Method Comparison Study Report for the ISO 16140-2:2016 validation of Compact Dry BC, for the detection of *Bacillus cereus* in a broad range of foods

MicroVal study number: 2019LR87

Method/Kit name: Compact Dry BC

Report version: Summary report

MicroVal Expert Laboratory: Campden BRI

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 BC

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 method: ISO 7932:2004 Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of presumptive *Bacillus cereus* – Colony count technique at 30ºC.

Scope of validation: A broad range of foods :

- Dairy products
- Fishery products
- Dried cereals, fruits, nuts seeds and vegetables
- Meat and poultry products
- Multicomponent 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
- $\overline{D}$ 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
- MRD Maximum Recovery Diluent
- NA Nutrient Agar
- PCA Plate count Agar
- SBA Sheep Blood Agar

Contents

1 Introduction

In this project a MicroVal validation study, based on ISO 16140-2:2016, of alternative method(s) for the enumeration of *Bacillus cereus* in a broad range of foods was carried out by Campden BRI as the MicroVal Expert Laboratory.

The alternative method used was: Compact Dry BC. The method is summarised below.

- *Dilute 10g portions of food in appropriate diluent*. Stomach 1 minute.*
- *Make further serial dilutions as required*
- *Enumeration of appropriate dilutions on Compact Dry BC by soaking into dehydrated film (1ml)*
- *Incubation at 30±1°C for 24h±2h (shortest time will be used)*

**according to ISO 6887*

The reference method used is: ISO 7932:2004 Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of presumptive Bacillus cereus – Colony count technique at 30ºC.

Scope of the validation study is: a broad range of foods Categories included:

- Dairy products
- Fishery products
- Dried cereals, fruits, nuts seeds and vegetables
- Meat and poultry products
- 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 Compact Dry BC shows comparable performance to the reference method ISO 7932:2004 for the enumeration of *Bacillus cereus* in a broad range of foods

2 Method protocols

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

The sample material was diluted in MRD or appropriate diluent from ISO 6887 and was carried out as a paired study.

2.1 Reference method

The reference method used was ISO 7932:2004 Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of presumptive *Bacillus cereus* – Colony count technique at 30ºC. See the flow diagram in Annex A.

In summary:

- 1ml samples of appropriate dilutions were spread plated with MYP and incubated under aerobic conditions at 30±1°C for 18-24h. Plates were re-incubated plates at 30±1°C for a further 24h if colonies were not clearly visible
- Up to 5 typical and 5 atypical colonies i.e. pink without halos were confirmed on sheep blood agar

Sample preparations used in the reference method and the alternative 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. Single plates of successive dilutions were done as a minimum. In order to increase the reliabillity, duplicate plates were carried out where considered necessary based on the expected contamination level and dilution plated. If only 1 dilution was plated then duplicate plates were used.

2.2 Alternative method

See the flow diagram of the alternative method in Annex A.

In summary

- 1ml samples of appropriate dilutions were plated into the centre of the Compact Dry BC plates. The lids were placed on the plates and the plates inverted and incubated at 30 ± 1°C for 24± 2h.
- Following incubation, light blue/blue colonies were counted as stipulated by the manufacturer's instructions, and the CFU/g was calculated for each sample.

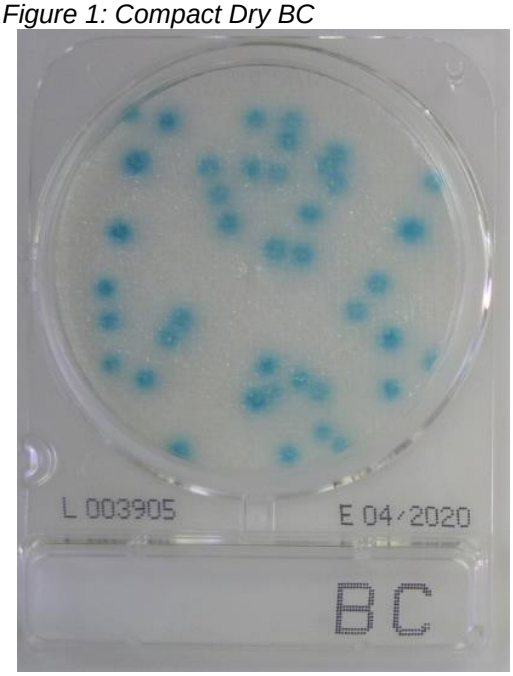
See the Compact Dry BC kit insert in Annex B.

