrene boxes to the participants by courier. All materials needed for analysis of the samples were provided by the participants. The participants had to start the analysis in a given time frame and to report the results before the appointed date. The results were statistically analyzed as described in Section 2.4.

2.2. Method for validation

The method was validated for test portions of 10 g or ml.

The method validated for detection of *Enterobacteriaceae* comprises of enrichment of the test portion (10 fold dilution) in non-selective medium (Buffered peptone water (BPW)), isolation on a selective solid medium (violet red bile glucose (VRBG) agar), subculturing of suspect colonies onto non-selective medium, and confirmation by testing for oxidase and glucose fermentation. Enrichment and plates were incubated at 37 °C for 18 h and 24 h, respectively.

The enumeration of *Enterobacteriaceae* comprised of preparation of a single decimal dilution series from the test portion if the product was liquid or from the initial suspension in the case of other products, using a diluent for general use (Anonymous, 2017c), followed by isolation from the dilution series using the pour plate technique with VRBG agar with a VRBG agar overlay. The dishes were incubated at 37 °C for 24 h. Confirmation was as described for the detection method.

2.3. Preparation and stability testing of the test materials

For every matrix the bulk was homogenized prior to packaging in portions of 10 g and the samples were stored at −20 °C before dispatching. All blank samples were gamma sterilized to guarantee the absence of *Enterobacteriaceae*. The samples to be artificially contaminated were tested for the level of *Enterobacteriaceae* present and this had to be sufficiently low (< 10 CFU mL^{-1}) in order to be included in the study for the detection method. Overnight cultures of *Escherichia coli* (NCCB 100297), *Salmonella* Enteritidis (NCCB 100284), *Klebsiella oxytoca* (ATCC 8724) and *Yersinia enterocolitica* (CCUG 8233) were prepared by culturing the cells in a suitable culturing medium and incubating them for the appropriate time and temperature (Biosisto b.v., Assen, The Netherlands). A single suspension of the four strains in a 1:1:1:1 ratio was prepared in milk with glycerol (15%) and stored at −80 °C before dispatching. The samples to be contaminated artificially had to be inoculated with 0.1 mL of the bacterial suspension by the participants themselves. The contamination level of the bacterial suspension was unknown to the participants of the study.

The homogeneity was determined according to ISO guide 34 (Anonymous, 2009). The homogeneity was determined for the matrix samples as well as for the bacterial inoculum, by analyzing 10 samples per matrix and, in case of multiple contamination levels, per contamination level. The content of each sample to be analyzed was homogenized and two sub-samples were taken from this homogenized sample and analyzed using ISO 21528-1 and/or ISO 21528-2 and ISO 4833 (Anonymous, 2004a; Anonymous, 2004b; Anonymous, 2013). All samples per matrix and contamination level were analyzed in one series. To calculate the homogeneity of the samples the between-group variation and the within-group variation was calculated, using ANOVA. If the obtained value was within two times the standard deviation the sample was considered homogeneous.

The stability was determined for the natural contaminated samples and the bacterial inoculum. The level of contamination was analyzed by taking one sub-sample, as described for homogeneity. The stability of the samples was determined on the date of the shipment of the samples to the participants by comparing the homogeneity data to the stability data. If the difference between both series was smaller than two times the standard deviation, the samples were considered stable.

2.4. Analysis of the data

Data obtained by some collaborators were excluded from the calculations only for clearly identified technical reasons. It was assessed whether enumeration data differed more than one log from the expected contamination level. In these cases technical faults were expected. Since not all labs were able to indicate technical faults, it was calculated using the Cochran test and Grubbs test if the data were outliers. Outliers were removed from the dataset.

For the detection method the sensitivity (percentage of true positive samples compared to the total number of samples analyzed), specificity (percentage of true negative samples compared to the total number of samples analyzed) and the detection level at 50% (LOD_{50}; the concentration in CFU per sample for which the probability of detection is 50%) were determined. The calculations for LOD_{50} were performed using a dedicated Excel sheet (Wilrich and Wilrich, 2009).

For the enumeration method the repeatability limit (r) and the reproducibility limit (R) were determined. The repeatability limit was derived from the mean of the variance estimates for all levels per matrix tested in the same laboratory and the reproducibility limit was derived from the mean of the variance estimates for all levels per matrix tested in all laboratories.

3. Results and discussion

3.1. Homogeneity and stability of the test materials

The samples were homogeneous after preparation and at the moment of shipping. No transportation and storage effects were therefore expected on the contamination level of the samples, since the samples were stored and transported on dry ice and all participants reported that they had received the samples in frozen condition. All participants started the analysis in the appointed week.

