Method Comparison Study Report for the ISO 16140-2:2016 validation of Compact Dry ETC, for the detection of Enterococcus in a broad range of foods

MicroVal study number: 2014LR48

Method/Kit name: Compact Dry ETC

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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

Company: Nissui Pharmaceutical Co. Ltd

Expert Laboratory: Campden BRI

Method/Kit name: Compact Dry ETC

Validation standard: ISO 16140-2:2016 Microbiology of the food chain —Method validation —Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method

Reference methods: NMKL Method No. 68 5th Edition 2011: Enterococcus. Determination in foods and feeds.

Scope of validation: A broad range of foods based on categories

1. Dairy products
2. Fruits and vegetables
3. Raw Meat and Poultry
4. Ready to eat foods
5. Multi component foods

Certification organisation: 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
- PSD Peptone salt diluent

Contents

1 Introduction

In this project a MicroVal validation study, based on ISO 16140-2:2016, of alternative method(s) for the enumeration of coagulase-positive *Enterococcus* in five different food categories was carried out by Campden BRI as the MicroVal Expert Laboratory.

The alternative method used was:

- Enumeration of *Enterococcus* on Compact Dry ETC, incubated at 37°C±1°C for 20 -24h

The reference method used was:

- NMKL Method No. 68 5th Edition 2011: Enterococcus. Determination in foods and feeds.

Categories included :

- Dairy products
- Fruits and vegetables
- Raw Meat and Poultry
- Ready to eat foods
- Multi component foods

Criteria evaluated during the study have been:

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

The final conclusion on the Method Comparison Study and ILS is summarised below:

The alternative method Compact Dry ETC shows comparable performance to the reference method (NMKL Method No. 68 5th Edition 2011) for the enumeration of *Enterococcus* in a broad range of foods.

2 Method protocols

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

According to ISO 16140-2 the reference method and alternative methods were performed with the same sample. The study was therefore a paired study design.

2.1 Reference method

The reference method was : NMKL Method No. 68 5th Edition 2011: Enterococcus. Determination in foods and feeds: 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 is used, then two plates of this dilution shall be 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 of the alternative method in Annex A.

The Compact Dry ETC method contains chromogenic medium and selective agents for the detection and enumeration of *Enterococcus* which according to the manufacturer's instructions appear as blue colonies after 20- 24hr incubation at 37±1ºC.

Samples of product containing the target organism were diluted 1 in 10 with an appropriate diluent according to ISO 6887 and homogenised in a stomacher.

Appropriate serial dilutions were made, and all relevant dilutions were analysed using the reference method and alternative method.

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

Categories	Types	No of samples analysed	Number of interpretable results
Dairy products	Dairy desserts e.g. chilled custard, trifle	8	5
	Soft cheese	11	5
	Hard cheese e.g. cheddar	9	5
	Total	28	15
Fruits and vegetables	Seasonings e.g. spices	5	5
	Sprouts e.g. mung beans	5	5
	Leafy greens e.g. parsley, lettuce	7	5
	Total	17	15
Raw poultry and meats	Fresh chicken cuts	6	5
	Fresh mince	7	5
	Frozen patties	7	6
	Total	20	16
Ready to eat foods	Ready to eat poultry e.g. turkey fillet	5	5
	Cooked fish products e.g. prawns	9	6
	Cooked meat e.g. ham	10	6
	Total	24	17
Multi component foods	Composite foods with raw ingredients e.g. sandwiches, pasta salads.	5	5
	Mayonnaise based salads	7	5
	Cooked chilled foods e.g. rice products	7	5
	Total	19	15
TOTAL		108	78

108 samples were analysed, leading to 78 interpretable results. The samples which were not used in the calculations are shown in Table 2:

Table 2 : Samples not used in the analysis

		Number of samples
Results below the detection limit	*With the reference method*	0
	With the alternative method	0
	With the two methods	30
Results above the detection limit	*With the reference method*	0
	With the alternative method	0
Presence of high background microflora on reference method plates		0
TOTAL		**30***

3.1.2 Test sample preparation

It is preferable to have naturally contaminated samples where possible, however, it is also necessary to artificially inoculate some samples where naturally contamianted samples cannot be sourced. Artificial contamination was carried out by spiking or seeding protocols. Injury efficiency was evaluated by enumerating the pure culture on selective and non-selective agars.

The observed injury measurements varied from 0.31 to more than 2 log cfu/g difference between non-selective and selective plates

30 samples were screened for natural contamination and 78 samples were artificially contaminated; only the 78 artificially contaminated gave interpretable results.

3.1.3 Protocols applied during the validation study

A single protocol was applied for the study.

Reference method plates were incubated at 37±1ºC for a total of 48±4h. Compact Dry ETC plates were incubated at 37±1ºC for 20-24h. In all cases the minimum incubation times were used.

3.1.4 Test results

The samples were analysed by the reference and the alternative methods in order to have at least 15 interpretable results per category, and at least 5 interpretable results per tested type by the two methods.

3.1.5 Calculation and interpretation of relative trueness study

The obtained data were analysed using the scatter plot. The graphs are provided with the line of identity (y = x). Figures 1 to 5 shows the data plotted per category and Figure 6 summarises all the data.

Figure 1 :

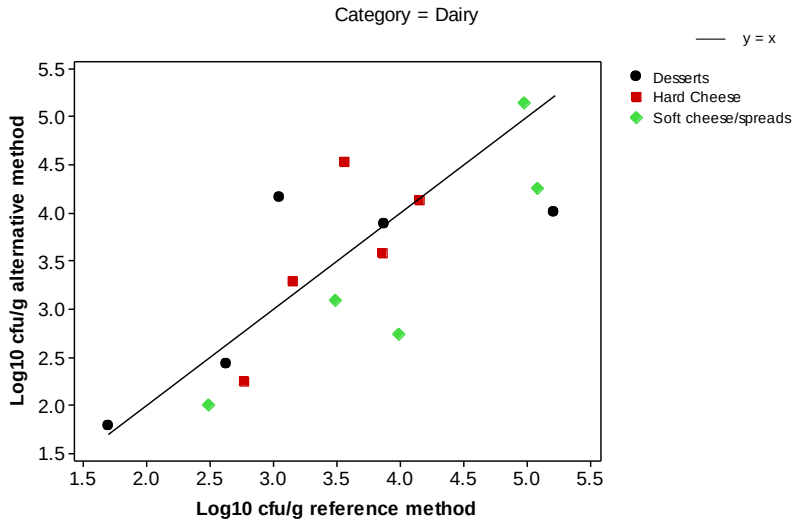


Figure 2:

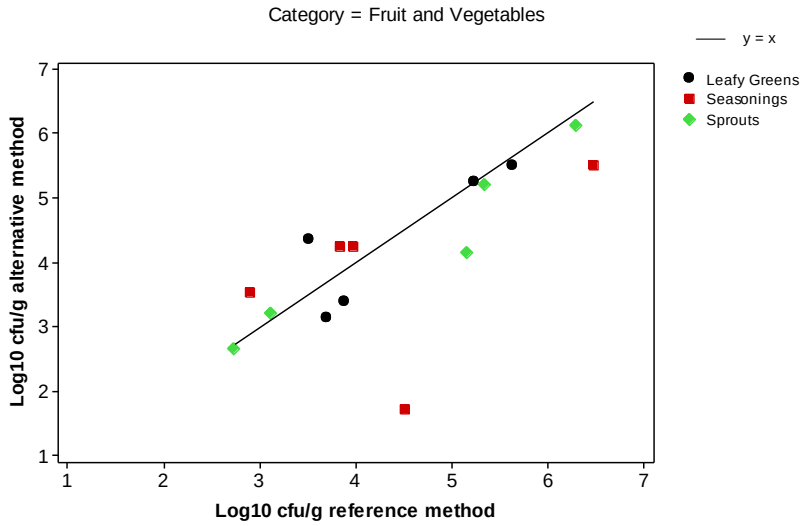


Figure 3:

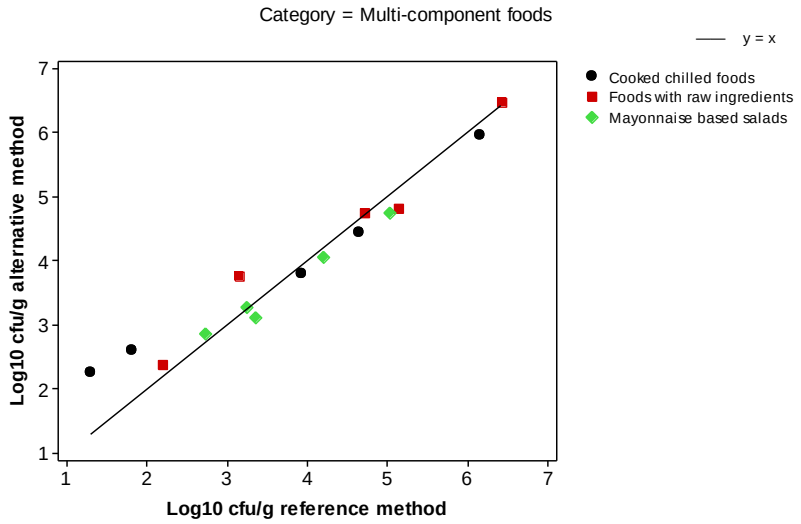


Figure 4

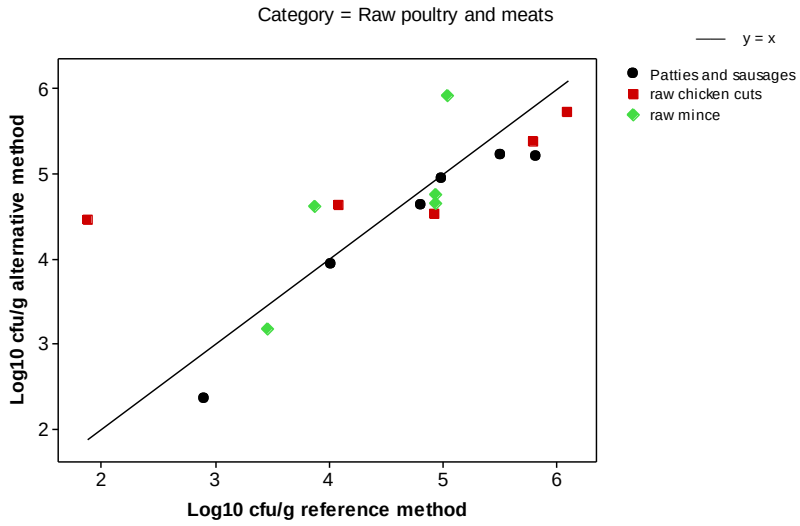

Category = Raw poultry and meats
y = x
Patties and sausages
raw chicken cuts
raw mince
Log10 cfu/g alternative method
Log10 cfu/g reference method
2
3
4
5
6

Figure 5

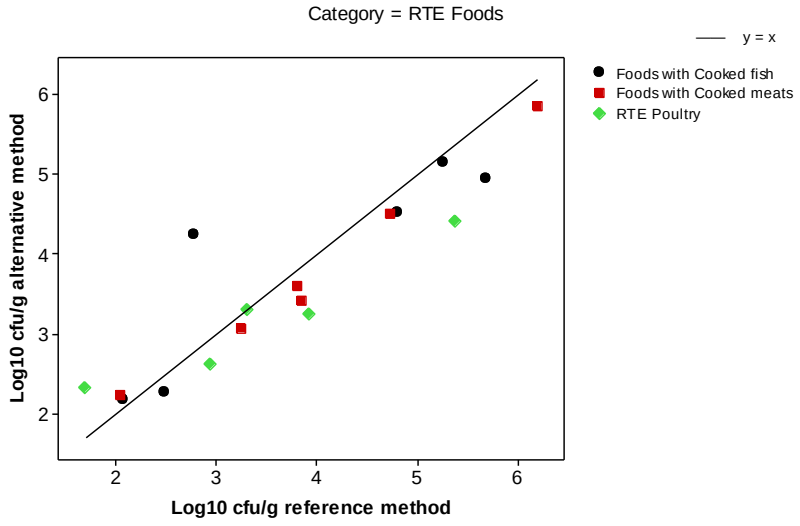
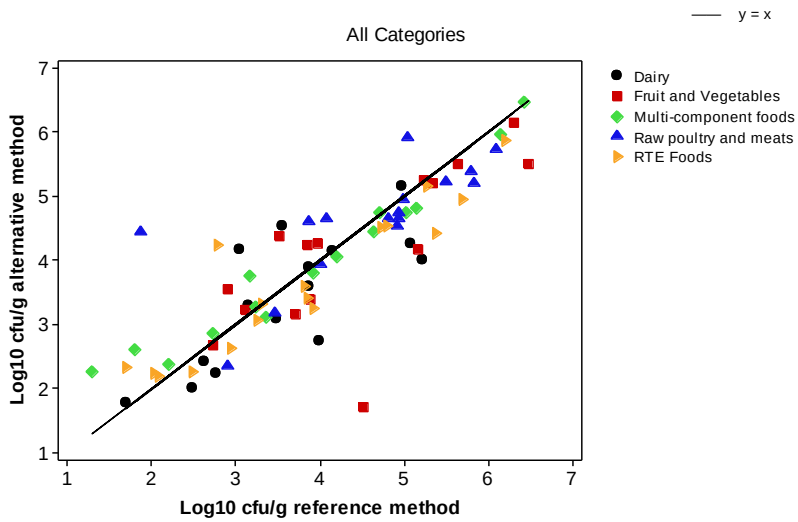

Category = RTE Foods
y = x
Foods with Cooked fish
Foods with Cooked meats
RTE Poultry
Log10 cfu/g alternative method
Log10 cfu/g reference method
2
3
4
5
6

Figure 6



According to ISO 16140-2:2016 6.1.2.3 the results of the scatter plot are interpreted based on a visual observation on the amount of bias and extreme results. The data appears acceptable on the whole with the exception of a cardamom sample with a large negative bias and a raw chicken sample with a large positive bias. There were no obvious reasons for these discrepancies.

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

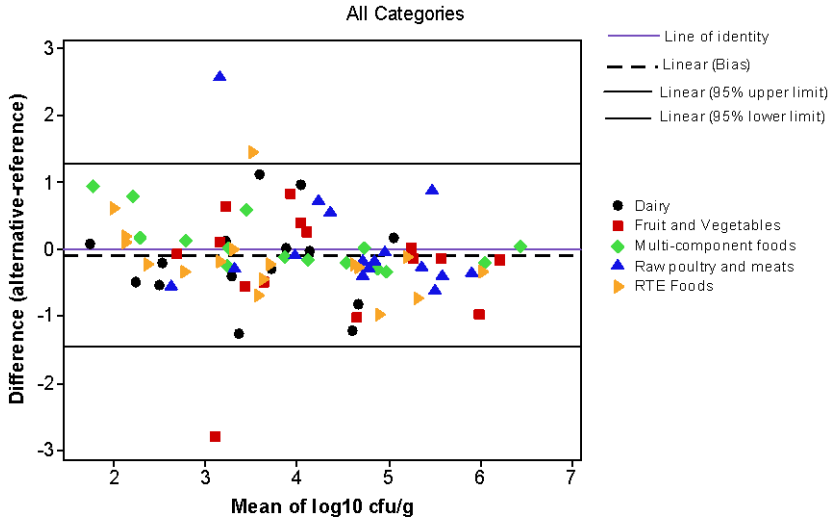
The Bland-Altman difference plot for all the samples is given Figure 7

Table 4 - Summary of the calculated values per category

Category.	n	$\overline{D}$	s_D	95% Lower limit	95% Upper limit
Dairy	15	-0.184	0.669	-1.665	1.297
Fruit and Vegetables	15	-0.276	0.876	-2.215	1.664
Multi-component	15	0.076	0.395	-0.798	0.951
Raw poultry and	16	0.064	0.801	-1.696	1.824
RTE Foods	17	-0.142	0.553	-1.349	1.065
All Categories	78	-0.092	0.676	-1.446	1.263

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

Figure 7 – Bland-Altman difference plot for all the 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 Table 5.