The alternative method principle is based on enumeration on a rehydratable media plate.

Compact Dry (Nissui Pharmaceutical Co. Ltd.) are ready-to-use dry media sheets comprising culture medium and a cold-soluble gelling agent, rehydrated by inoculating 1ml diluted sample into the centre of the self-diffusible medium. The Compact Dry X-BC method contains chromogenic medium and selective agents for the detection and enumeration of B.cereus, which according to the manufacturer's instructions appear as light blue/blue colonies after 24h incubation at 30ºC.

A picture is provided in Figure 1:

Figure 1: Compact Dry BC



2.3 Study design

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

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

The samples were prepared for analysis and diluted in accordance with ISO 6887 (parts 1, 2, 3, 4 and 5) unless specified differently in the alternative method.

See Table 1 below in section 3.1 for specific preparations used in the validation study.

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	Dry	milk powders, powders for milk-based desserts dried infant formula	5	6887-5
	Pasteurised dairy products	Ice-cream, drinks, cream, panna cotta	5	6887-5
	Pasteurised milk	Skimmed, full fat, flavoured milk, dairy sauces	5	6887-5
RTE Fishery products	Canned ambient stable fish	Canned fish, canned crab	5	6887-3
	Cooked fishery products	Cooked crustaceans, fish and seafood terrines	5	6887-3
	Smoked or cured	Smoked, dried or salted fish	5	6887-3
Dried cereals, fruits, nuts seeds and vegetables	Dried vegetables/seasonings	Dehydrated vegetables e.g. potato and seasonings	5	6887-4
	Dried cereals	Corn, oats, breakfast cereals, baby food	5	6887-4
	Nuts, seeds and flours	Wheat, nut butters seeds	5	6887-4
RTE meat and poultry products	RTE meat and poultry	Cooked turkey pate, sliced meats	5	6887-2
	Canned ambient stable	Canned (ambient) e.g. corned beef, duck pate	5	6887-2
	Fermented or dried	Salami, biltong, jerky	5	6887-2
Multicomponent Foods	RTE refrigerated	cooked chilled foods, rice and pasta, products	5	6887-2
	RTE frozen foods	e.g. fries, pizza, pies	5	6887-2
	Composite foods with substantial raw ingredients	pasta salads, sandwiches, deli-salads	5	6887-2

75 samples were analyzed, leading to 75 exploitable results.

3.1.2 Test sample preparation

No naturally contaminated samples were found in pre-screening studies. It was therefore necessary to use artificial contamination procedures. Artificial procedures used a range of seeding protocols and strains in order to examine a wide range of different conditions.

Artificial contaminations were obtained using a seeding protocol.
Samples were inoculated with *B.cereus* strains before storage of the inoculated samples, e.g. frozen foods were stored for at least 2 weeks at -20 °C, perishable foods were stored for at least 48 h at 2 – 8 °C, and shelf stable foods were stored for at least 2 weeks at room temperature. Dried products were preferentially inoculated with spores.

Sixteen strains were used for artificial inoculations. These cultures preferably originated from comparable sample types as the ones to be inoculated. Each particular strain was used to contaminate up to 5 different items.

Inoculation of samples was generally at the range usually associated with the test organisms and within the capabilities of the test methods. Enumeration methods will generally cover the range 10^2 cfu/g to 10^6cfu/g.

3.1.3 Protocols applied during the validation study

Incubation time

Incubation of the alternative method was done at 30°C for 22h (minimum of 24±2h)

Confirmations if required for the alternative method

No confirmations were done for the alternative method. For the reference method, presumptive *B.cereus* colonies on MYP were confirmed by stabbing onto Sheep Blood Agar and examined for zones of clearance after incubation at 30±1°C for 24±2h.

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.