3.2. Results of the interlaboratory studies

Fourteen laboratories participated in the detection trial. The raw data for all laboratories are presented in Table 1. One laboratory was excluded from the dataset for the matrix 'egg product', since the blanks for this matrix were contaminated and it was not possible to distinguish if the laboratory was able to detect the low inoculation level or whether the same contamination had occurred comparable to in the blanks.

Table 2
Average enumeration results of the participants for two biological replicates per matrix and contamination level.

Matrix	Inoculum level (log_{10} cfu/g)	Laboratory code												
		1	2	3	4	5	6	7	8	9	10	11	12	13
Egg product	Low	3.24	3.53	2.88	3.52	3.16	–*	3.53	3.83	3.76	3.98	3.18	3.64	3.51
	Middle	4.02	4.28	3.11	4.22	4.08	–	4.11	4.63	4.72	4.78	3.56	4.61	4.37
	High	5.55	6.13	5.16	5.78	5.46	–	5.84	6.16	6.13	–	4.96	6.23	6.16
Raw meat	Low	2.64	2.61	2.36	2.29	2.50	–	2.81	2.34	2.56	2.59	2.08	–	1.93
	Middle	4.07	3.93	3.35	3.63	4.07	–	3.76	3.96	–	4.02	3.36	3.73	3.00
	High	4.19	4.28	2.77	3.45	4.10	–	3.91	4.02	4.11	4.18	2.87	4.13	2.98
Animal feed	Low	1.60	1.57	1.40	1.48	–	–	1.35	1.80	1.51	1.64	1.8	1.7	1.6
	Middle	2.65	2.56	2.23	2.31	2.65	–	2.45	2.49	2.48	2.59	2.7	2.6	2.3
	High	3.70	3.65	3.15	3.32	–	–	3.38	3.65	3.45	3.74	3.7	3.6	3.5
Milk	Low	1.65	1.18	1.30	1.69	1.74	–	1.51	1.65	1.72	1.57	1.57	1.63	1.10
	Middle	2.63	2.32	2.30	2.16	2.60	–	2.19	2.56	2.41	2.33	2.56	2.49	2.23
	High	3.74	3.41	3.23	3.30	3.54	–	3.32	3.65	3.52	3.41	3.64	3.56	3.19
Tiramisu	Low	1.74	1.35	–	1.40	1.65	–	1.30	1.35	1.57	1.68	1.48	1.51	1.30
	Middle	2.72	2.28	2.05	2.36	2.50	–	2.34	2.47	2.39	2.51	–	2.40	2.18
	High	–	3.34	3.16	3.29	3.43	–	3.40	3.48	3.19	3.41	3.57	3.39	3.29

* Means no data obtained.

Thirteen laboratories participated in the enumeration trial. The raw data for all laboratories are presented in Table 2. One laboratory was totally excluded from the dataset since none of the their results were in line with the expected results, and their blanks were possibly contaminated.

3.3. Performance assessment of the method

The detection method was validated for test portions of 10 g or ml. A smaller test portion may be used, without the need for additional validation/verification, providing that the same ratio between enrichment broth and test portion is maintained. A larger test portion than that initially validated may be used, however enlarging the test portion may reduce the probability of detection. A validation/verification study should therefor show that there are no adverse effects on the detection of *Enterobacteriaceae*.

3.3.1. Specificity, sensitivity and LOD_{50}

The specificity and sensitivity for all matrices is presented in Table 3. For all matrices tested the specificity was above 95%. For all matrices and inoculation levels tested the sensitivity was above 95%. The sensitivity obtained was not used to assess the quality of the method, but can be used in the future to compare alternative methods to the standardised reference method.

LOD_{50} calculations for detection methods require a fractional recovery at the low level as a minimum. As can be seen from Table 1 there was a full recovery from the low contaminated samples by all laboratories. Since three of the low contaminated samples were contaminated at a level of 2 cfu/10 g sample, it can be concluded that the validated method is relatively sensitive. This therefore does not allow an LOD_{50} value to be calculated. As an indication, it can be concluded that the LOD_{50} values are lower than the low inoculum levels, meaning LOD_{50} is < 6 cfu/10 g for meat, < 12 cfu/10 g for egg product and < 2 cfu/10 g for tiramisu, smoked salmon and infant formula.