Table 5 - Data which are outside of the accepted limits -

Food Category	**Food type**	**Sample code**	**Food item**	**Strain**	**Spiking/seeding protocol**	**Difference log cfu/g (alternative – reference)**
RTE Foods	Foods with Cooked fish	49	Seafood terrine	*E. faecium* 9645	55°C/5min	1.49
Raw poultry and meats	Raw chicken cuts	46	Chicken mini fillets	*E.faecium* NCIMB 700580	Chill storage for 4 days	2.52
Fruits and vegetables	Seasonings	90	Whole cardamoms	*E.faecalis* 12672	Storage at ambient for 10days	-2.816

Comments

It is expected that not more than one in 20 data values will lie outside the CLs. Any disagreements with the expectation should be recorded.

For this data set there are 3 in 78 data values which lie outside the CLs (All categories plot). There were no identifiable trends in these data, and they covered 3 different food categories and 3 different inoculated strains.

3.1.6 Conclusion (RT study)

The relative trueness of the Alternative method (Compact Dry ETC) for *Enterococcus* is satisfied.

3.2 Accuracy profile study

The accuracy profile study is a comparative study between the results obtained by the reference method 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

For each of 5 food categories, one type of food was tested using 6 samples per type. Of the 6 samples, there were 2 at a low level, 2 at a medium level and 2 at a high level of contamination. For each of the 6 samples per category, 5 replicate test portions were tested.

According to ISO16140-2:2015 6.1.3.2, for each category being tested, at least one food type shall be tested but the six samples tested might belong to the same food item or to different food items. According to MicroVal discussions there are 2 options that may be used here. Either a single food item is used per type but 2 batches are tested, or 2 different food items are tested with one batch per item. So for example, for dairy desserts, it would be possible to test:

- chilled custard batch 1 and chilled custard batch 2, or
- chilled custard batch 1 and whipped cream batch 1

In order to evaluate the difference between the 2 options on the statistical analysis, this study tested both approaches.

The tested categories, types, items and inoculated strains are provided in the Table 6.

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

Category	Types	Strain	Item	Level
Dairy products	Dairy desserts	*E.mundtii* CRA 16812	Chilled custard Batch 1	Low:100cf/g
				Medium : 1000cfu/g
				High : 10,000cfu/g
			Chilled custard	Low:100cf/g

Category	Types	Strain	Item	Level
			Batch 2	Medium : 1000cfu/g
				High : 10,000cfu/g
			Whipped cream	Low:100cf/g
				Medium : 1000cfu/g
				High : 10,000cfu/g
Fruits and vegetables	Leafy greens e.g. parsley, lettuce	*E.faecium* NCIMB 9645	Parsley Batch 1	Low: 50cf/g
				Medium : 1000cfu/g
				High : 50,000cfu/g
			Parsley Batch 2	Low: 50cf/g
				Medium : 1000cfu/g
				High : 50,000cfu/g
			Shredded lettuce	Low: 50cf/g
				Medium : 1000cfu/g
				High : 50,000cfu/g
Raw poultry and meats	Fresh beef	*E.avium* NCIMB 702366	Fresh steak Batch 1	Low: 50cf/g
				Medium : 1000cfu/g
				High : 50,000cfu/g
			Fresh steak Batch 2	Low: 50cf/g
				Medium : 1000cfu/g
				High : 50,000cfu/g
			Patties	Low: 50cf/g
				Medium : 1000cfu/g
				High : 50,000cfu/g
Ready to eat foods	Cooked fish products e.g. prawns	*E. casseliflavus* CRA 16811	Tuna pate Batch 1	Low: 50cf/g
				Medium : 100cfu/g
				High : 1000cfu/g
			Tuna pate Batch 2	Low: 50cf/g
				Medium : 100cfu/g
				High : 1000cfu/g
			Fresh cooked prawns	Low: 50cf/g
				Medium : 100cfu/g
				High : 1000cfu/g
Multi component foods	Composite foods with raw ingredients	*E.hirae* CRA 15939	Pasta salad Batch 1	Low 500cf/g
				Medium : 5000cfu/g
				High : 50,000cfu/g
			Pasta salad Batch 1	Low 500cf/g
				Medium : 5000cfu/g
				High : 50,000cfu/g
			Sandwiches	Low 500cf/g
				Medium : 5000cfu/g
				High : 50,000cfu/g

Total number of samples tested= 225

3.2.2 Calculations and interpretation of accuracy profile study

The statistical results and the accuracy profiles are provided in Figures 8 to 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

Because the study design included 9 samples per category instead of 6, the statistical analysis was carried out 3 times for each category instead of once. For example for dairy products the analysis was carried out for

(i) custard batch 1 and custard batch 2)
(ii) custard batch 1 and cream
(iii) custard batch 2 and cream

