Quantitative methods - 2022LR108 Neogen OP-EBAC. Summary report.

Method Comparison Study Report for the ISO 16140-2:2016 validation of Neogen One Plate Enterobacteriacae OP-EBAC, for the enumeration of Enterobacteriacae in broad range of foods

MicroVal study number: 2022LR108

Method/Kit name: Neogen One Plate Enterobacteriacae OP-EBAC

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Foreword

This report is prepared in accordance with ISO 16140-2:2016 and MicroVal technical committee interpretation of ISO 16140-2 v.1.0

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Method/Kit name: Neogen One Plate Enterobacteriacae OP-EBAC

Validation standard: Microbiology of the food chain— Method validation

Part 1: Vocabulary (ISO 16140-1:2016) and

Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method (ISO 16140-2:2016)

Reference method:

ISO 21528-2:2017 Microbiology of the food chain - Horizontal method for the detection and enumeration of Enterobacteriaceae. Part 2: Colony- count technique

Scope of validation: Broad range of foods

Certification organization: Lloyd's Register

List of abbreviations

- AL Acceptability Limit
- AP Accuracy Profile
- Art. Cont. Artificial contamination
- CFU Colony Forming Units
- CL confidence limit (usually 95%)
- EL Expert Laboratory
- $\bar{D}$ Average difference
- g Gram
- h Hour
- ILS Interlaboratory Study
- Inc/Ex Inclusivity and Exclusivity
- LOQ Level of Quantification
- MCS Method Comparison Study
- min minute
- ml Millilitre
- MR (MicroVal) Method Reviewer
- MVTC MicroVal Technical Committee
- EL Expert Laboratory
- n number of samples
- na not applicable
- neg negative (target not detected)
- NG no growth
- nt not tested
- RT Relative Trueness
- SD standard deviation of differences
- 10^{-1} dilution 10-fold dilution of original food
- 10^{-2} dilution 100-fold dilution of original food
- MRD Maximum recovery diluent
- VRBGA Violet Red Bile Glucose Agar

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1 Introduction

In this project a MicroVal validation study, based on ISO 16140-2:2016, of alternative method for the enumeration of Enterobacteriaceae in 5 different food categories was carried out by Campden BRI as the MicroVal Expert Laboratory.

The alternative method used was:

Neogen One Plate Enterobacteriacae OP-EBAC. This media consists of a selective base which enables the enumeration of Enterobacteriaceae, by their ability to ferment glucose to produce acid visualised by an indicator dye. OP-EBAC allows the enumeration of Enterobacteriaceae in 18-22 hours for all food products, using only one plate incubated at 37°C ±1°C. For this validation study, the minimum incubation time of 18h was used for OP-EBAC plates. Colonies typical of Enterobacteriaceae gave yellow colonies against a purple-blue background, surrounded by a yellow halo.

The reference method used was: ISO 21528-2:2017 Microbiology of the food chain - Horizontal method for the detection and enumeration of Enterobacteriaceae. Part 2: Colony- count technique.

Scope of the validation study was: a broad range of foods

Categories included:

- Milk and dairy products
- Fresh produce and fruits
- Raw poultry and meats (Combined category raw/ RTC meats and poultry)
- Ready to eat foods (Combined category RTE/RTRH meats, poultry and fish)
- Multicomponent foods

Criteria evaluated during the study have been:

- Relative trueness study;
- Accuracy profiles;
- Limits of quantification (LOQ);
- Inclusivity and exclusivity.

The final conclusion on the Method Comparison study is summarized below:

The alternative method OP-EBAC shows comparable performance to the reference method for the enumeration of *Enterobacteriaceae* in a broad range of foods.

2 Method protocols

The Method Comparison Study was carried out using 10 gram portions of sample material.

According to ISO 16140-2 the reference method and alternative methods were performed with, as far as possible, exactly the same sample

2.1 Reference method

See the flow diagram in Annex A.

Sample preparations used in the reference method were done according to ISO 6887-series parts 1, 2, 3, 4 and 5. Plating was done according to ISO 7218:2007+A1:2013 section 10.2.2 which says at least one plate per dilution shall be used with at least two successive dilutions. Two plates per dilution may also be used to improve reliability. If only one dilution was used, then two plates of this dilution were used to improve reliability of the results. Depending on the sample being tested and the expected contamination level, single or multiple dilutions were used with single or duplicate plates if considered necessary to improve the reliability of the calculated result and ensure at least two relevant plates were available for use in calculations.

2.2 Alternative method

See the flow diagram in Annex A.

The alternative method principle is based on chromogenic media. This media consists of a selective base which enables the enumeration of *Enterobacteriaceae*, by their ability to ferment glucose to produce acid visualised by an indicator dye. OP-EBAC allows the enumeration of *Enterobacteriaceae* in 18-22 hours for all food products, using only one plate incubated at 37°C ±1°C. For this validation study, the minimum incubation time of 18h was used for OP-EBAC plates. Colonies typical of *Enterobacteriaceae* gave yellow colonies against a purple-blue background, surrounded by a yellow halo.

The counts for OP-EBAC (cfu/g) were calculated using the value obtained for a single plate. This was in contrast to the reference method where two or more plates were used as outlined in ISO 7218. For the purposes of the validation study, if multiple plates were within the range of 10-300, the plate which was closest to the median value of 150 was selected.

There are however potential issues with this rule for duplicate 10^{-1} plates, as it could bias the calculation towards selecting the highest value available. To take any possible bias into account, the method comparison study results were recalculated using two additional calculation strategies:

- Calculation based on random result. For each sample containing duplicate -1 plates, the 1st plate inputted to the results sheet was selected for calculation.
- Calculation based on the lowest result. In any case where there are multiple plates within the range of 10-300, the plate which produces the lowest value has been used.

Analysis of the data showed that the results produced in the MCS meet the ISO 16140-2 criteria with all calculation methods tested.

2.3 Study design

Samples of product containing the target organism were diluted 1 in 10 with an appropriate diluent according to ISO 6887 and homonegised in a stomacher. Appropriate serial dilutions were made and all relevant dilutions were analysed using the reference method and alternative method.

The reference method and alternative methods were performed with as far as possible, exactly the same sample.

3 Method comparison study

3.1 Relative trueness study

The trueness study is a comparative study between the results obtained by the reference method and the results of the alternative method. This study was conducted using naturally or artificially contaminated samples. Different categories, types and items were tested for this.

A total of 5 categories were included in this validation study. A minimum of 15 items for each category were tested by both the reference method and the alternative method in the relative trueness study, with a minimum of 15 interpretable results per category.

Each category was made up of 3 types, with at least 5 items representative for each type.

3.1.1 Number of samples
The categories, the types and the number of samples analyzed are presented in Table 1.

Table 1 – Categories, types and number of samples analyzed

Category	Types	Items	No of samples	ISO 6887
Dairy products (Combined raw milk and dairy products and heat processed milk and dairy products)	Pasteurised dairy products	Milk based desserts, cream	5	6887-5
	Milk based products (raw and pasteurized milk)	Yogurt, raw milk	5	6887-5
	Dry milk	Milk powder, powder for	5	6887-5

Category	Types	Items	No of samples	ISO 6887
		milk based desserts		
Meat and poultry products (Raw and RTC)	Fresh meats (unprocessed)	Pork, lamb beef cuts and poultry mince	5	6887-2
	Ready to cook (processed) meat	Pork sausage, Marinated beef, Burger patties	5	6887-2
	Ready to cook (processed) poultry	Marinated chicken, turkey meat balls, chicken goujons	5	6887-2
Ready to eat foods (Combined category RTE/RTRH meats, poultry and fish)	Ready to eat meat and poultry	turkey fillet, pate	5	6887-2
	Cooked and cured fish products	roll herring, seafood terrine	5	6887-2
	Raw cured meat and poultry	salami, ham	5	6887-2
Fresh Produce and fruits	Ready to eat fruit	Fruit mix, Fruit drinks	5	6887-4
	Cut ready to eat vegetables/sprouts	Bagged pre-cut salads Vegetable juices Bean sprouts	5	6887-4
	Leafy greens	Basil, Lettuce, Parsley	5	6887-4
Multi-component foods or meal components	Composite foods with substantial raw ingredients	Chilled pasta salad, sandwiches	5	6887-2
	Mayonnaise based deli-salads	Vegetable salad	5	6887-2
	Ambient stable acidified foods	Ketchup, mayonnaise and mustard	5	6887-2

3.1.2 Test sample preparation

Naturally contaminated samples were preferentially analyzed. 62 samples were screened for the presence of the target organism. The distribution of samples screened by category is shown in Table 2. From these samples 31 % were positive for the target organism and these samples were used in the data analysis. The remaining 69% were negative for the target organism. It was therefore necessary to use artificial contamination procedures

Table 2 – Screening of samples for target organism

Category	**Samples screened**	**Natural contamination – positive for target**	**% of category naturally contaminated**
Dairy products	5/15	0/5	0
Meat and poultry (raw and RTC)	15/15	8/15	53
Fresh produce and fruits	12/15	8/12	53
Ready to eat foods	15/15	1/15	7
Multi component foods or meal components	5/15	4/5	27

Artificial contaminations were obtained by:

- Spiking with contaminated injured cells (e.g. heat treatment, cold storage);

- Seeding with strains isolated from the same samples type, before storage under relevant conditions to stress the cells. The storage parameters used were: 48 h to 72h at 4°C for chilled foods, at -20°C for 72 h to 18 days for frozen foods, and lyophilised strains for dry foods.

Each strain was used to inoculate no more than 5 samples during the study. A total of 13 strains were used to inoculate relative trueness samples.

All strains used for sample spiking were stressed following an injury protocol approriate to the food item being inoculated. The injury efficiency was evaluated by comparing enumeration results onto selective and non-selective agars (respectively NA and NA + 3% salt).

25 % of the samples analysed in the relative trueness study were naturally contaminated.

3.1.3 Protocols applied during the validation study

Incubation time

The minimum incubation time 18h was used for the study for the alternative method.

3.1.4 Test results

The samples were analyzed by the reference and the alternative methods in order to have 15 interpretable results per incubation protocol, and 5 interpretable results per tested type.

The data are classified in three categories (See Table 3):

- Interpretable results by the two methods;

- Results with less than 4 colonies per plate with one of the two methods.These results were not kept for calculation in order to have the more precise result.

- Results below or above the quantification limit.

Table 3 - Classification of the samples

Category	**Number of samples with interpretable results**	**Number of samples with <4 CFU /plate**	**Number of samples below or above the quantification limit**	**Number of non-interpretable results**
Dairy products	15	0	5	5
Meat and poultry (raw and RTC)	15	2	5	7
Fresh produce and fruits	15	1	3	4
Ready to eat foods	15	0	15	15
Multi component foods or meal components	15	2	2	4
Total	**75**	**5**	**30**	**35**

3.1.5 Calculation and interpretation of relative trueness study
The calculations are provided in Annex B.

The obtained data were analyzed using the scatter plot. The graphs are provided with the line of identity (y = x).

Figure 1-5 shows the scatter plot for the individual categories.

Figure 6 shows the scatter plot for all categories.