3.3.2. Repeatability limit

The repeatability limit was calculated for the enumeration method and is presented in Table 4. The absolute difference between two independent single (log_{10}-transformed) test results (number of cfu per g or per mL) or the ratio of the higher to the lower of the two test results on the normal scale, obtained using the same method on identical test material in the same laboratory by the same operator using the same apparatus within the shortest feasible time interval, will, in not > 5% of cases, exceed the repeatability limit *r*.

As a general indication of repeatability limit (*r*), the following values (derived from the mean of the variance estimates for all levels per matrix tested in the interlaboratory study), may be used when testing samples:

$r = 0.37$ (expressed as a difference between log10-transformed test results); or $r = 2.33$ (expressed as a ratio between test results).

For example; a test result of 10,000 or 1.0×10^4 or log_{10} 4.0 cfu per g of sample material was observed in a given laboratory. Under repeatability conditions, the difference between log_{10}-transformed results should not be greater than ± 0.37 log_{10} units. So the result from a second test of the same sample should be between 3.63 (4.0–0.37) and 4.37 (4.0 + 0.37) log_{10} units.

For non-log-transformed results, the ratio between the first test result and the second test result from the same sample should not be > 2.33. So the second test result should be between 4290 (=10,000/2.33) and 23,300 (10,000 × 2.33) cfu per g.

3.3.3. Reproducibility limit

The repeatability limit was calculated for the enumeration method and is presented in Table 4. The absolute difference between two single (log_{10}-transformed) test results (number of cfu per g or per millilitre) or the ratio of the higher to the lower of the two test results on the normal scale, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will, in not > 5% of cases, exceed the reproducibility limit *R*.

As a general indication of reproducibility limit (*R*), the following values (derived from the mean of the variance estimates for all levels per matrix tested in the interlaboratory study), are used when testing food samples in general:

$R = 0.87$ (expressed as a difference between log_{10}-transformed test results); or $R = 7.38$ (expressed as a ratio between test results).

As a first example; a test result of 10,000 or 1.0×10^4 or log_{10} 4.0 cfu per g of sample material was observed in a first laboratory. Under reproducibility conditions, the difference between log_{10}-transformed results should not be more than ± 0.87 log_{10} units. So the result from a second laboratory should be between 3.13 (4.0–0.87) and 4.87 (4.0 + 0.87) log_{10} units.

For non-log-transformed results, the ratio between the test result from this first laboratory and a second laboratory should not be > 7.38. So the result from the second laboratory should be between 1360 (=10,000/7.38) and 73,800 (10,000 × 7.38) cfu per g.

As a second example; a laboratory wants to know the maximum value it may find for a sample, which is still in compliance with a preset limit (e.g. a limit of 1000 or log_{10} 3). For this, the *R* value (on the log scale) has to be multiplied by a factor of 0.59.

The factor 0,59 reflects the fact that a test with a one-sided 95% interval is used to test whether the limit is exceeded; it is obtained from

Table 3
Summary of the performance characteristics for the detection method for the matrices raw meat, tiramisu, smoked salmon, infant formula and egg product.

Matrix and parameters	Contamination level		
	Blank	Low	High
Meat			
Contamination level (cfu/10 g)	–	6	61
Number of participating collaborators	14	14	14
Number of collaborators retained after evaluation of the data	14	14	14
Number of samples	112	112	112
Number of samples retained after evaluation of the data	112	112	112
Test portion size in g	10	10	10
Sensitivity in %	–	99	100
Specificity in %	98	–	–
LOD_{50} (95% confidence interval) cfu/sample	NA[a]	NA[a]	NA[a]
Tiramisu			
Contamination level (cfu/10 g)	–	2	6
Number of participating collaborators	14	14	14
Number of collaborators retained after evaluation of the data	14	14	14
Number of samples	112	112	112
Number of samples retained after evaluation of the data	112	112	112
Test portion size in g	10	10	10
Sensitivity in %	–	100	100
Specificity in %	99	–	–
LOD_{50} (95% confidence interval) cfu/sample	NA[a]	NA[a]	NA[a]
Smoked salmon			
Contamination level (cfu/10 g)	–	2	6
Number of participating collaborators	14	14	14
Number of collaborators retained after evaluation of the data	14	14	14
Number of samples	112	112	112
Number of samples retained after evaluation of the data	112	112	112
Test portion size in g	10	10	10
Sensitivity in %	–	99	99
Specificity in %	96	–	–
LOD_{50} (95% confidence interval) cfu/sample	NA[a]	NA[a]	NA[a]
Infant formula			
Contamination level (cfu/10 g)	–	2	6
Number of participating collaborators	14	14	14
Number of collaborators retained after evaluation of the data	14	14	14
Number of samples	112	112	112
Number of samples retained after evaluation of the data	112	112	112
Test portion size in g	10	10	10
Sensitivity in %	–	98	100
Specificity in %	99	–	–
LOD50 (95% confidence interval) cfu/sample	NA[a]	NA[a]	NA[a]
Egg product			
Contamination level (cfu/10 g)	–	12	25
Number of participating collaborators 14	14	14	14
Number of collaborators retained after evaluation of the data	13	13	13
Number of samples	112	112	112
Number of samples retained after evaluation of the data	104	104	104
Test portion size in g	10	10	10
Sensitivity in %	–	100	100
Specificity in %	99	–	–
LOD_{50} (95% confidence interval) cfu/sample	NA[a]	NA[a]	NA[a]