Figure 8: Dairy

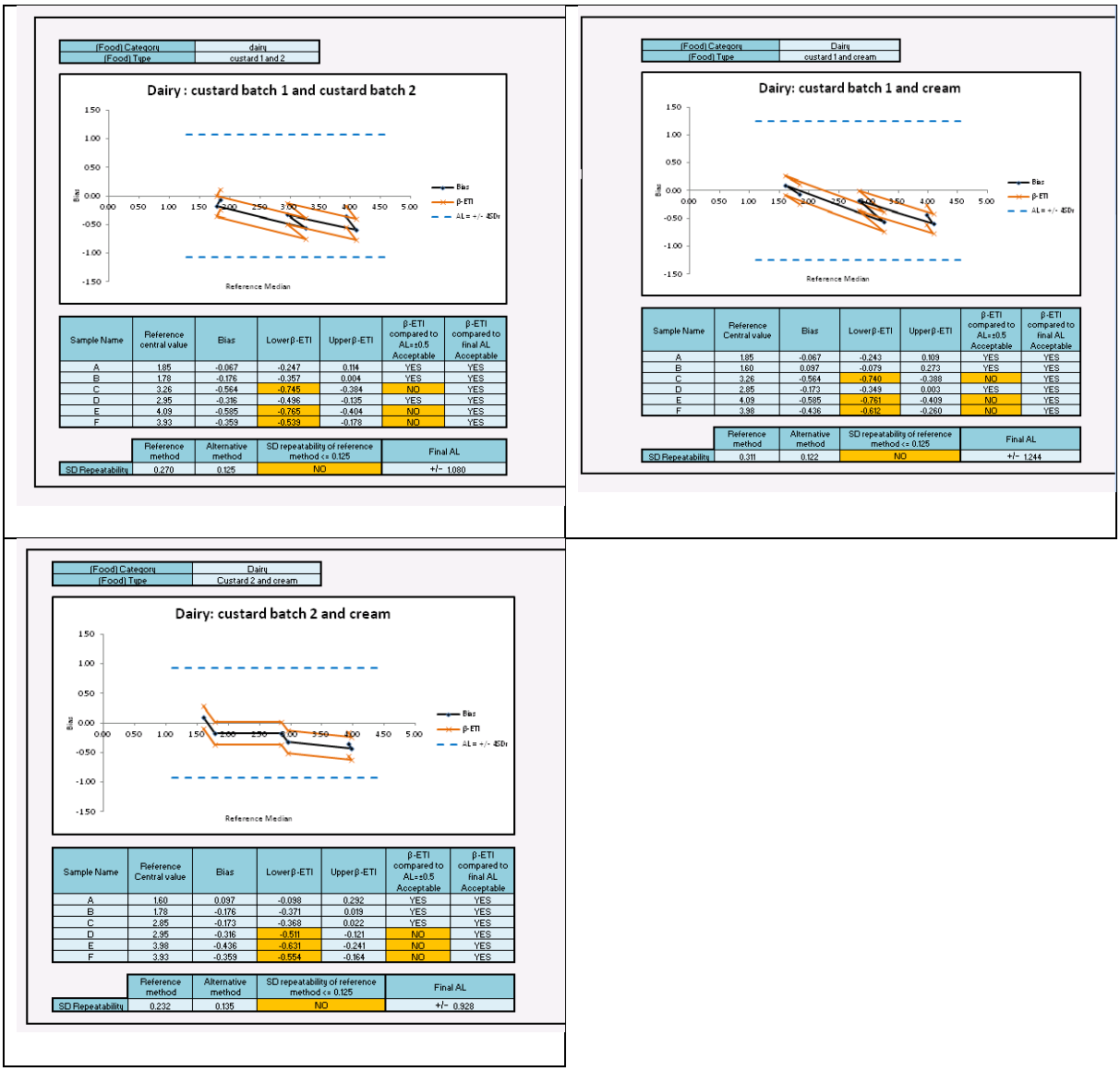
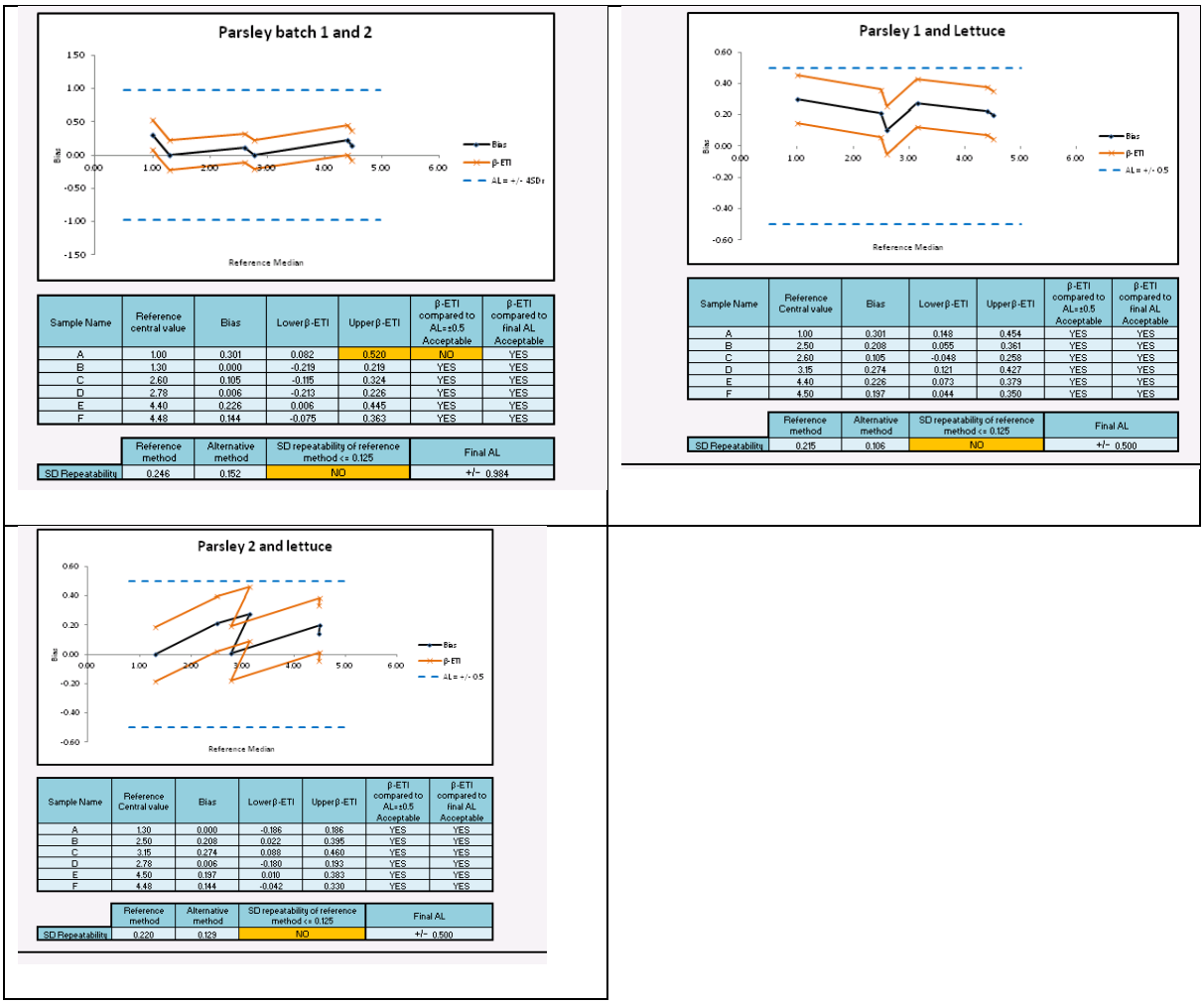


Figure 9: Fruits and vegetables



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
A	1.00	0.301	0.082	0.520	NO	YES
B	1.30	0.000	-0.219	0.219	YES	YES
C	2.60	0.105	-0.115	0.324	YES	YES
D	2.78	0.006	-0.213	0.226	YES	YES
E	4.40	0.226	0.006	0.445	YES	YES
F	4.48	0.144	-0.075	0.363	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.246	0.152	NO	+/- 0.984

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
A	1.00	0.301	0.148	0.454	YES	YES
B	2.50	0.208	0.055	0.361	YES	YES
C	2.60	0.105	-0.048	0.258	YES	YES
D	3.15	0.274	0.121	0.427	YES	YES
E	4.40	0.226	0.073	0.379	YES	YES
F	4.50	0.197	0.044	0.350	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.215	0.106	NO	+/- 0.500

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
A	1.30	0.000	-0.186	0.186	YES	YES
B	2.50	0.208	0.022	0.395	YES	YES
C	3.15	0.274	0.088	0.460	YES	YES
D	2.78	0.006	-0.180	0.193	YES	YES
E	4.50	0.197	0.010	0.383	YES	YES
F	4.48	0.144	-0.042	0.330	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.220	0.129	NO	+/- 0.500

Figure 10: Multi-component foods

(Food) Category	Pasta
(Food) Type	Pasta 1 and 2

Multi-component foods: pasta batch 1 and 2

Sample Name	Reference central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to ΔL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
A	1.98	0.136	0.058	0.214	YES	YES
B	1.78	0.222	0.144	0.300	YES	YES
C	3.95	-0.057	-0.135	0.021	YES	YES
D	4.03	-0.158	-0.236	-0.080	YES	YES
E	4.95	-0.078	-0.156	0.000	YES	YES
F	4.99	-0.131	-0.209	-0.053	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.170	0.054	NO	+/- 0.500

(Food) Category	Pasta
(Food) Type	Pasta 1 and sandwich

Multi-component foods: pasta batch 1 and sandwiches

Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to ΔL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
A	1.98	0.136	-0.004	0.276	YES	YES
B	1.80	0.301	0.161	0.441	YES	YES
C	3.95	-0.057	-0.197	0.083	YES	YES
D	3.78	0.072	-0.068	0.213	YES	YES
E	4.95	-0.078	-0.218	0.062	YES	YES
F	4.96	-0.131	-0.271	0.009	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.179	0.097	NO	+/- 0.500

(Food) Category	Pasta
(Food) Type	Pasta 2 and Sandwich

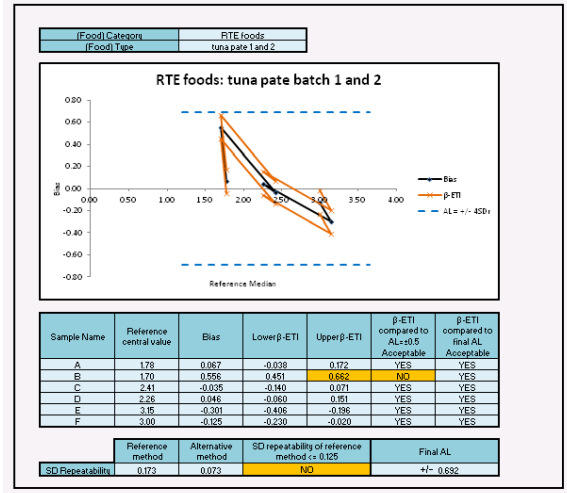
Multi-component foods: pasta batch 2 and sandwiches

Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to ΔL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
A	1.60	0.301	0.167	0.435	YES	YES
B	1.78	0.222	0.088	0.356	YES	YES
C	3.78	0.072	-0.062	0.207	YES	YES
D	4.03	-0.158	-0.292	-0.024	YES	YES
E	4.96	-0.131	-0.265	0.003	YES	YES
F	4.99	-0.131	-0.265	0.003	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.240	0.093	NO	+/- 0.500