Figure 1 - Scatter plot of the reference method versus alternative method results for the Dairy category

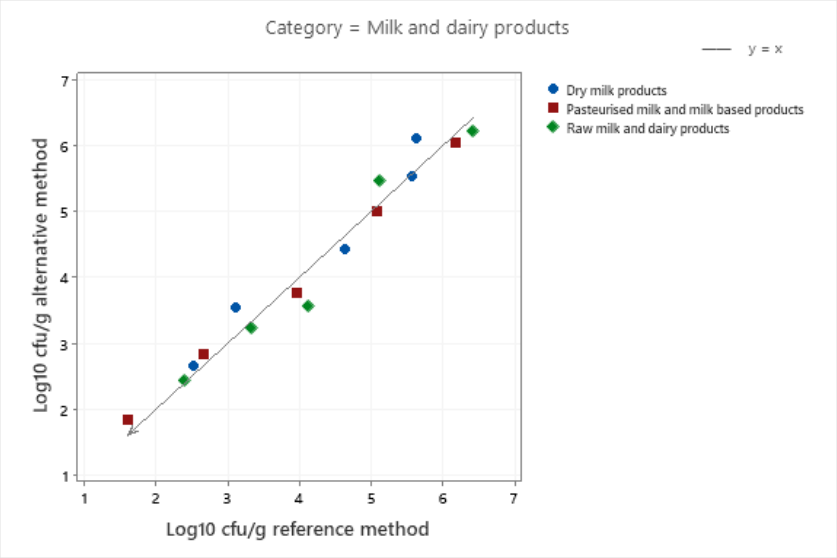


Figure 2- Scatter plot of the reference method versus alternative method results for the Fresh produce category

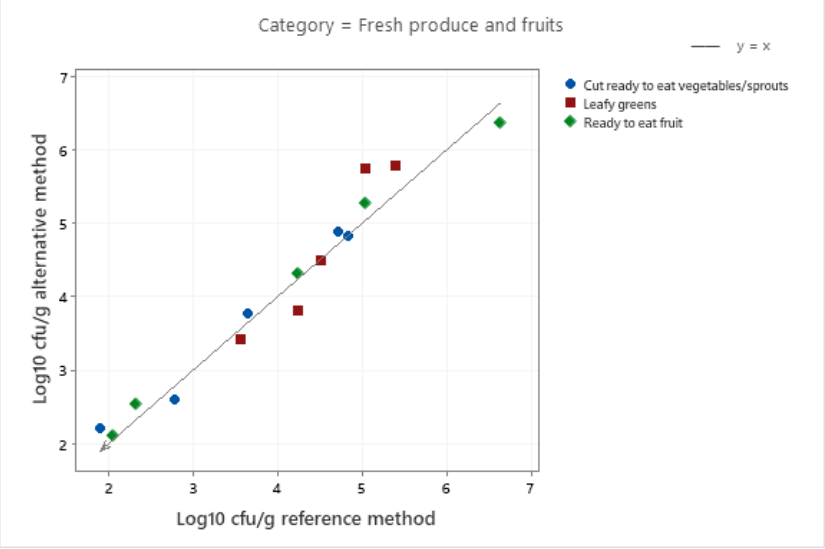


Figure 3- Scatter plot of the reference method versus alternative method results for the Ready to eat foods category

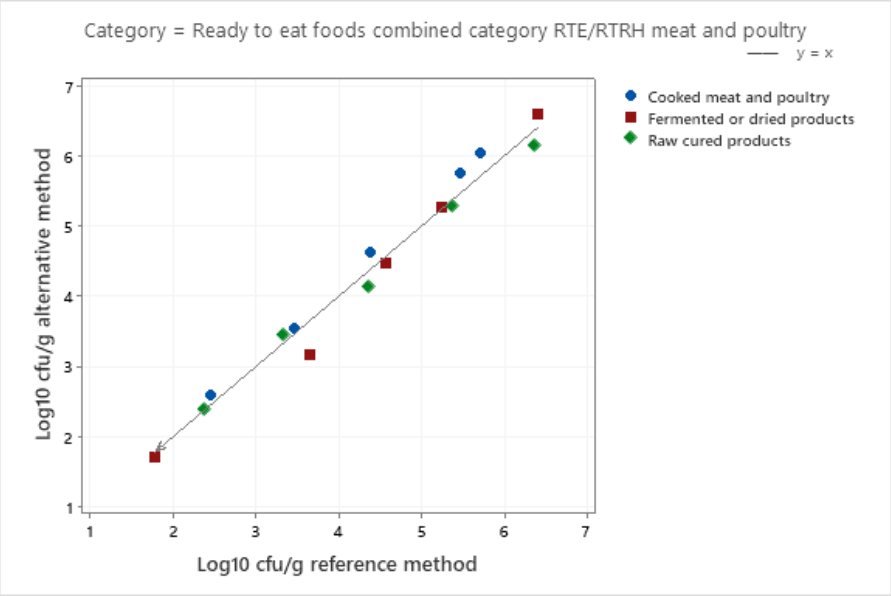


Figure 4- Scatter plot of the reference method versus alternative method results for the Meat and poultry (raw and ready to cook) category

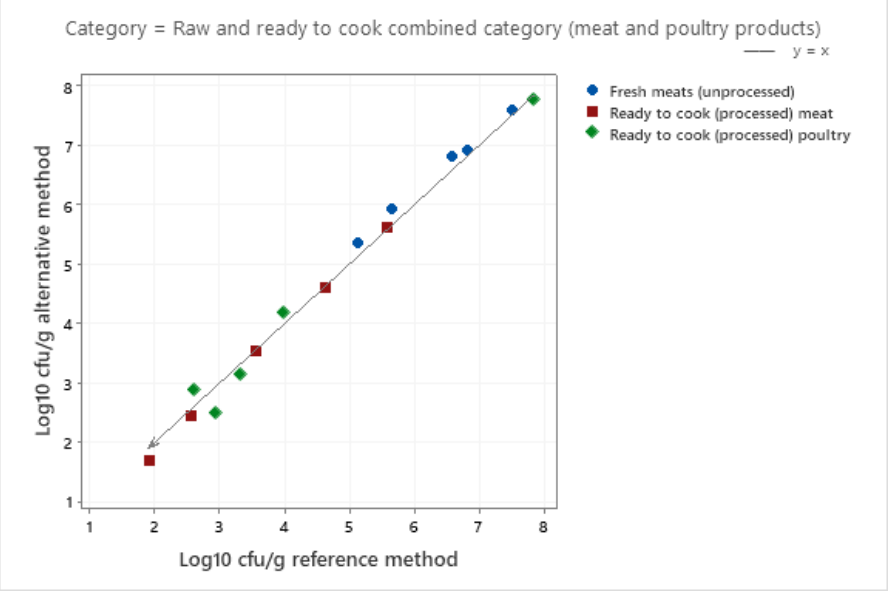


Figure 5- Scatter plot of the reference method versus alternative method results for the Multicomponent foods category

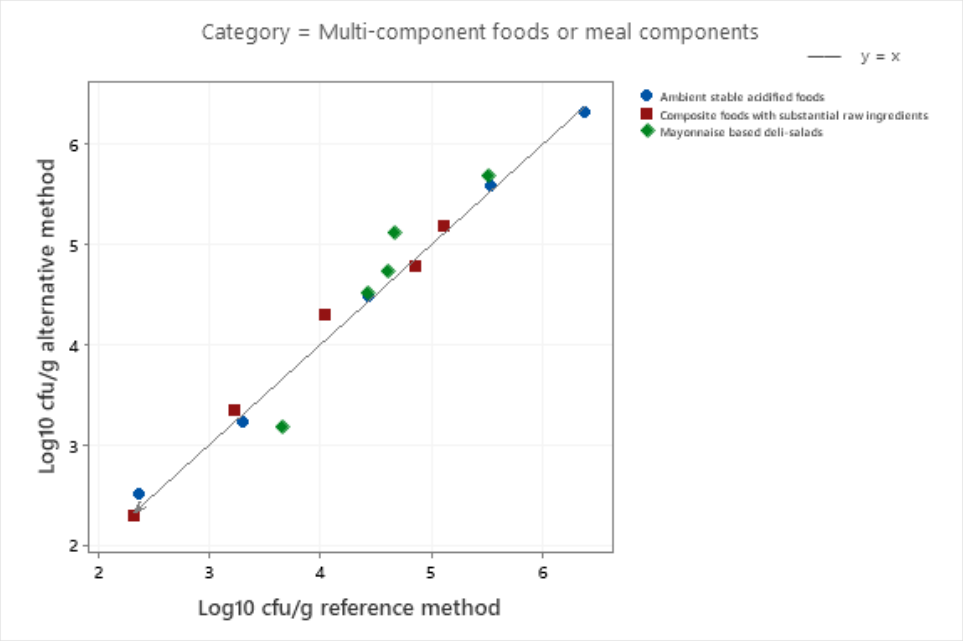
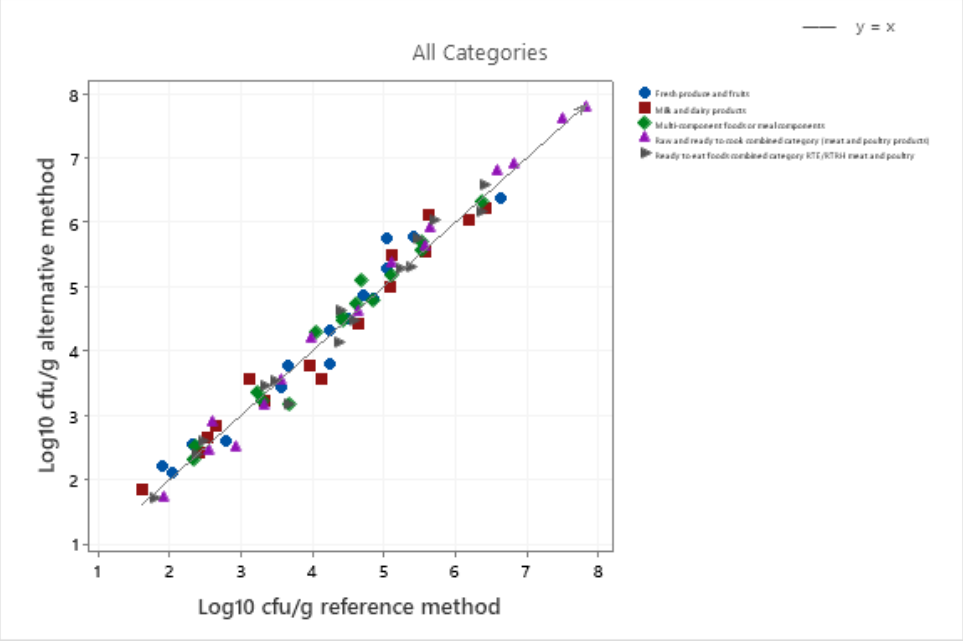


Figure 6 - Scatter plot of the reference method versus alternative method results for all the categories



According to ISO16140-2:2016 6.1.2.3, the results of the scatter plot are interpreted on the visual observation of the amount of bias and extreme results. The data in the scatter plots show no obvious disagreement.

A summary of the calculated values per category is provided in Table 4.