[a] Not applicable due to high contamination levels.

the following formula: $0.59 = 1.64/(1.96 \times \sqrt{2})$.

The maximum value is 0.51 (0.87×0.59) as a difference between $\log_{10}$-transformed test results or 3.26 (100.51) as a ratio between test results. So results up to $\log_{10}$ 3.51 ($\log_{10}$ 3 + $\log_{10}$ 0,51) or 3260 (1000×3.26) do not indicate non-compliance with the limit.

3.4. Discussion

The standard deviations for repeatability and reproducibility vary between the different matrices. The values reported were mostly around or below 0,10 for repeatability and mostly around 0,30 or even below for reproducibility. The performance characteristics for the ISO enumeration methods for e.g. *Listeria monocytogenes* (ISO 11290-2, Anon., 2017d) and for *Campylobacter* (ISO 10272-2, Anon., 2017e) were also established under the CEN mandate M381, following ISO 7218 (Anonymous, 2007, 2013). The precision data of *Enterobacteriaceae* are in line with the precision data of *Listeria monocytogenes*. For both microorganisms the data are generally lower than the data found in the *Campylobacter* study (ISO 10272-2, Anon., 2017e), where most likely these values are higher due to the complicated nature of *Campylobacter* in culturing methods.

Values found for repeatability and reproducibility in earlier European Union funded validation studies for the enumeration in foods of coagulase-positive staphylococci (Anonymous, 1999, 2003), presumptive *Bacillus cereus* (Anonymous, 2004c) and *Clostridium perfringens* (Anonymous, 2004d) were similar to those obtained for *Enterobacteriaceae* in this study.

Table 4
Summary of the performance characteristics for the enumeration method for the matrices egg product, raw meat, animal feed, tiramisu, and infant formula.