Figure 11: Raw poultry and meat

Figure 12: RTE foods

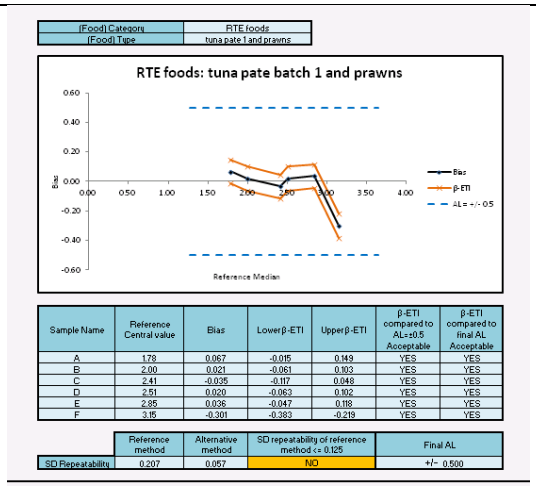


[Food] Category	RTE foods
[Food] Type	tuna pate 1 and 2

RTE foods: tuna pate batch 1 and 2

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
A	1.78	0.067	-0.038	0.172	YES	YES
B	1.70	0.556	0.451	0.662	NO	YES
C	2.41	-0.035	-0.140	0.071	YES	YES
D	2.26	0.046	-0.060	0.151	YES	YES
E	3.15	-0.301	-0.406	-0.196	YES	YES
F	3.00	-0.125	-0.230	-0.020	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.173	0.073	NO	+/- 0.692

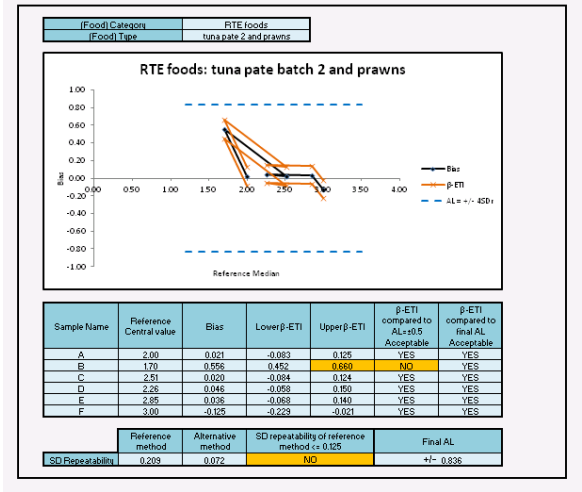


[Food] Category	RTE foods
[Food] Type	tuna pate 1 and prawns

RTE foods: tuna pate batch 1 and prawns

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
A	1.78	0.067	-0.015	0.149	YES	YES
B	2.00	0.021	-0.061	0.103	YES	YES
C	2.41	-0.035	-0.117	0.048	YES	YES
D	2.51	0.020	-0.063	0.102	YES	YES
E	2.85	0.036	-0.047	0.118	YES	YES
F	3.15	-0.301	-0.383	-0.219	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.207	0.057	NO	+/- 0.500



[Food] Category	RTE foods
[Food] Type	tuna pate 2 and prawns

RTE foods: tuna pate batch 2 and prawns

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
A	2.00	0.021	-0.083	0.125	YES	YES
B	1.70	0.556	0.452	0.660	NO	YES
C	2.51	0.020	-0.084	0.124	YES	YES
D	2.26	0.046	-0.059	0.150	YES	YES
E	2.85	0.036	-0.069	0.140	YES	YES
F	3.00	-0.125	-0.229	-0.021	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.209	0.072	NO	+/- 0.836

According to ISO 16140, if any of the upper or lower limits for the six samples exceeds the 0.5log Acceptability Limits (ALs) and the standard deviation, *S*ref > 0,125, then an additional evaluation procedure

is followed: New ALs are calculated as a function of the standard deviation: AL *s* = 4_ *s*ref. If for all *i* in the accuracy profile *Ui* ≤ AL*s* and *Li* _ −AL*s* , the alternative method is accepted as being equivalent to the reference method for the given combination category and type.

For some of the food categories the additional AL calculation was required. This was for the dairy products, fruit and vegetables products and RTE foods.

For the dairy product, 5 of the 9 samples showed an AL above 0.5logcfu/g. These were for custard batch 1 medium level, custard batch 1 high level, custard batch 2 medium level, custard batch 2 high level, and cream high level. These levels showed a negative bias i.e. a lower level on the alternative method compared to the reference method. The samples were inoculated with *E.mundtii* 16812.

For the fruit and vegetables, only 1 of the 9 samples (parsley batch 1 low level inoculated with *E.faecium* NCIMB 9645) had a slight positive bias of 0.520. All other samples were within the recalculated ALs

For the RTE foods, only 1 of the 9 samples (tuna pate batch 2 low level inoculated with *E.casseliflavus* CRA 16811) had a positive bias of 0.660. All other samples were within the ALs.

After the AL values were recalculated, all the data for the dairy, fruit and vegetables and RTE foods fell within the new ALs the alternative method was accepted as being equivalent to the reference method.

For 2 categories, multi-component foods and raw meat and poultry the AL of 0.5 was achieved and the alternative method was accepted as being equivalent to the reference method without the need for the additional calculation.

3.3 Inclusivity / exclusivity

The inclusivity study is a study involving pure target strains to be detected or enumerated by the alternative method

3.3.1Protocol

After being grown according to appropriate conditions, decimal dilutions were made, and the 50 target strains and 30 non-target strains were enumerated by the alternative method, the reference method and a non selective agar (TSA).

3.3.2 Results

Inclusivity

Of the 50 inclusivity strains tested 36 strains were detected and 14 were not detected using the alternative method. For the reference method 33 of the strains were detected and 17 were not detected.

The strains not detected for either method were*: E. cecorum*, 16849; *E. aquamarinus*, 16813; *E. dispar*, 16850, *E. columbae*, 16851; *E. pseudoavium*, 16852; *E. sulfureus*, 16853; *E. seriolicida*, 16854; *E. flavescens*, 16855; *E. sacharolyticus*, 16863; *E. dispar*, 16864; *E. xiangfangensis*,16865; *E. solitarus*, 16867.

Those not detected by the alternative method but detected by the reference method were: *E. durans*, 16810; *E. porcinus*16857.

Those not detected by the reference method but detected by the alternative method were: *E. durans*, 16464; *E. haemoperoxidus*, 16858; *E. thailandicus*, 16859; *E.malodoratus*, 16860; *E. gallinarum*, 16861.

It would appear that both methods were good at detecting the more usual Enterococcus species, i.e. *E. faecalis* and *E. faecium*, but less good at detecting other species. In the inclusivity study there were 50 strains of Enterococci covering 23 different species. The Compact Dry ETC method was more specific as it detected 11 of the 23 different species whereas the reference method only detected 8 of the different species.

Exclusivity
Of the 30 exclusivity strains tested, 28 were not detected and 2 were detected using both the reference and alternative methods. The 2 detected cultures were *Lactobacillus gasseri CRA 6804* and *Streptococcus lactis* CRA 527.

3.4 Limit of quantification (LOQ)

The limit of Quantification (LOQ) is only required for instrumental measurements. It was not done in this study

3.5 Conclusion (MCS)

Overall, the conclusions for the Method Comparison are:

- The Compact Dry ETC for enumeration of *Enterococcus* in foods method shows satisfying trueness
- The Compact Dry ETC for enumeration of *Enterococcus* in foods method shows satisfactory and accuracy profile.
- The Compact Dry ETC for enumeration of *Enterococcus* in foods method was shown to be specific and selective. Compared to the Reference method it was able to detect more inclusivity cultures covering a wider range of species.

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 organization

There were 5 organisations used in this study representing 3 different countries. The number of collaborators from each organisation varied from 1 to 3 (according to ISO16140-2:2016 6.2.2) giving a maximum of 11 potential data sets. Three of the data sets were not used in the analysis due to incomplete data for the reference method, even though the alternative method performed well. So finally, there were 8 valid data sets from 4 different organisations and 3 different countries

4.2 Matrix and strain used

Chilled salmon pâté was inoculated with *Enterococcus faecalis* NCIMB 775. For each of the 11 collaborators participating in the interlaboratory study 7 x 10g samples of salmon pâté were weighed into sterile stomach bags. One sample of pâté remained uninoculated. For the remaining six samples, appropriate dilutions of the *E.faecalis* culture were used to individually inoculate 2 x 10g samples at the low (~10^2 cfu/ml), middle (~10^4cfu/ml) and high (~10^6cfu/ml) contamination levels.