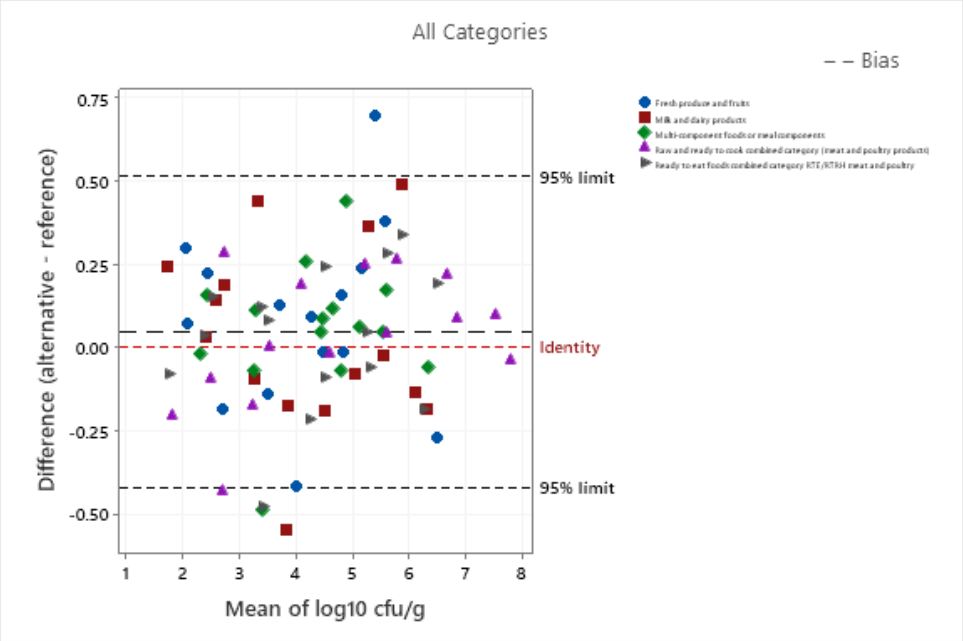
Table 4 - Summary of the calculated values per category

Category	n	$\bar{D}$	SD	95 % low limit	95 % upper limit
Fresh produce and fruits	15	0.08	0.28	-0.53	0.70
Milk and dairy products	15	0.03	0.28	-0.59	0.65
Multi-component foods or meal components	15	0.05	0.20	-0.39	0.50
Raw and ready to cook combined category (meat and poultry products)	15	0.03	0.20	-0.42	0.48
Ready to eat foods combined category RTE/RTRH meat and poultry	15	0.03	0.22	-0.45	0.51
All products	75	0.05	0.23	-0.42	0.51

$\bar{D}$: Average difference SD: standard deviation of differences n: number of samples

The Bland-Altman difference plot for all the samples is given Figure 7.

Figure 7 – Bland-Altman difference plot for all samples



Samples for which the difference between the result observed with the reference and the alternative methods is above or lower than the limits are listed in the Table 5.

Table 5 - Data which are outside of the accepted limits

Category	**Type**	**N° Sample**	**Reference method Log cfu/g**	**Alternative method Log cfu/g**	**Mean Log cfu/g**	**Difference Alternative – reference)**	**Lower / Upper limits**	**Comments**
Fresh produce and fruits	Leafy greens	D11	5.04	5.74	5.39	0.70	Upper limit 0.51	Naturally contaminated
Dairy products	Raw milk and dairy products	A3	4.11	3.57	3.84	-0.55	Lower limit -0.42	Seeding protocol, inoculated with *Escherichia adecarboxylata* and chilled for 72 hours
Multicomponent foods	Mayonnaise-based deli salads	E7	3.66	3.18	3.42	-0.49	Lower limit -0.42	Naturally contaminated
RTE/RTRH foods	Cooked and cured fish products	C7	3.65	3.18	3.41	-0.48	Lower limit -0.42	Inoculated with heat stressed *Proteus mirabilis*

It is expected that not more than one in 20 data values will lie outside the CLs. In this study, there were 4 data points from a total of 75 data points which were outside of the accepted limits.

Each data point that lies outside the acceptability limits belongs to a different food category, suggesting that the results are random outliers in the study. Further analysis of the results outside the limits revealed that two samples were naturally contaminated, and the remaining two samples were artificially contaminated. The results indicate that the type of contamination did not influence the likelihood of obtaining a result outside of the calculated limits.

The two artificially contaminated samples with points outside of the limits were inoculated following different protocols, One sample (A3, raw milk) was inoculated with a heat stressed organism and the other sample (C7, salmon pate) was inoculated and then stored chilled for 72 hours.

Additional analysis showed that two of the four samples with differences outside the calculated limits are within -0.5 log. There is no indication of systematic bias in this study, with a slight overall positive bias of 0.05 obtained for all categories tested. As a result of the good agreement between the reference and alternative methods, the calculated acceptability limits are relatively narrow at -0.42 and 0.51.

3.1.6 Conclusion (RT study)

The relative trueness of the Alternative method is satisfied as it shows comparative performance to the reference method. Whilst the expectation of not more than 1 in 20 data points outside of the acceptability limits was not met there is no trend indication of systematic bias regarding sample type or contamination procedures. The bias on each category is minimal.

3.2 Accuracy profile study

The accuracy profile study is a comparative study between the results obtained by the reference and the results of the alternative method. This study is conducted using artificially contaminated samples, using one type per category.

3.2.1 Categories, sample types and strains

Five food categories were tested with a single batch of two different food types using 6 samples per type.

Two samples were contaminated at a low level, 2 at intermediate level, 2 at a high level. For each sample, 5 replicates (5 different test portions) were tested. A total of 30 samples were analysed per food type. The following food type/strain pairs were studied (See Table 6):

Each sample was bulk inoculated and five replicate test portions examined from the bulk sample.

A 100g sample was inoculated with 1ml of appropriate dilution of inoculating strain and homogenised by hand massaging or stomaching to evenly distribute the inoculum. For all matrices, the 100g samples will be inoculated and stored at 2-8°C for 48-72h prior to analysis.

***Table 6* - Categories, types, items, strains and inoculation levels for accuracy profile study**

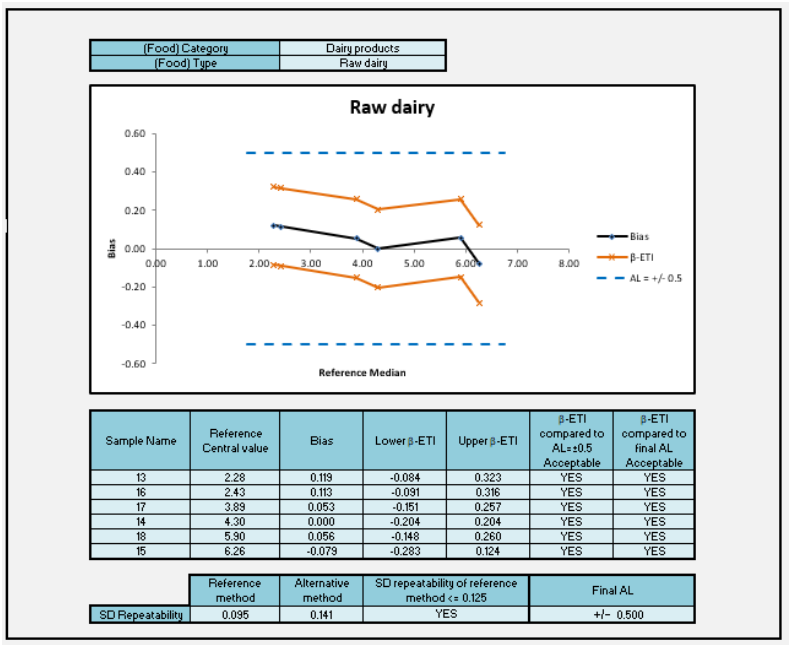
Category	Types	Strain Enterobacteriaceae	Item
Dairy products Combined raw milk and dairy products and heat processed milk and dairy products	Milk based products (raw and pasteurized milk)	*Leclercia adecarboxylata* CRA 5501 Isolated from skimmed milk powder	Raw milk
			Raw milk cheese
Ready to eat foods (Combined category RTE/RTRH meats, poultry and fish)	Cooked and cured fish products	*Lelliottia amnigena* NCIMB 2118 isolated from seawater	Seafood terrine
			Salmon pate
Produce and fruits (combined category fresh and processed)	Cut ready to eat vegetables	*Citrobacter amalonaticus* CRA 7458 isolated from beansprouts	Lettuce
			Spinach
Meat and poultry products (Raw and RTC)	Fresh meats (unprocessed)	*Escherichia fergusonii* CRA 7522 isolated from sausages	Raw ground beef
			Chicken breast fillets
Multicomponent	Composite foods with raw /processed ingredients	*Atlantibacter hermanii* CRA 7477 isolated from sesame seeds	Sandwich
			Pasta salad

3.2.2 Calculations and interpretation of accuracy profile study

The summary tables (in log CFU/g) are provided in Annex C. The statistical results and the accuracy profiles are provided in Figures 8-12.

The calculations were done using the AP Calculation Tool MCS (Clause 6-1-3-3 calculation and interpretation of accuracy profile study) available on http://standards.iso.org/iso/16140

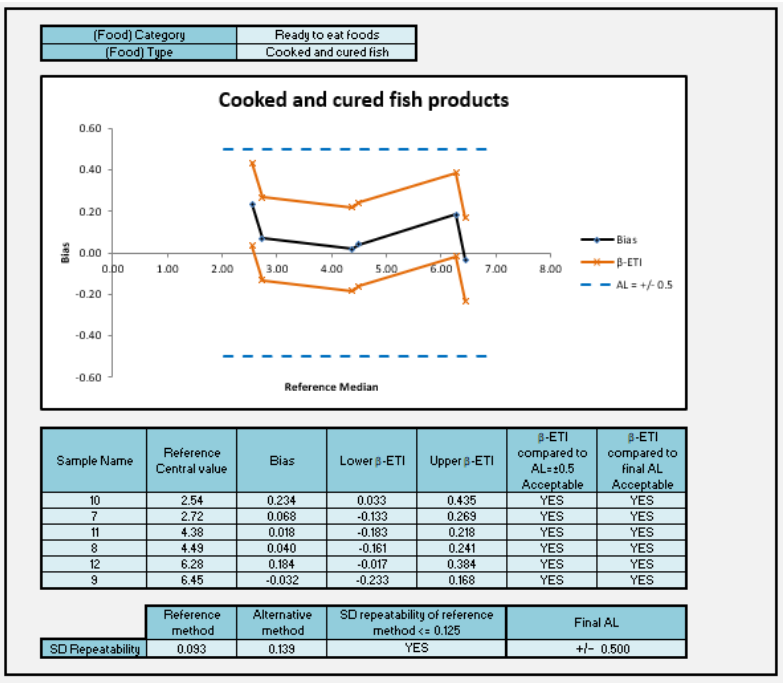
Figure 8 – Accuracy Profile graph for dairy category



Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
13	2.28	0.119	-0.084	0.323	YES	YES
16	2.43	0.113	-0.091	0.316	YES	YES
17	3.89	0.053	-0.151	0.257	YES	YES
14	4.30	0.000	-0.204	0.204	YES	YES
18	5.90	0.056	-0.148	0.260	YES	YES
15	6.26	-0.079	-0.283	0.124	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.095	0.141	YES	+/- 0.500