Summarised data is given in Annex C. Raw data is given in Excel sheet : 2019LR87 Relative Trueness

3.1.5 Calculation and interpretation of relative trueness study

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

Figure 1 shows the scatter plot for the Dairy products
Figure 2 shows the scatter plot for the RTE Fishery products
Figure 3 shows the scatter plot for the Dried cereals, fruits, nuts seeds and vegetables
Figure 4 shows the scatter plot for the RTE Meat and poultry products
Figure 5 shows the scatter plot for the Multicomponent foods
Figure 6 shows the scatter plot for all the categories

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

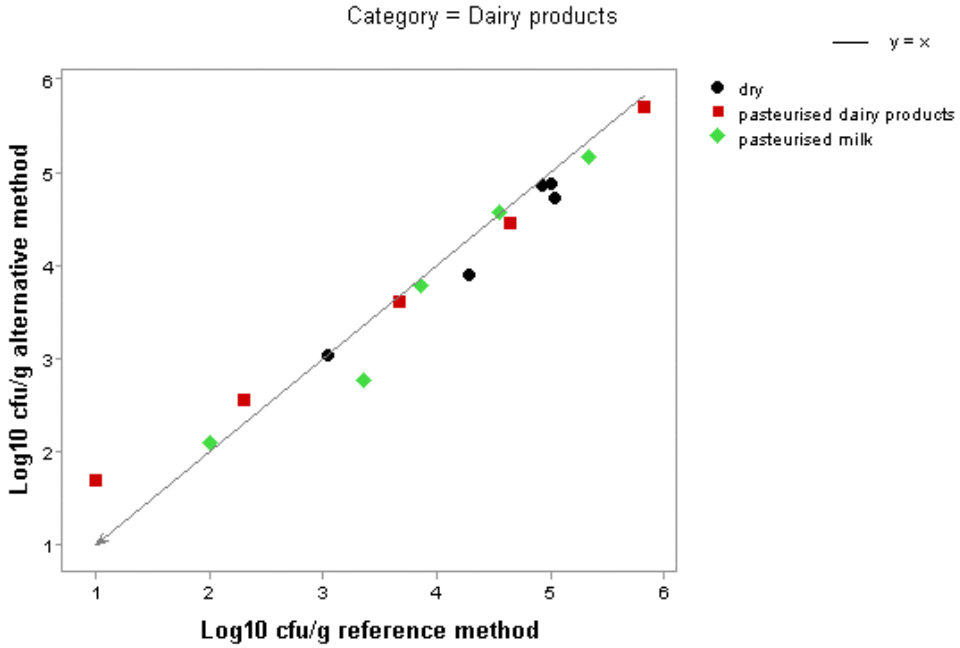


*Figure 2- Scatter plot of the reference method versus alternative method results for the **Fishery products***

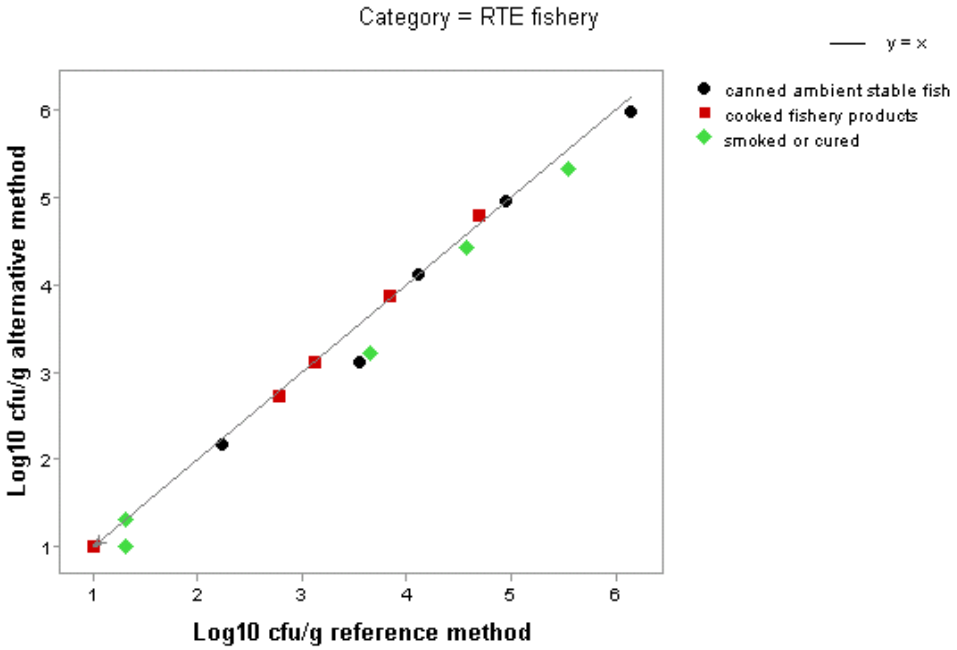


Figure 3- Scatter plot of the reference method versus alternative method results for the ***Dried cereals, fruits, nuts seeds and vegetables***