4.3 Shipping of samples

Prior to despatch, each set of samples was removed from the freezer and packed into plastic containers (Air-Sea Containers Limited, code 490). These plastic containers were then placed inside a thermal control unit (Air-Sea Containers Limited, TC-20 code 802) with cool packs (Air-Sea Containers Limited, CP-20 code 405). Each laboratory also received an additional vial containing water “temperature control sample” which was packed with the test samples.
This was used to enable the laboratory to take a temperature measurement, representative of the samples, upon receipt. In addition to this a continuous electronic temperature monitor (Thermochron iButton) was placed in the sample packages. The laboratories were requested to return the ibuttons to the expert laboratory upon receipt. The target storage conditions were for the temperature to stay lower or equal to 8°C during transport, and between 0°C – 8°C in the labs.

Shipping was arranged so that each laboratory would receive their samples within 24 to 72h dependent on location and speed of the International courier service. The condition of the samples was recorded by each laboratory on a supplied form.

The analyses were started on Tuesday 29th November 2016

4.4 Calculation and interpretation of data

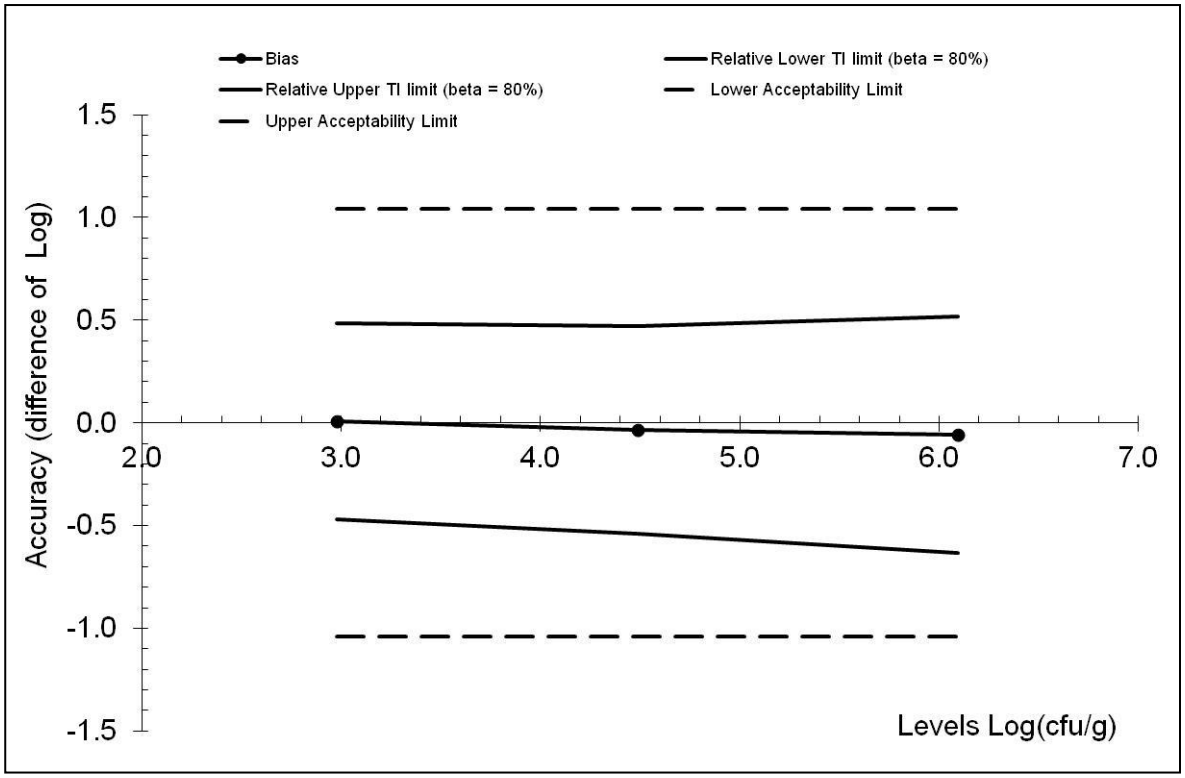
The data from the collaborative trial were calculated and interpreted according to section 6.2.3 of ISO 16140-2:2016 using the freely available Excel® spreadsheet (http://standards.iso.org/iso/16140). Version 14-03-2016 was used for these calculations.

The results obtained by the collaborators are shown in Tables 7. The accuracy profile plot is shown in Figures 13 and the statistical analysis of the data is shown in Tables 8.

Table 7: Summary of the results of the interlaboratory study per analyte level

		Reference method x_{ijk}		**Alternative method k_{ijk}**	
Collaborators (i)	**Level (k)**				
A	Blank	<10		<10	
B	Blank	<10		<10	
C	Blank	<10		<10	
D	Blank	<10		<10	
E	Blank	<10		<10	
I	Blank	<10		<10	
J	Blank	<10		<10	
K	Blank	<10		<10	
		Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
A	Low	2.699	2.568	2.550	2.561
B	Low	2.491	2.672	2.380	2.630
C	Low	3.204	3.369	3.320	3.490
D	Low	3.196	3.294	3.339	3.249
E	Low	3.324	3.163	3.031	3.048
I	Low	2.602	3.076	3.000	3.059
J	Low	2.845	3.072	3.038	3.000
K	Low	2.954	3.134	3.114	2.963
A	Medium	4.111	4.277	4.079	4.194
B	Medium	4.140	4.244	4.123	4.173
C	Medium	4.862	4.834	4.862	4.959
D	Medium	4.963	4.778	4.967	4.810
E	Medium	4.765	4.878	4.649	4.785
I	Medium	4.138	4.287	4.214	4.406
J	Medium	4.436	4.699	4.320	4.357
K	Medium	4.260	4.105	4.226	4.102
A	High	5.778	5.791	5.699	5.751
B	High	5.751	5.737	5.631	5.744
C	High	6.342	6.362	6.350	6.322
D	High	6.633	6.643	6.826	6.663
E	High	6.102	6.152	6.186	6.279
I	High	6.105	6.008	5.729	5.822
J	High	6.135	6.260	5.751	5.839
K	High	6.041	5.691	6.301	5.707

Figure 13. Accuracy profile of Compact Dry ETC from the ILS



The statistical analysis of the ILS data is shown in Table 8 below. It can be seen that the repeatability standard deviation (S_r) was similar for the alternative and reference method ranging from 0.087 to 0.162 for ETC and 0.097 to 0.162 for the reference method.

The between-labs standard deviation (S_L) was microbiologically similar for the alternative method (0.309 to 0.355) and the reference method (0.252 to 0.315) as was the reproducibility standard deviation (S_R) showing (0.321 to 0.391) for the alternative method and (0.300 to 0.312) for the reference method.

According to the ISO 16140-2:2016 standard, if any of the values of the β-ETI fall outside of the Acceptability Limits AL (±0.5log units)then a further calculation is done to calculate the pooled average SR of the reference method. This was done and gave an SR value of 0.315. This value was used to recalculate the new AL as a function of the standard deviation (ALs) using the formula 3.3 x SR,ref which gives new ALs values of +1.04 and -1.04.

Whilst quite large, the re-calculated AL is similar to those found in the methods comparison study where the AL's ranged from 0.500 to 1.244 for the 5 different product categories, with an average of 0.78

Looking at Figure 13, it can be seen that no values lie outside of these new ALs values and therefore the alternative method is accepted as being equivalent to the reference method.