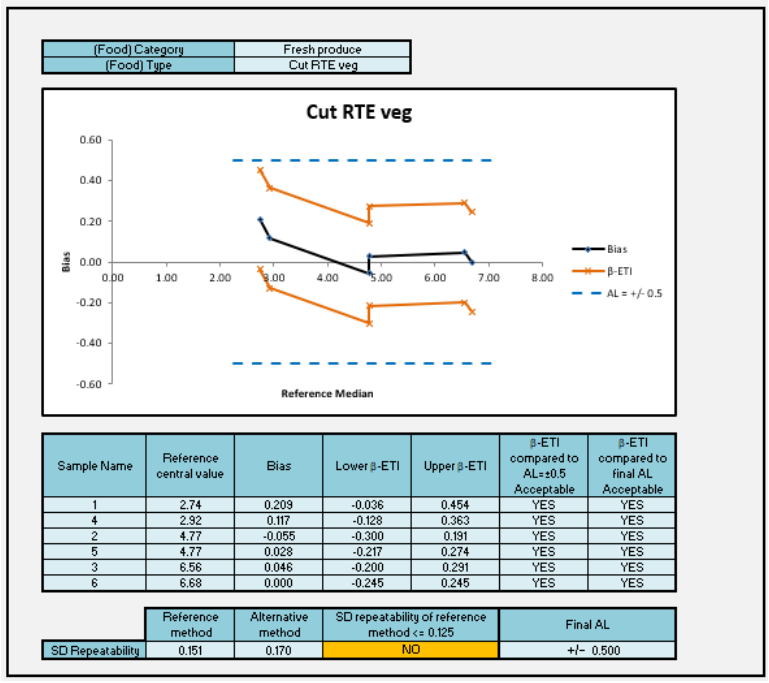
Figure 9 – Accuracy Profile graph for ready to eat foods



Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
10	2.54	0.234	0.033	0.435	YES	YES
7	2.72	0.068	-0.133	0.269	YES	YES
11	4.38	0.018	-0.183	0.218	YES	YES
8	4.49	0.040	-0.161	0.241	YES	YES
12	6.28	0.184	-0.017	0.384	YES	YES
9	6.45	-0.032	-0.233	0.168	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.093	0.139	YES	+/- 0.500

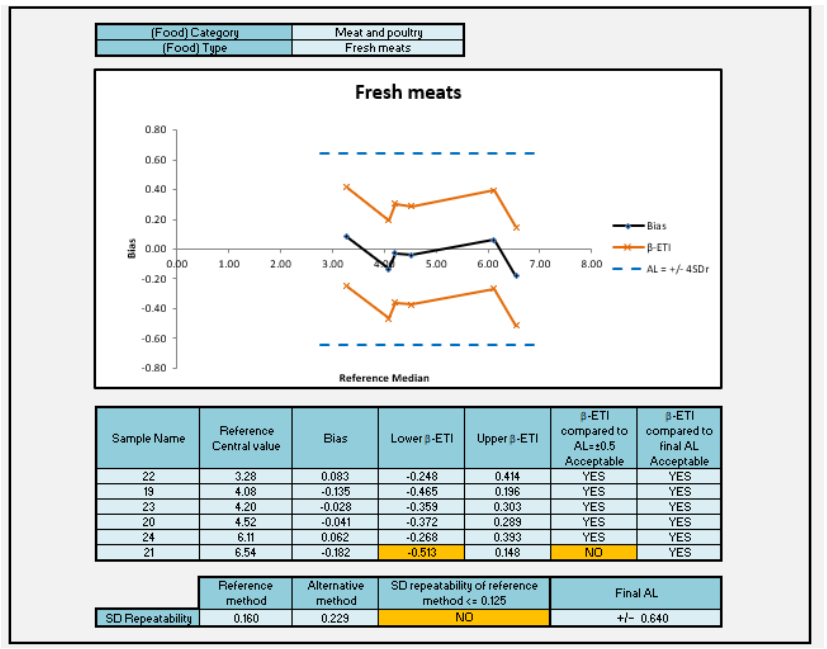
Figure 10 – Accuracy Profile graph for produce and fruits



Sample Name	Reference central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
1	2.74	0.209	-0.036	0.454	YES	YES
4	2.92	0.117	-0.128	0.363	YES	YES
2	4.77	-0.055	-0.300	0.191	YES	YES
5	4.77	0.028	-0.217	0.274	YES	YES
3	6.56	0.046	-0.200	0.291	YES	YES
6	6.68	0.000	-0.245	0.245	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.151	0.170	NO	+/- 0.500

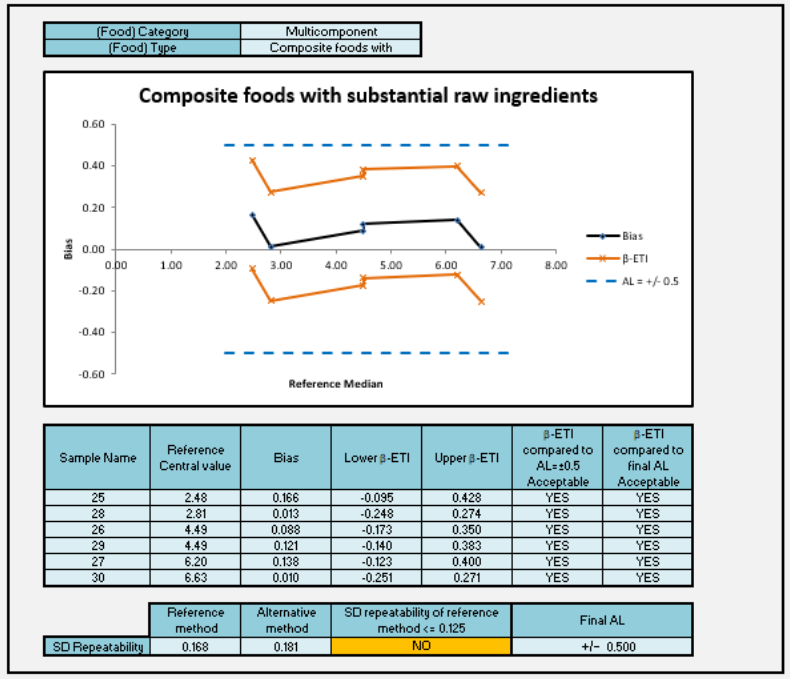
Figure 11 – Accuracy Profile graph for meat and poultry products (raw and ready to cook)



Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
22	3.28	0.083	-0.248	0.414	YES	YES
19	4.08	-0.135	-0.465	0.196	YES	YES
23	4.20	-0.028	-0.359	0.303	YES	YES
20	4.52	-0.041	-0.372	0.289	YES	YES
24	6.11	0.062	-0.268	0.393	YES	YES
21	6.54	-0.182	-0.513	0.148	NO	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.160	0.229	NO	+/- 0.640

Figure 12 – Accuracy Profile graph for multicomponent



Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
25	2.48	0.166	-0.095	0.428	YES	YES
28	2.81	0.013	-0.248	0.274	YES	YES
26	4.49	0.088	-0.173	0.350	YES	YES
29	4.49	0.121	-0.140	0.383	YES	YES
27	6.20	0.138	-0.123	0.400	YES	YES
30	6.63	0.010	-0.251	0.271	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.168	0.181	NO	+/- 0.500

Comments

In this study the following categories met the AL of 0.5log: multicomponent foods, ready to eat foods, fresh produce and dairy products.

In this study, the following categories required the new AL to be calculated: Meat and poultry (Raw and RTC), this category met the new AL value of 0.640 log.

The accuracy of the Alternative method is satisfied as all categories met the 0.5log AL or the re-calculated AL.

3.3 Inclusivity / exclusivity

Inclusivity is the ability of the alternative method to detect the target analyte from a wide range of strains. Exclusivity is the lack of interference from a relevant range of non-target strains of the alternative method.

3.3.1 Protocols

- Inclusivity

51 strains were grown in NB medium at 37°C overnight. Each strain was tested once with the alternative method, the reference method and a non-selective agar.

- Exclusivity

30 cultures were grown in an appropriate non-selective medium at under appropriate conditions. Each strain was tested once with the alternative method, the reference method and a non-selective agar.

3.3.2 Results

All raw data are given in Annex D.

- Inclusivity

A total of 51 strains were tested for inclusivity. 51 of these strains showed a positive result with the reference and alternative methods.

- Exclusivity

A total of 30 strains were tested for exclusivity. 29 of these strains showed a negative result on the reference and alternative methods. The remaining strain (*Acinetobacter calcoaceticus*, CRA 7421) gave growth on VRBGA and OP-EBAC, although the colony morphology was atypical on both media types. Additional analysis of the colonies by MALDI ToF identified the isolates as *Acinetobacter* species,verifying that this is a negative result.

3.3.3 Conclusion
The alternative method, Neogen OP-EBAC, for enumeration of Enterobacteriaceae is selective and specific.

3.4 Conclusion (MCS)

Overall, the conclusions for the Method Comparison are:

- The alternative method, Neogen OP-EBAC, for enumeration of Enterobacteriaceae shows satisfactory results for relative trueness;

- The alternative method, Neogen OP-EBAC, for enumeration of Enterobacteriaceae shows satisfactory results for accuracy profile;
- The alternative method, Neogen OP-EBAC, for enumeration of Enterobacteriaceae is selective and specific.

4 Interlaboratory study

The inter-laboratory study is a study performed by multiple laboratories testing identical samples at the same time, the results of which are used to estimate alternative-method performance parameters.

4.1 Study organisation

4.1.1 Collaborators
Samples were sent to 12 laboratories; each laboratory had one participant. The collaborators were from four countries: England, Italy, Slovakia, Austria

4.1.2 Matrix and strain used
Breaded vegetable sticks were inoculated with *E.coli* CRA 108 (isolated from breaded fishcakes).

4.1.3 Sample preparation
Samples were prepared and inoculated on 18th March 2024 as described below:

For each collaborator, a set of samples was prepared containing 2 samples at a low level, two samples at a medium level, two samples at a high level and a single uninoculated blank sample. The samples were blind-coded so that the collaborators did not know the intended contamination level. For laboratories where there were two different collaborators, a different set of codes were used for each collaborator. A set of samples was also prepared for the EL although the data from these was not used in the data analysis

The target levels and codes are shown in Table 7.

Table 7 : Contamination levels

Contamination level	Sample code Collaborator
Uninoculated	4
Low (10^2 cfu/g)	1
Low (10^2 cfu/g)	5
Medium (10^4 cfu/g)	2
Medium (10^4 cfu/g)	6
High (10^6 cfu/g)	3
High (10^6 cfu/g)	7

4.1.4 Labelling and shipping
Blind coded samples were placed in isothermal boxes, which contained cooling blocks, and express-shipped to the different laboratories.

A temperature control flask containing a sensor was added to the package in order to register the temperature profile during the transport, the package delivery and storage until analyses.

Samples were shipped in 24 h to 48 h to the involved laboratories. The temperature conditions were required to stay lower or equal to 8°C during transport, and ≤-18°C during storage in the laboratories.

4.1.5 Analysis of Samples
Collaborative study laboratories and the expert laboratory carried out the analyses on either Thursday 21st March or Monday 25th March with the alternative and reference methods. The majority of participants performed the analysis on Monday 25th March. Three participants performed the testing on Thursday 21st March due to staffing availability. The expert lab performed the analysis on both testing days. The analyses by the reference method and the alternative method were performed on the same day.

4.2 Experimental parameters controls

4.2.1 Detection of Enterobacteriaceae in the matrix before inoculation
In order to detect the presence of *Enterobacteriaceae,* the reference method was performed on five portions (10 g) before the inoculation. All the results were negative.

4.2.2 Strain stability during transport

Duplicate samples inoculated at two levels (10^2 and 10^6 cfu/g) were tested for the enumeration of total viable count after 7 days of storage at ≤-18°C (Table 8). Frozen samples were thawed under controlled conditions prior to analysis.