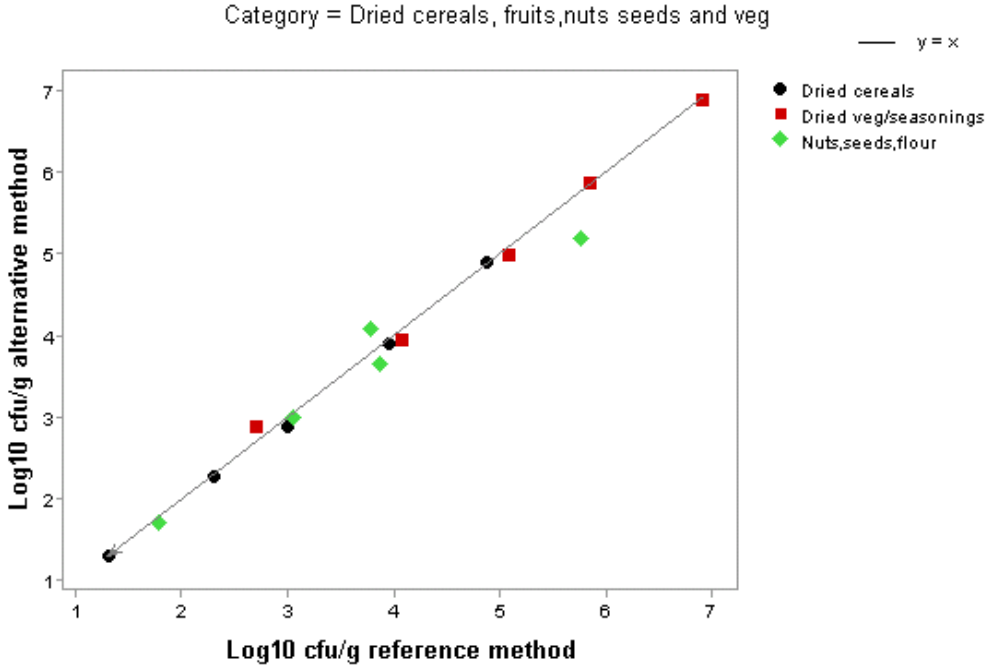


Figure 4- Scatter plot of the reference method versus alternative method results for the ***Meat and poultry products***