Table 8. Statistical analysis of the ILS data according to the ISO spreadsheet

Accuracy profile	0.5						
Study Name	Hyserve Compact Dry ETC						
Date	22/12/2016						
Coordinator	Campden BRI						
Tolerance probability (beta)	80%	80%	80%				
Acceptability limit in log (lambda)	1.04	1.04	1.04				
	Alternative method				**Reference method**		
Levels	**Low**	**Medium**	**High**		**Low**	**Medium**	**High**
Target value	**2.979**	**4.486**	**6.096**				
Number of participants (K)	8	8	8		8	8	8
Average for alternative method	2.986	4.452	6.038		2.979	4.486	6.096
Repeatability standard deviation (sr)	0.089	0.087	0.162		0.162	0.112	0.097
Between-labs standard deviation (sL)	0.309	0.328	0.355		0.252	0.315	0.296
Reproducibility standard deviation (sR)	0.321	0.339	0.391		0.300	0.334	0.312
Corrected number of dof	7.563	7.480	8.319		9.364	7.838	7.717
Coverage factor	1.487	1.489	1.470				
Interpolated Student t	1.405	1.406	1.392				
Tolerance interval standard deviation	0.3402	0.3591	0.4122				
Lower TI limit	2.508	3.947	5.464				
Upper TI limit	3.464	4.956	6.611				
Bias	**0.007**	**-0.034**	**-0.058**				
Relative Lower TI limit (beta = 80%)	**-0.471**	**-0.539**	**-0.632**	TRUE			
Relative Upper TI limit (beta = 80%)	**0.485**	**0.471**	**0.516**	TRUE			
Lower Acceptability Limit	**-1.04**	**-1.04**	**-1.04**				
Upper Acceptability Limit	**1.04**	**1.04**	**1.04**				
New acceptability limits may be based on reference method pooled variance							
Pooled repro standard dev of reference	0.315						

TRUE

Application of clause 6.2.3
Step 8: If any of the values for the β-ETI fall outside the acceptability limits, calculate the pooled average reproducibility standard deviation of the reference method.
Step 9: Calculate new acceptability limits as a function of this standard deviation.

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

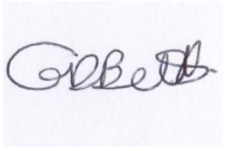
5 Overall conclusions of the validation study

Based on the results of the Methods comparison study (MCS) and the Inter-laboratory study (ILS):

- The Compact Dry ETC for enumeration of *Enterococcus* in foods method shows satisfying trueness from the MCS
- The Compact Dry ETC for enumeration of *Enterococcus* i in foods method shows satisfactory accuracy profile from the MCS
- The Compact Dry ETC for enumeration of *Enterococcus* in foods method was shown to be specific and selective from the MCS. Compared to the Reference method it was able to detect more inclusivity cultures covering a wider range of species.
- From the ILS it would appear that in the hands of the eight collaborators, the performance of Compact Dry ETC was not substantially different from the Reference method as shown by accuracy profile study.

The alternative Compact Dry ETC shows comparable performance to the reference method: NMKL Method No. 68 5th Edition 2011: Enterococcus. Determination in foods and feeds, for enumeration of Enterococcus in a broad range of foods

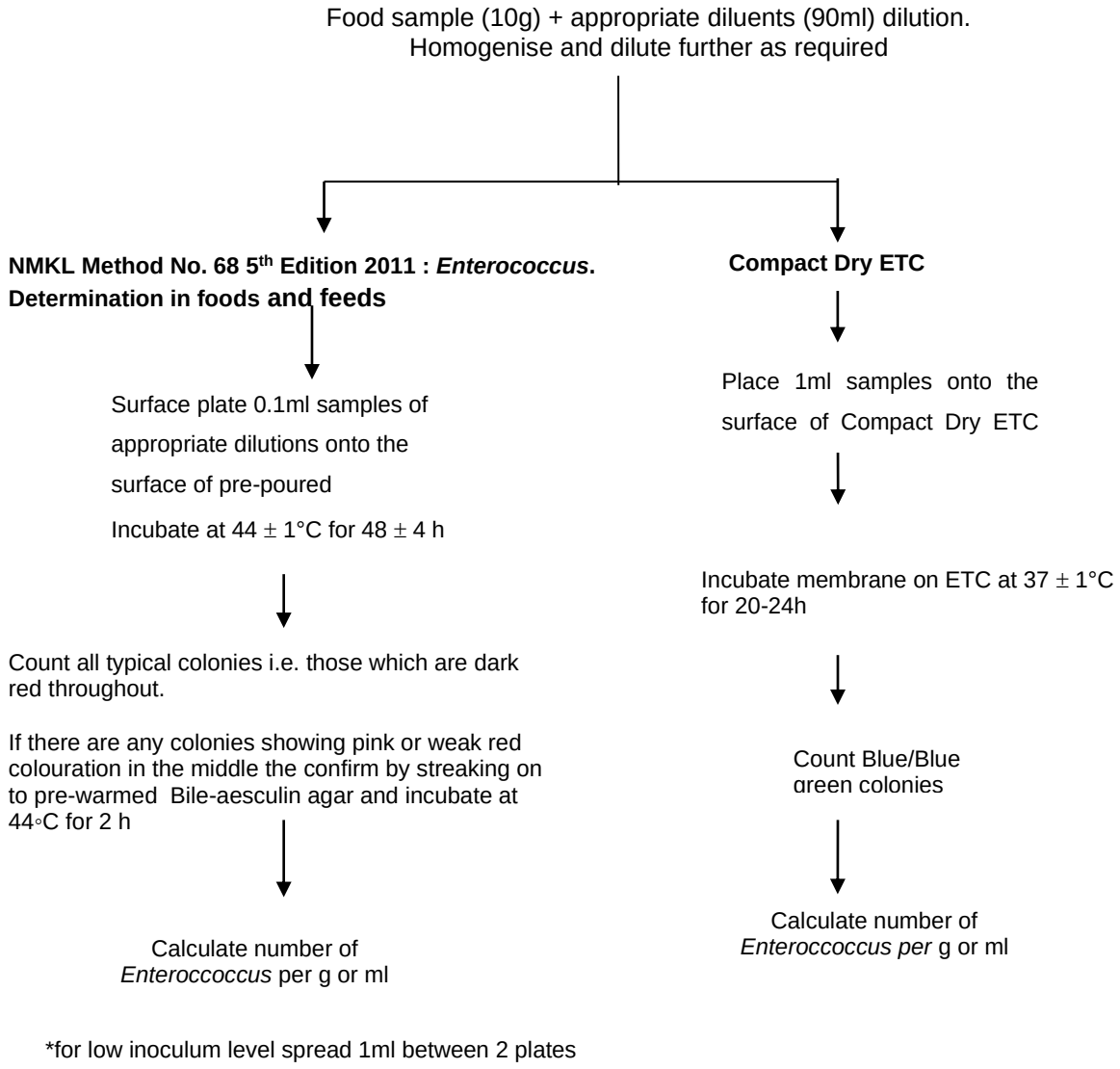
Date : 03/03/2019

Signature: 

Annexes A: Flow diagram of the reference and alternative method. B: Test kit insert

ANNEX A: Flow diagram of the alternative method and reference methods

Reference method (NMKL 68) and Candidate method (Compact Dry ETC) for enumeration of Enterococci



*for low inoculum level spread 1ml between 2 plates

ANNEX B Kit insert

HyServe

Compact Dry ETC medium for enterococcus/enterococcuse/milieu pour nombre enterococcus selectifs /medio para enterococcus/ medio per conta enterococcus /meio para contagem enterococcus

40 plates/Platten/plaques/placas/piastre/placas	ID-No. 1 002 944
240 plates/Platten/plaques/placas/piastre/placas	ID-No. 1 002 945
500 plates/Platten/plaques/placas/piastre/placas	ID-No. 1 402 945
1200 plates/Platten/plaques/placas/piastre/placas	ID-No. 1 002 993

English

Compact Dry ETC is a ready to use, selective and chromogene plate for the detection and enumeration of Enterococcus

Specimen pretreatment

Viable count in water or liquid foodstuff
Drop 1 ml of specimen (dilute if necessary) on the middle of the Compact Dry plate.

Viable count in solid foodstuff
Add buffer solution to the specimen and homogenize by stomacher®. Drop 1 ml of specimen (dilute if necessary) on the middle of the dry sheet of the Compact Dry plate.

Viable count in swab test specimen
Use the swab to wipe the surface, put into the device with wiping solution. Drop 1 ml of wiping solution (dilute if necessary) on the middle of the Compact Dry plate. It is recommended to use "Swab for Compact Dry" offered by HyServe Id-No. 1 002 952/3 (40/240 pieces).