Table 8 – E.coli stability in the matrix

Day	Alternative method (log cfu/g)				Reference method (log cfu/g)			
	Level 1		Level 2		Level 1		Level 2	
	A	B	A	B	A	B	A	B
Day 0	3.8	3.8	6.6	6.7	3.8	3.7	6.7	6.4
Day 7	3.6	3.8	6.4	6.7	3.7	3.7	6.2	6.5

4.2.3 Logistic conditions

The temperatures measured at receipt by the collaborators, the temperatures registered by the thermo-probe, and the receipt dates are given in Table 9.

Table 9 - Sample temperatures at receipt

Collaborator	Average Temperature measured by the probe (°C)	Temperature measured at receipt (°C)	Receipt date and time	Analysis date
1	-9.1	-1.6	20/03/2024 9:00	21/3/2024
2	-14.5	-1.4	20/03/2024 10:00	25/3/2024
3	-16.7	-1.8	20/03/2024 10:00	25/3/2024
4	-14.5	1.2	20/03/2024 15:06	25/3/2024
5	-13.5	-1.0	20/03/2024 10:50	25/3/2024
6	-11.3	-1.7	20/03/2024 10:30	25/3/2024
7	No data received			

Collaborator	Average Temperature measured by the probe (°C)	Temperature measured at receipt (°C)	Receipt date and time	Analysis date
8	-4.9°C	0.0	21/03/2024 14:00	21/03/2024
9	-8.5	1.1	22/03/2024 15:00	25/03/2024
10	No data received			
11	-8.9	-4	20/03/2024 14:00	21/03/2024
12	-9.2	-0.1	20/03/2024 9:50	25/3/2024

No issues was encountered during the transport or at receipt for the collaborators. All the samples were delivered on time and in appropriate conditions. Temperatures during shipment and at receipt were all correct.

Two participants did not return the data in time for the analysis.

4.3 Calculation and summary of data

4.3.1 MicroVal Expert laboratory results

The results obtained by the expert laboratory are given in Tables 10 and 11.

Table 10 – Results obtained by the expert lab – stored in transport box

Level	Reference method	Alternative method
Blank	4.1	3.5
Low	3.1	3.7
Low	3.7	3.1
Medium	4.8	4.9
Medium	5.4	5.4
High	6.7	6.8
High	7.0	7.0

Table 11 – Results obtained by the expert lab – stored at ≤-18≤for 7 days

Level	Reference method	Alternative method
Blank	4.7	4.5
Low	4.8	4.1
Low	3.8	4.4
Medium	5.0	5.0
Medium	5.0	5.1
High	6.8	7.0
High	6.9	6.9

4.3.2 Results obtained by the collaborative laboratories

The data from the collaborative trial were calculated and interpreted according to section 6.2.3 of ISO 16140-2:2016 using the freely available Excel® spreadsheet (http://standards.iso.org/iso/16140). Version 14-03-2016 was used for these calculations.

The results obtained by the collaborators are shown in Table 12.

The accuracy profile plot is shown in Figure 13 and the statistical analysis of the data shown in Table 13.

Table 12. Summary of the results of the interlaboratory study per analyte level.

Collaborator	**Level**	**Reference method (Log cfu/g)**		**Alternative method (Log cfu/g)**	
		Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
01	low	4.9	4.7	4.3	3.7
02	low	3.0	3.0	3.1	3.0
03	low	3.5	3.6	3.1	3.2
04	low	3.3	3.6	3.5	3.4
05	low	3.7	2.6	3.6	2.6
06	low	3.3	3.4	3.2	3.2
07	low	3.4	3.3	3.3	3.4
08	low	4.1	4.3	4.1	4.7

Collaborator	Level	Reference method (Log cfu/g)		Alternative method (Log cfu/g)	
		Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
01	medium	4.7	5.1	4.4	5.1
02	medium	4.7	4.7	4.8	4.8
03	medium	4.8	4.7	4.6	4.8
04	medium	4.8	5.3	4.7	5.1
05	medium	4.9	3.2	4.6	2.9
06	medium	4.6	4.4	4.5	4.7
07	medium	4.7	5.1	4.7	5.0
08	medium	5.1	5.1	5.2	5.2
01	high	6.7	6.5	6.8	6.5
02	high	6.6	7.0	6.8	7.0
03	high	6.8	7.8	6.5	7.2
04	high	6.8	7.3	6.9	7.1
05	high	6.8	6.8	6.8	5.8
06	high	6.4	6.9	6.6	6.9
07	high	6.7	7.2	6.1	7.0
08	high	7.0	7.3	7.0	7.5
01	blank	3.1		3.6	
02	blank	<1		<1	
03	blank	<1		<1	
04	blank	3.8		<1	
05	blank	<1		<1	
06	blank	1.9		1.3	
07	blank	2.7		2.5	
08	blank	3.1		3.2	

Two labs have been excluded from the data analysis. This is because the uninoculated sample was at 10^4 cfu/g.

The high level of natural contamination observed in uninoculated samples explored in Section 4.4.

Lab number 4 observed a discrepant result for the blank sample. These results are not consistent with the results of the uninoculated sample for the other participants. The other samples plated by this lab showed good agreement between alternative and reference methods. It is therefore likely that this sample was not plated on the One Plate EBAC agar.

Figure 13. Accuracy profile of One Plate EBAC from the ILS

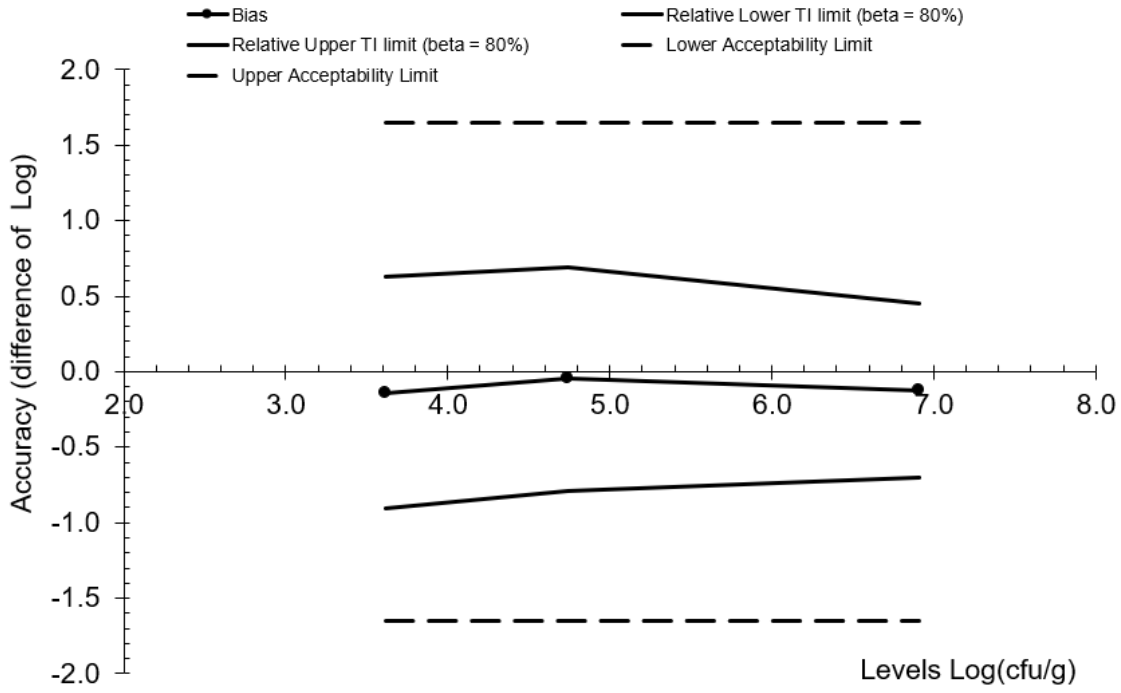


Table 13. Statistical analysis of the ILS data according to the ISO spreadsheet

	Alternative method			Reference method		
Levels	**Low**	**Medium**	**High**	**Low**	**Medium**	**High**
Target value	3.616	4.742	6.911			
Number of participants (K)	8	8	8	8	8	8
Average for alternative method	3.477	4.692	6.786	3.616	4.742	6.911
Repeatability standard deviation (sr)	0.320	0.487	0.416	0.301	0.480	0.341
Between-labs standard deviation (sL)	0.425	0.212	0.044	0.557	0.000	0.050
Reproducibility standard deviation (sR)	0.532	0.531	0.419	0.633	0.480	0.345
Corrected number of dof	10.004	14.270	14.909	8.773	14.933	14.885
Coverage factor	1.441	1.392	1.383			
Interpolated Student t	1.372	1.344	1.341			
Tolerance interval standard deviation	0.5590	0.5500	0.4319			
Lower TI limit	2.710	3.953	6.207			
Upper TI limit	4.244	5.431	7.365			
Bias	-0.138	-0.049	-0.125			
Relative Lower TI limit (beta = 80%)	-0.906	-0.789	-0.704			
Relative Upper TI limit (beta = 80%)	0.629	0.690	0.454			
Lower Acceptability Limit	-1.65	-1.65	-1.65			
Upper Acceptability Limit	1.65	1.65	1.65			
New acceptability limits may be based on reference method pooled variance						
Pooled repro standard dev of reference	0.500					

TRUE
TRUE

Select ALL blue lines to draw the accuracy profile as illustrated in the worksheet "Graph Profile"

The data falls within the acceptability limits and meets the ISO 16140-2 criteria. A review of the accuracy profile and statistical analysis revealed that there was a high acceptability limit of 1.65 and -1.65 observed in

the ILS. To investigate possible reasons for the high AL seen in the ILS, a root cause analysis was carried out.

4.4 Root cause analysis

4.4.1 Natural contamination in blank samples

Data revealed that natural contamination was observed in the blank samples tested by the participants. The natural contamination was highly variable between laboratories, ranging from <1 log cfu/g to 4.4 log cfu/g.

The product was breaded vegetable sticks, a frozen ready-to-cook multicomponent product.

The ingredients are as follows: Vegetables (50%) (Peas, Carrot, Potato, Sweetcorn), Wheat Flour (**Wheat** Flour, Calcium Carbonate, Iron, Niacin, Thiamin), Water, Dried Potato, Sunflower Oil, Salt, Rapeseed Oil, Yeast, White Pepper.

Natural contamination of sample was not observed in the stability or screening study.

Additional analysis of the colonies growing on the blank sample plates was carried out as part of the investigation. The colonies were identified as *Pantoa agglomerans* using MADLI-ToF by the expert laboratory and 1 of the participants.

Further work revealed that colonies isolated from the inoculated samples on both reference and alternative plates were identified as a mixture of *Pantoa agglomerans* and the inoculating strain, *E. coli*.

4.4.2 Large acceptability limits

The acceptability limits for the accuracy profile were recalculated to ±1.65 log cfu/g. The possible reasons for this are explored below.

The same batches of media were used by all participants. The incubation times and temperatures were correct.

Table 15 shows the repeatability of the reference and alternative methods.

Table 15. Repeatability of the reference and alternative methods

Method	Low	Medium	High
Reference	0.301	0.480	0.341
Alternative	0.320	0.487	0.416

The repeatability of the reference and alternative methods is consistently high at all levels of contamination.

Table 16 shows the standard deviation between labs.

Table 16. Standard deviation between labs of the reference and alternative methods

Method	Low	Medium	High
Reference	0.633	0.480	0.345
Alternative	0.532	0.531	0.419

The standard deviation between labs is very high for both reference and alternative methods, particularly at the low and medium levels. This is likely to be the cause of the large, recalculated acceptability limits.