Matrix and parameters	Contamination level		
	Low	Middle	High
Egg product			
Contamination level (log_{10} cfu/g)	3,5	4,2	5,8
Number of participating collaborators	13	13	13
Number of collaborators retained after data evaluation	12	12	11
Number of total samples (2 per level per collaborator)	26	26	26
Number of samples retained after evaluation of the data	24	24	22
Mean value contamination level (Σa (log_{10} cfu/g))	3,48	4,21	5,78
Repeatability standard deviation s_r (log_{10} cfu/g)	0,12	0,12	0,25
Repeatability limit r			
as difference on log_{10} scale (log_{10} cfu/g)	0,32	0,33	0,70
as ratio on normal scale (cfu/g)	2,10	2,14	5,03
Reproducibility standard deviation s_R (log_{10} cfu/g)	0,32	0,50	0,48
Reproducibility limit R			
as difference on log_{10} scale (log_{10} cfu/g)	0,91	1,39	1,33
as ratio on normal scale (cfu/g)	8,12	24,66	21,48
Raw meat			
Contamination level (log_{10} cfu/g)	2,4	3,7	3,8
Number of participating collaborators	13	13	13
Number of collaborators retained after data evaluation	11	11	12
Number of total samples (2 per level per collaborator)	26	26	26
Number of samples retained after evaluation of the data	22	22	24
Mean value contamination level (Σa (log_{10} cfu/g))	2,43	3,72	3,75
Repeatability standard deviation s_r (log_{10} cfu/g)	0,15	0,13	0,10
Repeatability limit r			
as difference on log_{10} scale (log_{10} cfu/g)	0,42	0,36	0,27
as ratio on normal scale (cfu/g)	2,65	2,28	1,86
Reproducibility standard deviation s_R (log_{10} cfu/g)	0,28	0,36	0,57
Reproducibility limit R			
as difference on log_{10} scale (log_{10} cfu/g)	0,79	1,02	1,61
as ratio on normal scale (cfu/g)	6,10	10,38	40,7
Animal feed			
Contamination level (log_{10} cfu/g)	1,5	2,5	3,5
Number of participating collaborators	13	13	13
Number of collaborators retained after data evaluation	10	12	11
Number of total samples (2 per level per collaborator)	26	26	26
Number of samples retained after evaluation of the data	20	24	22
Mean value contamination level (Σa (log_{10} cfu/g))	1,60	2,50	3,53
Repeatability standard deviation s_r (log_{10} cfu/g)	0,12	0,08	0,08
Repeatability limit r			
as difference on log_{10} scale (log_{10} cfu/g)	0,34	0,23	0,24
as ratio on normal scale (cfu/g)	2,19	1,68	1,72
Reproducibility standard deviation s_R (log_{10} cfu/g)	0,18	0,17	0,20
Reproducibility limit R			
as difference on log_{10} scale (log_{10} cfu/g)	0,50	0,46	0,56
as ratio on normal scale (cfu/g)	3,14	2,90	3,65
Pasteurized milk			
Contamination level (log_{10} cfu/g)	1,5	2,5	3,5
Number of participating collaborators	13	13	13
Number of collaborators retained after data evaluation	12	12	12
Number of total samples (2 per level per collaborator)	26	26	26
Number of samples retained after evaluation of the data	24	24	24
Mean value contamination level (Σa (log_{10} cfu/g))	1,53	2,40	3,46
Repeatability standard deviation s_r (log_{10} cfu/g)	0,14	0,08	0,11
Repeatability limit r			
as difference on log_{10} scale (log_{10} cfu/g)	0,40	0,24	0,30
as ratio on normal scale (cfu/g)	2,50	1,72	2,00
Reproducibility standard deviation s_R (log_{10} cfu/g)	0,24	0,18	0,19
Reproducibility limit R			
as difference on log_{10} scale (log_{10} cfu/g)	0,67	0,49	0,54
as ratio on normal scale (cfu/g)	4,66	3,10	3,45
Tiramisu			
Contamination level (log_{10} cfu/g)	2,4	3,7	3,8
Number of participating collaborators	13	13	13
Number of collaborators retained after data evaluation	11	11	11
Number of total samples (2 per level per collaborator)	26	26	26
Number of samples retained after evaluation of the data	22	22	24
Mean value contamination level (Σa (log10 cfu/g))	1,48	2,38	3,36
Repeatability standard deviation s_r (log_{10} cfu/g)	0,21	0,07	0,06
Repeatability limit r			

(*continued on next page*)

Table 4 (*continued*)

Matrix and parameters	Contamination level		
	Low	Middle	High
as difference on $\log_{10}$ scale ($\log_{10}$ cfu/g)	0,59	0,18	0,16
as ratio on normal scale (cfu/g)	2,93	1,53	1,45
Reproducibility standard deviation s_R ($\log_{10}$ cfu/g)	0,22	0,18	0,13
Reproducibility limit R			
as difference on $\log_{10}$ scale ($\log_{10}$ cfu/g)	0,61	0,52	0,36
as ratio on normal scale (cfu/g)	4,08	3,28	2,28

4. Conclusion

The method for detection of *Enterobacteriaceae* was validated for the matrices meat, tiramisu, infant formula, liquid egg and smoked salmon and showed a specificity and sensitivity above 95% for all matrices. An accurate LOD_{50} value could not be established for this method. The method for enumeration was validated for the matrices meat, tiramisu, infant formula, liquid egg and animal feed.

As a general indication of repeatability limit (r) and reproducibility limit (R), the following values (derived from the mean of the variance estimates for all levels per matrix tested in the interlaboratory study), may be used when testing samples: $r = 0.37$ (expressed as a difference between log10-transformed test results) and $R = 0.87$ (expressed as a difference between $\log_{10}$-transformed test results).

The precision data resulting from this interlaboratory studies indicate a sufficient precision of the qualitative and quantitative methods for *Enterobacteriaceae* and these data were therefore incorporated in the newly published ISO standards EN ISO 21528:2017 - Microbiology of the food chain - Horizontal methods for the detection and enumeration of *Enterobacteriaceae* - Part 1: Detection method, and Part 2: Colony-count technique (Anonymous, 2017a, 2017b).

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