Test instructions
1. Open the cap and drop 1 ml of specimen on the middle of the Compact Dry plate.
2. Specimen diffuses automatically and evenly into the sheet and transforms the dried sheet into a gel within seconds.
3. Put the cap again on the plate and write the information needed on the memorandum section.
4. Turn over the capped plate and put in the incubator.
5. After incubation count the number of **blue/blue green** colored colonies underneath the plate. White paper placed under the plate helps to count the colonies.

Incubation time 20-24 hours
Incubation temperature 37 ± 1 °C
Please use the incubation time/temperature according to the national food analysis recommended for *Enterococci*.

Interpretation of the results
Colonies grown are almost all **blue/blue-green**. Bacteria other than Enterococcus are inhibited to grow and they do not form any colonies.

Storage and shelf life
Keep at room temperature (+1 to +30 °C).
Total shelf life 18 months after manufacturing.

Notes
- Some colonies might not be clearly blue/blue-green colored.
- High concentrations on plates (> 300 CFU) will cause the entire growth area to become blue/blue-green. In this case dilute the specimen.
- After use please follow the current disposal regulations.
- The growth area is 20 cm². The back of the plate has a grid carved of 1 cm x 1 cm to make the colony counting easier. In case of any difficulties to count colonies due to large number of colonies grown, total viable count can be obtained by multiplying 20 by an average number of colonies per grid counted from several grids.
- Compact Dry plates are produced at an ISO 9001 certified site.

Deutsch

Compact Dry ETC ist eine gebrauchsfertige, selektive und chromogene Platte zum Nachweis von Enterococcus

Probenvorbereitung

Lebendkeimzahl in Wasser oder flüssigen Lebensmitteln
1 ml der Probe (evtl. verdünnen) in der Mitte der Compact Dry Platte aufbringen.

Lebendkeimzahl in festen Lebensmitteln
Zugabe von Pufferlösung und Homogenisierung der Lebensmittelprobe im Stomacher® ist erforderlich. 1 ml der Probe (evtl. verdünnen) in der Mitte der Compact Dry Platte aufbringen.

Lebendkeimzahl aus Tupfer-Proben
Mit dem sterilen, feuchten Wattetupfer kann z.B. die Oberfläche gewischt werden. Der Tupfer wird zurück in die Aufnahmeflüssigkeit überführt. Nach Schütteln wird die gesamte Lösung (1 ml) in der Mitte der Compact Dry Platte aufgebracht. Es wird empfohlen den Swab für Compact Dry von HyServe, Id-No. 1 002 952/3 (40/240 Stück) zu verwenden.

Testanweisung
1. Öffnen des Deckels und Auftropfen von 1 ml Probenmaterial in die Mitte der Compact Dry Platte.
2. Das Probenmaterial diffundiert automatisch und gleichmäßig in die Nährsubstanz und rehydriert das Gewebe innerhalb von Sekunden zu einem Gel.
3. Platte mit Deckel verschließen und beschriftbare Fläche zur Kennzeichnung verwenden.
4. Geschlossene Platte umdrehen und in einen Brutschrank legen.
5. Nach Inkubation die Anzahl der **blau-, blau-grün** farbigen Kolonien von der Rückseite der Platte her zählen. Ein weißes Papier als Unterlage erleichtert den Zählvorgang.

Inkubationszeit 20 - 24 Stunden
Inkubationstemperatur 37 ± 1 °C
Sie können auch die von nationalen Reglementierungen empfohlene Inkubations-temperatur zur Analyse von *Enterococci* in Lebensmitteln benutzen.

Interpretation des Ergebnisses
Nahezu alle Kolonien nehmen die **blau-/blaugrüne** Farbe an. Das Wachstum anderer Bakterien außer Enterococcus ist inhibiert.

Lagerung und Haltbarkeit
Bei Raumtemperatur aufbewahren (+1 bis +30 °C). Haltbarkeit bis 18 Monate nach Herstellung.

Bemerkungen
- Nicht alle Kolonien zeigen möglicherweise eine eindeutige blau/blau-grüne Färbung.
- Extreme hohe Bakterienanzahl in der Probe (> 300 KBE) wird zu einer blau-blaugrün Gesamtfärbung der Platte führen.
- Nach Gebrauch entsprechend der gültigen Abfall-regelung die Platten entsorgen.
- Die Plattenfläche beträgt 20 cm². Auf der Plattenrückseite ist ein Raster mit 1cm x 1cm eingraviert, um die Koloniezählung zu erleichtern. Sollte es problematisch sein auf Grund hoher Koloniedichte eine ganze Platte auszuzählen, sind einzelne Quadrate auszuzählen und der Mittelwert mit 20 zu multiplizieren.
- Compact Dry Platten können bis zu 300 Kolonien pro Platte nachweisen. Daher ist es erst nötig Kontaminationen, die diese Lebendkeimzahl überschreiten, zu verdünnen und die Verdünnungen auf die Platte aufzubringen.
- Compact Dry Platten werden in einem ISO 9001 zertifizierten Betrieb gefertigt.

Français

Compact Dry ETC est une plaque prête à l'utilisation pour détecter le nombre de Enterococcus selectifs

Traitement préliminaire de l'échantillon

Nombre de germes revivifiables dans l'eau ou dans des aliments liquides
Appliquer 1 ml de l'échantillon (le diluer si nécessaire) au centre de la plaque Compact Dry.

Nombre de germes revivifiables dans des aliments solides
Il est nécessaire d'ajouter une solution tampon à l'échantillon et de l'homogénéiser par Stomacher®. Appliquer 1 ml de l'échantillon (le diluer si nécessaire) au centre de la plaque Compact Dry.

Nombre de germes revivifiables dans des échantillons prélevés
Utiliser le tampon pour essuyer la surface, le placer dans l'unité avec la solution d'essuyage. Appliquer 1 ml de la solution d'essuyage (le diluer si nécessaire) au centre de la plaque Compact Dry. Il est recommandé d'utiliser le tampon 'Swab for Compact Dry' distribué par la société HyServe Id-No. 1 002 952/3 (40/240 pièces).

Instructions pour le test
1. Ouvrir le couvercle et appliquer 1 ml de l'échantillon sur la plaque Compact Dry.
2. L'échantillon se répand automatiquement et uniformément sur la feuille et en l'espace de quelques secondes, il transforme la feuille sèche en un gel.
3. Refermer le couvercle de la plaque et inscrire les informations nécessaires dans la partie correspondante.
4. Retourner la plaque fermée et la placer dans l'incubateur.
5. Après le temps d'incubation, compter le nombre de colonies **bleu/bleu-vert** au dos de la plaque. Les colonies peuvent être comptées plus simplement en plaçant du papier blanc sous la plaque.

Temps d'incubation 20 - 24 heures
Température d'incubation 37 ± 1 °C
Il faut toujours utiliser le temps/la température d'incubation conformément à l'analyse nationale des aliments recommandée pour calculer le nombre total de germes revivifiables.

Interprétation des résultats
Pratiquement toutes les colonies se colorent en **bleu/bleu-vert**. La croissance de bactéries non Enterococci est principalement interdite.

Stockage et durée de conservation
Stockage à température ambiante (+1 à +30 °C). Durée totale de conservation 18 mois après fabrication.

Remarques
- Quelques colonies risquent de ne pas se colorer nettement en bleu/bleu-vert.
- Des concentrations élevées sur les plaques (> 300 CFU) entraînent une coloration bleu/bleu-vert de toute la surface. Dans un tel cas, il faut diluer l'échantillon.
- Après l'utilisation, éliminer les plaques en respectant les règlements correspondants en vigueur.
- La surface de la plaque est de 20 cm². Une grille de 1 cm x 1 cm est taillée dans le dos de la plaque afin de faciliter le calcul des colonies. S'il est toutefois difficile de compter le nombre de colonies, suite à un grand nombre de colonies, il est possible de déterminer le nombre total de germes revivifiables dans certains carrés de la grille et d'en multiplier par 20 la valeur moyenne obtenue.
- Les plaques Compact Dry sont fabriquées dans une usine certifiée conforme à ISO 9001.