The large standard deviation between labs can be explained by the high variability in natural contamination of *Enterobacteriaceae* present in the samples.

An alternative explanation for the large standard deviation is the potential variation in temperature and transport conditions between participants. The sample is a frozen, ready to cook product and deviations in temperature could impact the overall microflora of the sample, including the level of *Enterobacteriaceae*.

4.4.3 Impact of natural contamination on low-level samples

The level of *Enterobacteriaceae* enumerated in low-level samples is higher than the anticipated level of 10^2 cfu/g. This was due to natural contamination of *Pantoa agglomerans* in the sample leading to an increased count of 10^3 cfu/g.

The performance of the alternative media at the lower range of enumeration has been demonstrated in the Method Comparison Study. 21% of samples tested for relative trueness contained natural or artificial contamination at a level of 10^2 cfu/g. 25% of samples tested for accuracy profile contained artificial contamination at a level of 10^2 cfu/g. In these parts of the study, good agreement between the reference and alternative methods was demonstrated.

A previous interlaboratory study was performed where both reference and alternative methods experienced zero counts in the low level samples due to instability of the strain in the matrix. Despite the issues in the inoculation of samples in the previous interlaboratory study, the available results demonstrated good agreement between the reference and alternative methods within the 10^2-10^3 cfu/g range.

4.4.4 Performance of media
The accuracy profile interlaboratory study data shows agreement between reference and alternative methods. A slight negative bias was observed and low and high levels: -0.138 and -0.125. The bias of the medium level was minimum (-0.049).

4.4.5 Conclusions of root cause analysis
There is a large amount of natural contamination in samples which has been identified as *Pantoa agglomerans*. The natural contamination observed is variable between labs which has caused large re-calculated acceptability limits and higher levels of *Enterobacteriaceae* than inoculated.

It has been accepted by the MicroVal Technical Committee that the recalculated acceptability limits are due to an issue with the preparation of samples, rather than an issue with the performance of the alternative method.

The accuracy profile data meets the ISO 16140-2 requirements and shows agreement between reference and alternative methods.

5 Overall conclusions of the validation study

- The alternative method, One Plate EBAC, for enumeration of *Enterobacteriaceae* shows satisfactory results for relative trueness;
- The alternative method, One Plate EBAC, for enumeration of *Enterobacteriaceae* shows satisfactory results for accuracy profile;
- The alternative method, One Plate EBAC, for enumeration of *Enterobacteriaceae* is selective and specific.
- The alternative method, One Plate EBAC, for enumeration of *Enterobacteriaceae* shows satisfactory performance in the ILS
- The alternative method, One Plate EBAC, for enumeration of *Enterobacteriaceae* shows comparable performance to the reference method ISO 21528-2

10 June 2024

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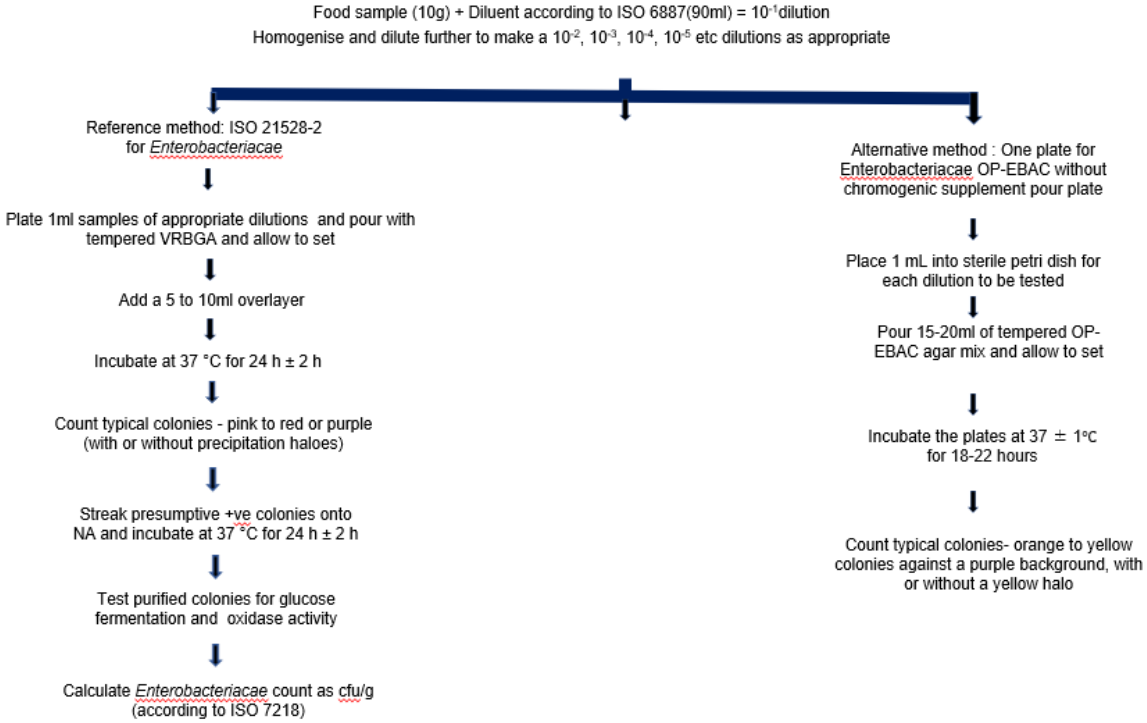
ANNEX A: Flow diagram of the reference and alternate method



ANNEX B: Calculation and interpretation of relative trueness

Type	Code	Sample	log(Ref)	log(Alt)	Mean	Difference
Category: Milk and dairy products						
Raw milk and dairy	**A1**	Raw milk 1	2.40	2.43	2.41	0.03
Raw milk and dairy	**A2**	Raw milk 2	3.32	3.23	3.28	-0.09
Raw milk and dairy	**A3**	Raw milk 3	4.11	3.57	3.84	-0.55
Raw milk and dairy	**A4**	Roquefort	5.11	5.48	5.30	0.36
Raw milk and dairy	**A5**	Raw milk cheese 2	6.41	6.23	6.32	-0.18
Pasteurised milk and milk products	**A6**	Cheese original	1.60	1.85	1.72	0.24
Pasteurised milk and milk products	**A7**	Organic free range double cream	2.65	2.84	2.75	0.19
Pasteurised milk and milk products	**A8**	Double organic cream	3.95	3.78	3.87	-0.18
Pasteurised milk and milk products	**A9**	British double cream	5.08	5.00	5.04	-0.08
Pasteurised milk and milk products	**A10**	Clotted cream	6.18	6.04	6.11	-0.13
Dry milk products	**A11**	Dried skimmed milk	2.52	2.66	2.59	0.14
Dry milk products	**A12**	Skimmed milk powder	3.11	3.56	3.34	0.44
Dry milk products	**A13**	Custard powder	4.62	4.43	4.53	-0.19
Dry milk products	**A14**	Dried skimmed milk powder 2	5.57	5.54	5.56	-0.02
Dry milk products	**A15**	Dried skimmed milk powder 3	5.62	6.11	5.87	0.49
Category: Ready to eat/ready to reheat meat and poultry (combined category)						
Cooked meat and poultry	**C1**	Chicken poppers	2.45	2.60	2.52	0.15
Cooked meat and poultry	**C2**	Cocktail sausages	3.46	3.54	3.50	0.08
Cooked meat and poultry	**C3**	Coronation chicken	4.38	4.62	4.50	0.24
Cooked meat and poultry	**C4**	Ready to eat BBQ chicken pieces	5.46	5.75	5.61	0.29

Type	Code	Sample	log(Ref)	log(Alt)	Mean	Difference
Cooked meat and poultry	**C5**	Zingy sweet chilli chicken	5.70	6.04	5.87	0.34
Fermented or dried products	C6	Roll herring	1.78	1.70	1.74	-0.08
Fermented or dried products	C7	Salmon pate 1	3.65	3.18	3.41	-0.48
Fermented or dried products	C8	Salmon pate 2	4.57	4.48	4.52	-0.09
Fermented or dried products	C9	Anchovy	5.23	5.28	5.25	0.05
Fermented or dried products	C10	Seafood terrine	6.40	6.59	6.49	0.19
Raw cured products	**C11**	Dried steak strips	2.36	2.40	2.38	0.04
Raw cured products	**C12**	Beef jerky	3.32	3.45	3.38	0.12
Raw cured products	**C13**	Seak strips 2	4.36	4.15	4.25	-0.22
Raw cured products	**C14**	Tender jerky	5.36	5.30	5.33	-0.06
Raw cured products	**C15**	Biltong	6.36	6.18	6.27	-0.19
Category: Fresh produce and fruits						
Ready to eat fruit	**D1**	Apples, mango strawberry and raspberry	2.32	2.54	2.43	0.22
Ready to eat fruit	**D2**	Mango	2.04	2.11	2.08	0.07
Ready to eat fruit	**D3**	Pomegrante seeds	4.23	4.32	4.28	0.09
Ready to eat fruit	**D4**	Mango	5.04	5.28	5.16	0.24
Ready to eat fruit	**D5**	Grapes and berries	6.63	6.36	6.50	-0.27
Cut ready to eat vegetables/sprouts	D6	Fine beans & tenderstem broccoli	4.72	4.88	4.80	0.16
Cut ready to eat vegetables/sprouts	D7	Tenderstem broccoli	2.78	2.60	2.69	-0.18
Cut ready to eat vegetables/sprouts	**D8**	Mixed vegetables	3.64	3.77	3.71	0.13
Cut ready to eat vegetables/sprouts	**D9**	Carrot batons	4.84	4.83	4.83	-0.01
Cut ready to eat vegetables/sprouts	D10	Broccoli florets	1.90	2.20	2.05	0.30
Leafy greens	D11	Italian wild rocket	5.04	5.74	5.39	0.70

Type	Code	Sample	log(Ref)	log(Alt)	Mean	Difference
Leafy greens	D12	Babyleaf salad	4.51	4.49	4.50	-0.01
Leafy greens	D13	Mixed leaf salad	3.57	3.43	3.50	-0.14
Leafy greens	D14	Butterhead salad	4.23	3.81	4.02	-0.42
Leafy greens	D15	Spinach, watercress & rocket salad	5.40	5.78	5.59	0.38
Category: Multicomponent foods and meal components						
Composite foods with substantial raw ingredients	**E1**	Ham sandwich no mayo	2.32	2.30	2.31	-0.02
Composite foods with substantial raw ingredients	**E2**	Seafood cocktail sandwich	3.23	3.34	3.29	0.11
Composite foods with substantial raw ingredients	**E3**	Red leicester ploughman's sandwich	4.04	4.30	4.17	0.26
Composite foods with substantial raw ingredients	**E4**	Pomodorino tomato and sweet pepper salad	5.11	5.18	5.15	0.06
Composite foods with substantial raw ingredients	**E5**	Mediterranean salad	4.85	4.78	4.81	-0.07
Mayonnaise based deli-salads	E6	Potato salad	4.67	5.11	4.89	0.44
Mayonnaise based deli-salads	E7	Potato salad	3.66	3.18	3.42	-0.49
Mayonnaise based deli-salads	**E8**	Potato and egg salad	4.43	4.52	4.47	0.09
Mayonnaise based deli-salads	**E9**	Ham egg and coleslaw salad	4.61	4.73	4.67	0.12
Mayonnaise based deli-salads	**E10**	Coleslaw	5.52	5.69	5.60	0.17
Ambient stable acidified foods	**E11**	Mayonnaise squeezy	2.36	2.52	2.44	0.16
Ambient stable acidified foods	**E12**	Sweet chilli sauce	3.30	3.23	3.27	-0.07
Ambient stable acidified foods	**E13**	Tartare sauce	4.43	4.48	4.45	0.05
Ambient stable acidified foods	**E14**	BBQ sauce	5.53	5.58	5.56	0.05
Ambient stable acidified foods	**E15**	Tomato ketchup	6.38	6.32	6.35	-0.06
Category: raw and ready to cook meat and poultry (combined category)						
Fresh meats (unprocessed)	T1	Chicken breast fillets	6.82	6.91	6.86	0.09
Fresh meats (unprocessed)	T2	Lean diced beef	5.11	5.36	5.24	0.25