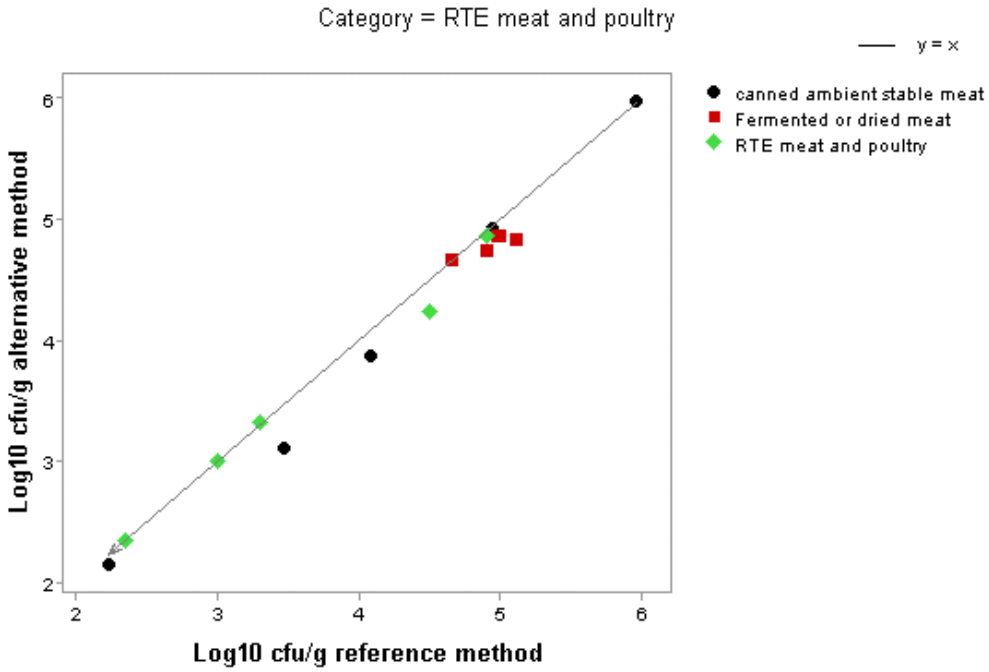


Figure 5- Scatter plot of the reference method versus alternative method results for the ***Multicomponent foods***

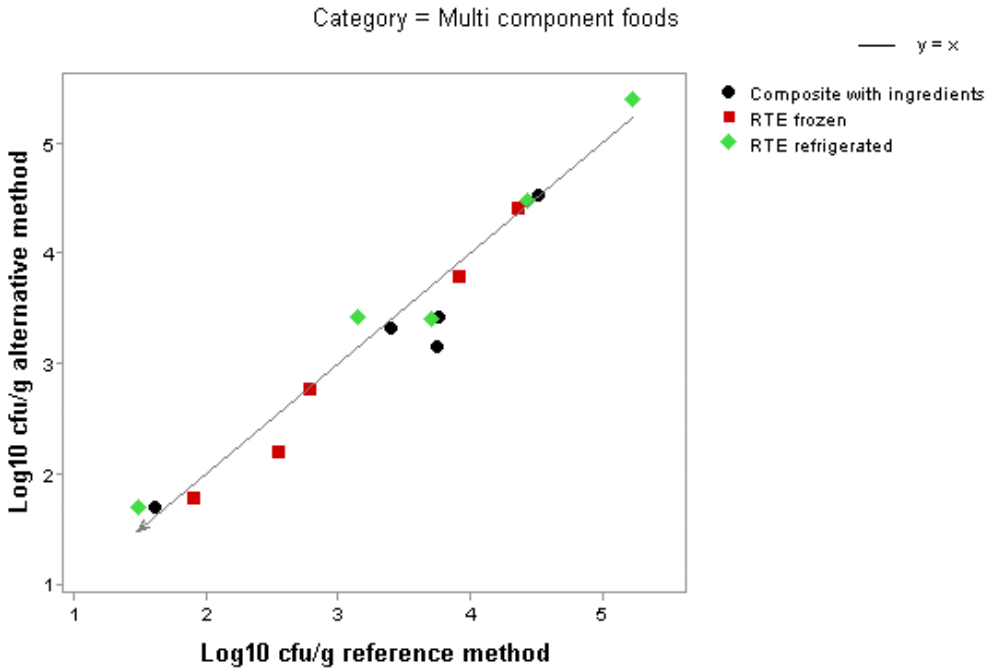
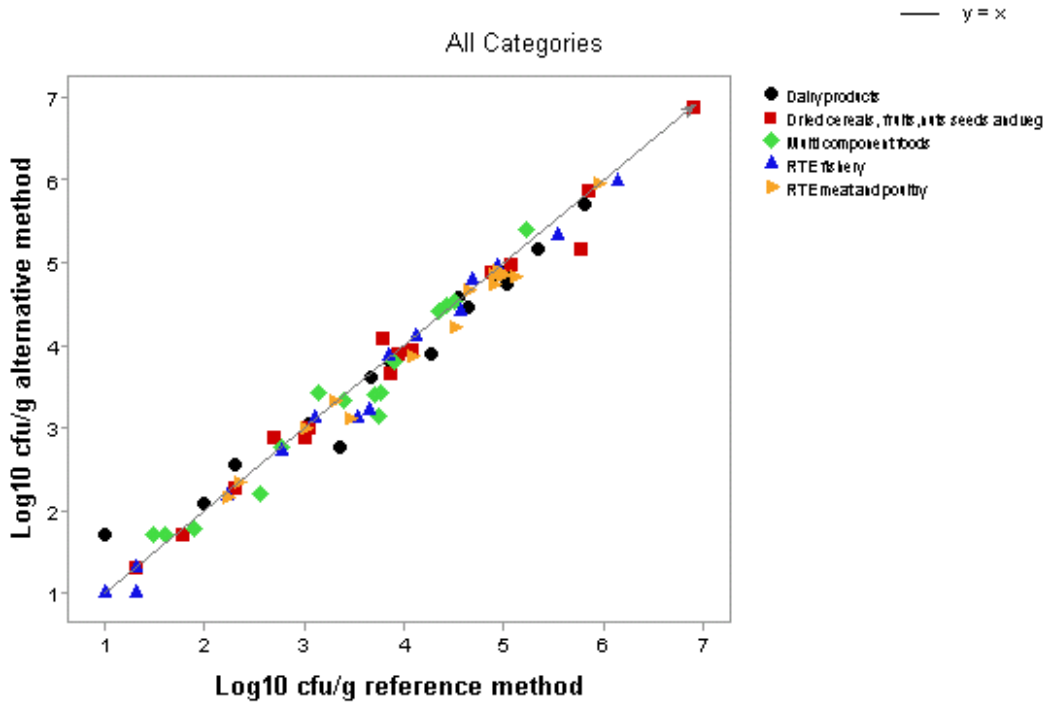