Type	Code	Sample	log(Ref)	log(Alt)	Mean	Difference
Fresh meats (unprocessed)	T3	Fresh lamb chops	5.65	5.92	5.79	0.27
Fresh meats (unprocessed)	T4	Turkey thigh mince 7% fat	6.58	6.80	6.69	0.22
Fresh meats (unprocessed)	T5	Pork loin steaks	7.51	7.60	7.55	0.10
Ready to cook (processed) meat	**T6**	BBQ pork riblets	5.58	5.62	5.60	0.04
Ready to cook (processed) meat	**T7**	Pork shoulder in a BBQ sauce	4.62	4.60	4.61	-0.02
Ready to cook (processed) meat	**T8**	Fire pit sweet and smoky beef kebabs	3.54	3.54	3.54	0.00
Ready to cook (processed) meat	**T9**	Fire pit beef burgers	2.54	2.45	2.50	-0.10
Ready to cook (processed) meat	**T10**	Pork sausages	1.90	1.70	1.80	-0.20
Ready to cook (processed) poultry	T11	Breaded chicken goujons	3.32	3.15	3.23	-0.18
Ready to cook (processed) poultry	T12	Brtish turkey meatballs	7.83	7.79	7.81	-0.04
Ready to cook (processed) poultry	T13	BBQ roast chicken wings	3.99	4.18	4.08	0.19
Ready to cook (processed) poultry	**T14**	Chicken kiev bites	2.92	2.49	2.71	-0.43
Ready to cook (processed) poultry	**T15**	Southern fried breaded chicken mini fillets	2.59	2.88	2.73	0.28

ANNEX C: Summary tables accuracy profile study.

(Food) Category 2	Dairy products
(Food) Type 2	Raw dairy

			Reference method result					Alternative method result				
Sample Name	(Food) item	Level	rep 1	rep 2	rep 3	rep 4	rep 5	rep 1	rep 2	rep 3	rep 4	rep 5
13	raw milk	low	200	190	200	180	190	270	260	230	250	150
16	raw milk cheese	low	250	460	270	320	200	350	490	290	490	220
17	raw milk cheese	intermediate	7100	7700	8700	10000	7700	7300	14000	8700	11000	8300
14	raw milk	intermediate	20000	20000	17000	19000	21000	22000	31000	20000	10000	14000
18	raw milk cheese	high	610000	1300000	550000	800000	990000	910000	1300000	740000	710000	1200000
15	raw milk	high	1500000	2100000	2300000	1500000	1800000	1400000	2400000	2900000	1300000	1500000

(Food) Category 1	Fresh produce
(Food) Type 1	Cut RTE veg

			Reference method result					Alternative method result				
Sample Name	(Food) item	Level	rep 1	rep 2	rep 3	rep 4	rep 5	rep 1	rep 2	rep 3	rep 4	rep 5
1	Lettuce	low	430	550	270	1200	1400	730	840	890	1500	1300
4	Spinach	low	790	900	840	840	890	1100	940	1100	970	1100
2	Lettuce	intermediate	59000	40000	49000	64000	60000	68000	35000	40000	52000	54000
5	Spinach	intermediate	65000	54000	110000	55000	59000	75000	56000	12000	81000	63000
3	Lettuce	high	3600000	5800000	4300000	3100000	3000000	3000000	5800000	3400000	4600000	4000000
6	Spinach	high	4800000	5900000	7300000	4600000	4800000	5800000	7400000	4800000	4400000	3900000

(Food) Category 3	Multicomponent
(Food) Type 3	Composite foods with substantial raw ingredients

			Reference method result					Alternative method result				
Sample Name	(Food) item	Level	rep 1	rep 2	rep 3	rep 4	rep 5	rep 1	rep 2	rep 3	rep 4	rep 5
25	sandwich	low	540	740	170	300	235	980	1200	440	390	280
28	pasta salad	low	530	650	680	670	610	670	670	950	750	650
26	sandwich	intermediate	64000	31000	45000	26000	19000	61000	38000	93000	34000	20000
29	pasta salad	intermediate	31000	25000	74000	26000	35000	29000	30000	75000	41000	46000
27	sandwich	high	1600000	2100000	1400000	1900000	1500000	1700000	2400000	1800000	2400000	2200000
30	pasta salad	high	4300000	2900000	3500000	4300000	6200000	4400000	4000000	3400000	6200000	7500000

(Food) Category 4	Meat and poultry
(Food) Type 4	Fresh meats

			Reference method result					Alternative method result				
Sample Name	(Food) item	Level	rep 1	rep 2	rep 3	rep 4	rep 5	rep 1	rep 2	rep 3	rep 4	rep 5
22	chicken breast	low	2800	1900	980	1500	3300	3200	2300	1500	2300	2500
19	raw ground beef	low	19000	12000	9000	7600	14000	4300	10000	8800	8600	20000
23	chicken breast	intermediate	12000	15000	18000	25000	16000	13000	15000	15000	29000	18000
20	raw ground beef	intermediate	31000	28000	33000	35000	40000	24000	41000	31000	30000	21000
24	chicken breast	high	1300000	2900000	2400000	990000	1000000	1500000	3000000	1600000	1500000	1200000
21	raw ground beef	high	6000000	3400000	2600000	3500000	4800000	5900000	3200000	2300000	1700000	400000

(Food) Category 5	Ready to eat foods
(Food) Type 5	Cooked and cured fish products

			Reference method result					Alternative method result				
Sample Name	(Food) item	Level	rep 1	rep 2	rep 3	rep 4	rep 5	rep 1	rep 2	rep 3	rep 4	rep 5
10	salmon pate	low	330	350	290	380	410	600	540	570	800	620
7	seafood terrine	low	540	530	590	520	530	620	910	910	500	600
11	salmon pate	intermediate	27000	24000	22000	35000	22000	21000	25000	38000	37000	16000
8	seafood terrine	intermediate	34000	31000	23000	26000	40000	47000	34000	15000	43000	34000
12	salmon pate	high	2400000	2600000	1500000	1800000	1900000	4400000	2300000	3000000	2900000	2400000
9	seafood terrine	high	2100000	2800000	3700000	1600000	3400000	2600000	2200000	2500000	3400000	5100000

ANNEX D: Raw data inclusivity and exclusivity study

Inclusivity

Code	Strain	Source	Alternative OP EBAC						Reference VRBGA						Non-selective PCA				
			-5	-6	-7	-8	Calculated count (cfu/ml)	log cfu/ml	-5	-6	-7	-8	Calculated count (cfu/ml)	log cfu/ml	-6	-7	-8	Calculated count (cfu/ml)	log cfu/ml
1	*Buttiauxella warmboldiae*	Rainwater		66	4	0	6.60E+07	7.8		38	2	0	3.60E+07	7.6	79	8	1	7.90E+07	7.9
2	*Citrobacter amalonaticus*	Beansprouts		T	100	7	1.00E+09	9.0		T	73	9	7.50E+08	8.9	T	75	3	7.10E+08	8.9
3	*Citrobacter braakii*	Industrial isolate		T	55	6	5.50E+08	8.7		T	39	4	3.90E+08	8.6	T	68	5	6.60E+08	8.8
4	*Citrobacter diversus*	Industrial isolate		T	54	5	5.40E+08	8.7		35	4	1	3.50E+07	7.5	T	59	2	5.50E+08	8.7
5	*Cronobacter sakazakii*	Dried milk		T	22	1	2.20E+08	8.3		T	25	1	2.40E+08	8.4	T	31	2	3.00E+08	8.5
6	*Cronobacter universalis*	fresh water		T	44	5	4.40E+08	8.6		T	56	6	5.60E+87	87.7	T	70	4	6.70E+08	8.8
7	*Enterobacter aerogenes*	Sesame seeds		T	24	1	2.40E+08	8.4		T	17	1	5.60E+08	8.7	T	18	3	1.90E+08	8.3
8	*Enterobacter agglomerans*	Dried milk		T	161	19	1.60E+09	9.2		T	118	14	1.20E+09	9.1	T	146	13	1.40E+09	9.1
9	*Enterobacter amnigenus*	Mushrooms	T	73	8	2	7.30E+07	7.9	T	80	5	0	7.70E+07	7.9	109	6	0	1.00E+08	8.0
10	*Enterobacter asburiae*	Clinical		T	28	1	2.80E+08	8.4		T	22	4	2.40E+08	8.4	T	25	1	2.40E+08	8.4
11	*Enterobacter cloacae*	Tomato salad		T	104	9	1.00E+09	9.0		T	67	12	7.20E+08	8.9	T	107	13	1.10E+09	9.0

Code	Strain	Source	Alternative OP EBAC						Reference VRBGA						Non-selective PCA				
			-5	-6	-7	-8	Calculated count (cfu/ml)	log cfu/ml	-5	-6	-7	-8	Calculated count (cfu/ml)	log cfu/ml	-6	-7	-8	Calculated count (cfu/ml)	log cfu/ml
12	*Enterobacter dispar*	unkown		T	38	6	3.80E+08	8.6		T	15	1	1.50E+08	8.2	T	40	4	4.00E+08	8.6
13	*Enterobacter gergoviae*	Clinical		T	103	9	5.00E+08	8.7		T	28	4	2.90E+08	8.5	T	32	5	3.40E+08	8.5
14	*Enterobacter intermedius*	Surface water	T	35	1	0	3.50E+07	7.5	T	39	3	0	3.80E+07	7.6	46	2	0	4.40E+07	7.6
15	*Enterobacter taylorae*	Bird seed	T	T	53	3	5.30E+08	8.7	T	T	40	6	4.20E+08	8.6	T	59	6	5.90E+08	8.8
16	*Enterobacter xiangfangensis*	NCIMB 14836	T	T	58	1	5.80E+08	8.8	T	T	92	13	9.50E+08	9.0	T	95	9	9.50E+08	9.0
17	*Erwinia amylovorans*	Industrial isolate	T	T	115	10	1.10E+09	9.0	T	T	97	9	9.70E+08	9.0	T	150	17	1.50E+09	9.2
18	*Escherichia adecarboxylata*	Skim milk powder	T	T=177	12	1	1.20E+08	8.1	T	148	14	3	1.50E+08	8.2	T	15	3	1.60E+08	8.2
19	*Escherichia vulneris*	Vegetables	T	50	7	1	5	0.7	T	43	3	0	4.20E+07	7.6	95	5	0	9.10E+07	8.0
20	*Hafnia alvei*	Prawn coleslaw	T	T	16	2	1.60E+08	8.2	T	T	19	1	1.80E+08	8.3	T	19	1	1.80E+08	8.3
21	*Klebsiella aerogenes*	Water	T	29	0	0	2.90E+07	7.5	T	T	19	3	2.00E+08	8.3	T	20	2	2.00E+08	8.3
22	*Klebsiella oxytoca*	Pharyngeal tonsil	T	T	37	2	3.70E+08	8.6	T	T	21	1	2.00E+08	8.3	T	33	2	3.20E+08	8.5

Code	Strain	Source	Alternative OP EBAC						Reference VRBGA						Non-selective PCA				
			-5	-6	-7	-8	Calculated count (cfu/ml)	log cfu/ml	-5	-6	-7	-8	Calculated count (cfu/ml)	log cfu/ml	-6	-7	-8	Calculated count (cfu/ml)	log cfu/ml
23	*Klebsiella pneumoniae*	Industrial isolate	T	150	10	1	1.50E+08	8.2	112	19	0	0	1.20E+07	7.1	123	5	0	1.20E+08	8.1
24	*Klebsiella rhinoscleromatis*	Industrial isolate	16	0	0	0	1.60E+06	6.2	10	1	0	0	1.00E+06	6.0	45	6	1	4.60E+07	7.7
25	*Klebsiella trevisanii*	Industrial isolate	T	T	19	2	1.90E+08	8.3	T	105	11	0	1.20E+08	8.1	T	15	3	1.60E+08	8.2
26	Kluyvera ascorbata	industrial	T	T	26	3	2.60E+08	8.4	T	T	31	1	2.90E+08	8.5	T	32	4	3.30E+08	8.5
27	*Leclercia ardecarboxyla*	Oregano	T	T	17	0	1.70E+08	8.2	T	T	19	0	1.90E+08	8.3	247	21	4	2.40E+08	8.4
28	Lelliottia amnigena	sea water	T	85	3	0	8.50E+07	7.9	T	104	10	0	1.00E+08	8.0	87	10	0	8.80E+07	7.9
29	*Methanolibacter arachdis*	Groundnut root	T	T	41	1	4.10E+08	8.6	T	T	20	5	2.30E+08	8.4	T	50	4	4.90E+08	8.7
30	*Morganella morganii*	Pork	T	T	55	7	5.50E+08	8.7	T	T	49	3	4.70E+08	8.7	T	64	6	6.40E+08	8.8
31	*Pantoea agglomerans*	Pasteurised milk	97	5	0	0	9.70E+06	7.0	T	36	3	0	3.50E+07	7.5	42	3	0	4.10E+07	7.6
32	*Proteus mirabilis*	Poultry		T	25	0	2.50E+08	8.4		T	16	4	1.80E+08	8.3	T	72	2	6.70E+08	8.8
33	*Proteus vulgaris*	Poultry		T	30	3	3.00E+08	8.5		T	36	3	3.50E+08	8.5	T	43	5	4.40E+08	8.6
34	*Providencia alcalifaciens*	Chicken	T	T	22	0	2.20E+08	8.3	T	T	17	2	1.70E+08	8.2	T	33	4	3.40E+08	8.5

Code	Strain	Source	Alternative OP EBAC						Reference VRBGA						Non-selective PCA				
			-5	-6	-7	-8	Calculated count (cfu/ml)	log cfu/ml	-5	-6	-7	-8	Calculated count (cfu/ml)	log cfu/ml	-6	-7	-8	Calculated count (cfu/ml)	log cfu/ml
35	*Providencia rettgeri*	Human faeces		T	18	1	1.80E+08	8.3		T	24	2	2.40E+08	8.4	T	37	2	3.50E+08	8.5
36	*Raoutella planticola*	Raw tuna		T	21	4	2.10E+08	8.3		123	8	0	1.20E+08	8.1	189	20	0	1.90E+08	8.3
37	*Salmonella bongori*	outbreak isolate		T	37	2	3.70E+08	8.6		T	42	4	4.20E+08	8.6	T	48	1	4.50E+08	8.7
38	*Salmonella enterica subsp arizonae*	DSMZ 14955		T	29	1	2.90E+08	8.5		T	32	1	3.00E+08	8.5	T	36	2	3.50E+08	8.5
39	*Salmonella enterica subsp houtenae*	NCTC 10401		T	45	4	4.50E+08	8.7		T	38	3	3.70E+08	8.6	T	62	7	6.30E+08	8.8
40	*Salmonella enterica subsp java*	NCTC 5706		T	70	4	7.00E+08	8.8		T	55	6	5.50E+08	8.7	T	62	2	5.80E+05	5.8
41	*Salmonella enterica subsp schwarzengrund*	NCTC 6756		T	53	4	5.30E+08	8.7		T	66	9	6.80E+08	8.8	T	80	5	7.70E+08	8.9
42	*Serratia fonticola*	Chicken	T	57	5	0	5.70E+07	7.8	T	57	5	0	5.70E+07	7.8	134	7	0	1.30E+08	8.1
43	*Serratia liquifaciens*	Mince	T	T	13	4	1.30E+08	8.1	T	T	13	0	1.30E+08	8.1	T	29	1	2.70E+08	8.4
44	*Serratia marcescens*	Raw mince	T	T	33	2	3.30E+08	8.5	T	T	33	2	3.20E+08	8.5	T	38	5	3.90E+08	8.6
45	*Shigella dysenteriae*	Industrial isolate	T	T	11	3	1.10E+08	8.0	T	T	16	1	1.50E+08	8.2	T	23	0	2.30E+08	8.4

Quantitative methods - 2022LR108 Neogen OP-EBAC. Summary report.

Code	Strain	Source	Alternative OP EBAC						Reference VRBGA						Non-selective PCA				
			-5	-6	-7	-8	Calculated count (cfu/ml)	log cfu/ml	-5	-6	-7	-8	Calculated count (cfu/ml)	log cfu/ml	-6	-7	-8	Calculated count (cfu/ml)	log cfu/ml
46	*Shimwellia blattae*	cockroach	T	T	15	2	1.50E+08	8.2	T	T	13	3	1.50E+08	8.2	T	15	2	1.60E+08	8.2
47	*Escherichia coli*	Salmon fish cakes	T	T	32	3	3.20E+08	8.5	T	T	12	4	1.50E+08	8.2	T	23	5	2.50E+08	8.4
48	*Escherichia fergusonii*	Sausages	T	T	26	2	2.60E+08	8.4	T	T	14	3	1.50E+08	8.2		19	7	1.90E+08	8.3
49	*Escherichia hermanii*	Seasame seeds	T			37	3.70E+08	8.6	T	T	T	28	2.80E+09	9.4		T	34	3.40E+09	9.5
50	*Citrobacter gillenii*	raw mince	T	96	9	0	9.60E+07	8.0	T	T	72	9	7.40E+08	8.9		32	3	3.20E+08	8.5
51	*Citrobacter freundii*	coleslaw	T	T	26	9	2.60E+08	8.4	T	T	29	6	3.20E+08	8.5		34	0	3.40E+08	8.5

Exclusivity

Number	Organism	Source	Alternative method: OPEBAC	Reference method: VG	Non-selective agar (MRSA, MEA, PCA, NA+ salt, TSA)						
			-1	-1	-4	-5	-6	-7	-8	cfu/ml	log cfu/ml
1	*Acinetobacter calcoaceticus**	sesame seeds	0	0	T	T	41	2	1	3.90E+07	7.6
2	*Acinetobacter lwoffii*	Tomatoes	0	0	T	T	39	3	0	3.80E+07	7.6
3	*Aeromonas salmonicida*	NCTC10402	0	0	T	56	9	0	0	6.70E+07	7.8
4	*Avibacterium avium*	Chicken	0	0	T	T	12	3	0	1.40E+07	7.1
5	*Bacillus coagulans*	Sterilised milk	0	0	T	123	70	7	0	1.80E+07	7.3
6	*Bacillus subtilis*	Custard	0	0	T	T	45	5	0	4.50E+07	7.7
7	*Brochothrix thermosphacta*	Fresh pork sausage	0	0	T	T	16	6	0	2.00E+07	7.3
8	*Burkholderia gladioli*	Industrial	0	0	T	T	13	1	0	1.30E+07	7.1

Number	Organism	Source	Alternative method: OPEBAC	Reference method: VG	Non-selective agar (MRSA, MEA, PCA, NA+ salt, TSA)						
			-1	-1	-4	-5	-6	-7	-8	cfu/ml	log cfu/ml
9	*Burkholderia stabilis*	soft drinks environment	0	0	T	T	44	4	0	4.40E+07	7.6
10	*Candida magnoliae*	Strawberries	0	0	T	T	17	1	nt	1.60E+07	7.2
11	*Flavobacterium indologenes*	bamboo shoots	0	0	T	T	52	2	0	5.20E+07	7.7
12	*Flavobacterium resinovorum*	NCIMB 8767	0	0	T	T	37	6	1	3.90E+07	7.6
13	*Lactobacillus brevis*	Fermenting olives	0	0	T	T	T	14	2	1.50E+08	8.2
14	*Lactobacillus casei*	Industrial isolate	0	0	T	T	T	T	39	3.90E+08	8.6
15	*Listeria innocua*	Mammal Brain	0	0	T	T	86	6	0	8.40E+07	7.9
16	*Listeria monocytogenes*	Soft cheese	0	0	T	T	63	10	2	6.60E+07	7.8

Number	Organism	Source	Alternative method: OPEBAC	Reference method: VG	Non-selective agar (MRSA, MEA, PCA, NA+ salt, TSA)						
			-1	-1	-4	-5	-6	-7	-8	cfu/ml	log cfu/ml
17	*Novosphingobium capsulatum*	Distilled water	0	0	T	10	1	0	0	1.00E+06	6.0
18	*Pasteurella multocida*	Cattle	0	0	T	T	51	9	0	5.50E+07	7.7
19	*Pediococcus pentasaceus*	Brine	0	0	T	T	T	17	1	1.60E+08	8.2
20	*Pseudomonas aeruginosa*	Blood	0	0	T	T	95	6	0	9.20E+07	8.0
21	*Pseudomonas fluorescens*	Soil	0	0	19	1	0	0	0	1.80E+05	5.3
22	*Shewanella putrefaciens*	Industrial isolate	0	0	30	3	0	0	0	3.00E+05	5.5
23	*Acinetobacter tolerans*	NCIMB 8551	0	0	T	T	T	T	33	3.30E+09	9.5
24	*Staphylococcus epidermis*	NCIMB 8853	0	0	T	T	54	8	0	5.60E+07	7.7
25	*Stenotrophomonas maltophila*	NCIMB 9428	0	0	T	T	T	64	4	6.20E+08	8.8
26	*Streptococcus agalactiae*	Milk	0	0	T	T	59	7	0	6.00E+07	7.8

Number	Organism	Source	Alternative method: OPEBAC	Reference method: VG	Non-selective agar (MRSA, MEA, PCA, NA+ salt, TSA)						
			-1	-1	-4	-5	-6	-7	-8	cfu/ml	log cfu/ml
27	*Streptococcus pyogenes*	Clinical	0	0	T	T	32	3	0	3.20E+07	7.5
28	*Vibrio parahaemolyticus*	Human faeces	0	0	50	5	0	0	0	5.00E+05	5.7
29	Xanthomonas maltophilia	Bamboo shoots	0	0	T	T	52	6	0	5.30E+07	7.7
30	Zygosaccharomyces bailii	Fruit Jam	0	0	T	20	2	0	nt	2.00E+06	6.3

* colonies atypical on both media types