Figure 6 - Scatter plot of the reference method versus alternative method results for all the categories



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 scatter plots show good agreement between the reference method and alternative method.

There are no obvious disagreements between the two methods although there was a very slight negative bias observed on the scatterplot for the alternative method. This is further described in the Bland Altman plot analysis.

A summary of the calculated values per category is provided in Table 2.

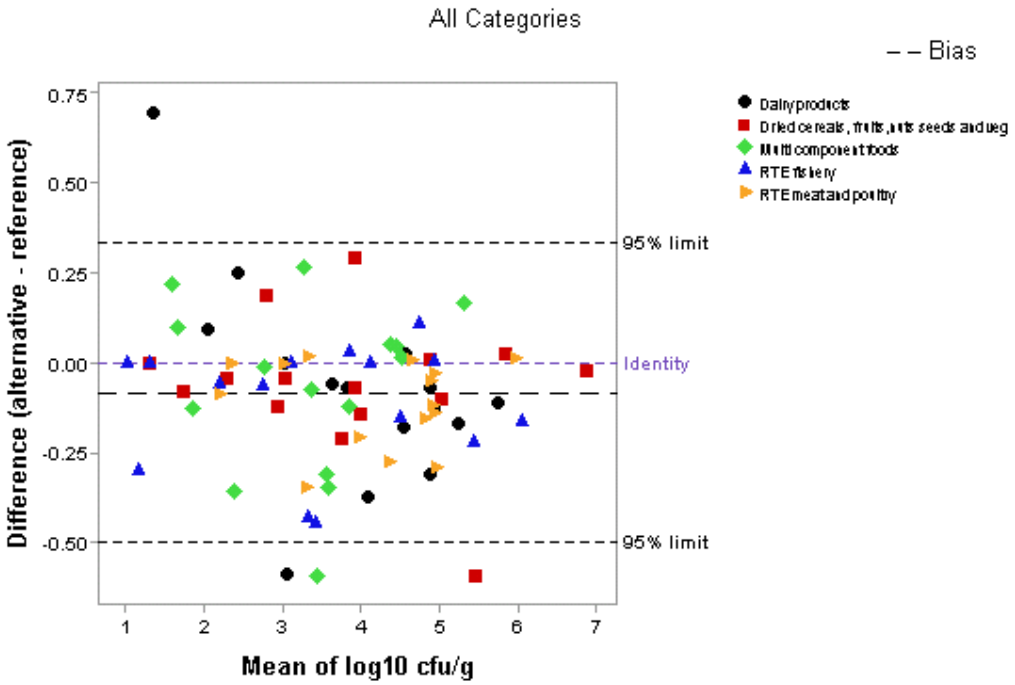
Table 2 - Summary of the calculated values per category

Row	Category.	n	Dbar	sD	95% Lower limit	95% Upper limit
1	Dairy products	15	-0.066	0.290	-0.710	0.577
2	Dried cereals, fruits,nuts seeds and veg	15	-0.060	0.194	-0.490	0.369
3	Multi component foods	15	-0.072	0.242	-0.609	0.465
4	RTE fishery	15	-0.113	0.170	-0.490	0.264
5	RTE meat and poultry	15	-0.109	0.122	-0.380	0.162
6	All Categories	75	-0.084	0.207	-0.500	0.332

$\overline{D}$: 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 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 the Table 3.

Table 3 - Data which are outside of the accepted limits

Category	Type	Code	Reference method Log cfu/g	Alternative method Log cfu/g	Mean Log cfu/g	Difference Alternative – reference)	Lower / Upper limits
Dried cereals, fruits,nuts seeds and veg	Nuts,seeds, flour	45	5.77	5.18	5.47	-0.59	-0.500
Multi component foods	Composite with ingredients	75	3.74	3.15	3.44	-0.59	-0.500
Dairy products	pasteurised milk	12	3.36	2.77	3.07	-0.59	-0.500
Dairy products	pasteurised dairy products	6	1.00	1.70	1.35	0.70	0.332

Comments

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. This meets the expectation

Three points were below the lower AL of -0.500. These samples were only just below the lower AL and were from three different categories; Dried cereals, fruits,nuts seeds and vegetables, Multi component foods and for different strains.

One sample was above the 0.322 upper AL and this was for pasteurised dairy products where the count on the reference method was based on <4 colonies.

3.1.6 Conclusion (RT study)

The relative trueness of the Alternative method is satisfied as there were only four data points outside of the acceptability limits and there was no major bias in the Bland Altman plot. There was a slight negative bias in the overall data set of -0.084

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 was 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 4):

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, except dry products, the 100g samples were inoculated and stored at 2-8°C for 48-72h prior to analysis. For dried products, a lyophilised culture was used and mixed into the samples prior to testing.

***Table 4* - Categories, types, items, strains and inoculation levels for accuracy profile study**

Category	Types	Ioculated Strain	Item	Inoculation levels
Dairy products	Pasteurised dairy products	*B.weihenstephanensis* CRA 16578 isolated from pasteurised milk	Panna cotta	Level 1x5: 10^3 cfu/g
				Level 2x5: 10^4 cfu/g
				Level 3x5: $5x10^5$ cfu/g
			Cream	Level 1x5: 10^3 cfu/g
				Level 2x5: 10^4 cfu/g
				Level 3x5: $5x10^5$ cfu/g
Dried cereals, fruits, nuts seeds and vegetables	Dehydrated vegetables/ seasonings	*B.cereus* CRA 8711 isolated from baby milk (Spores were used)	Dried baby food no probiotics	Level 1x5: 10^4 cfu/g
				Level 2x5: 10^5 cfu/g
				Level 3x5: 10^6 cfu/g
			Dehydrated veg	Level 1x5: 10^4 cfu/g
				Level 2x5: 10^5 cfu/g
				Level 3x5: 10^7 cfu/g
RTE Fishery products	Cooked fishery products	*B.cereus* CRA6295 isolated from flavouring	Seafood terrine	Level 1x5: 10^2 cfu/g
				Level 2x5: $5x10^3$ cfu/g
				Level 3x5: $5x10^5$ cfu/g
			Salmon pate	Level 1x5: 10^2 cfu/g
				Level 2x5: $5x10^3$ cfu/g
				Level 3x5: $5x10^5$ cfu/g
RTE meat and poultry products	Cooked chilled meats	*B.cereus* CRA16569 isolated from meat loaf	Sliced ham	Level 1x5: 10^2 cfu/g
				Level 2x5: $5x10^3$ cfu/g
				Level 3x5: 10^5 cfu/g
			Pork liver pate	Level 1x5: 10^2 cfu/g
				Level 2x5: $5x10^3$ cfu/g
				Level 3x5: 10^5 cfu/g
Multi component foods	Products with substantial raw ingredients	*B.thuringiensis* CRA 1744 isolated from flour	Sandwich	Level 1x5: 10^2 cfu/g
				Level 2x5: $5x10^3$ cfu/g
				Level 3x5: $5x10^5$ cfu/g
			Pasta salad	Level 1x5: 10^2 cfu/g
				Level 2x5: $5x10^3$ cfu/g
				Level 3x5: $5x10^5$ cfu/g

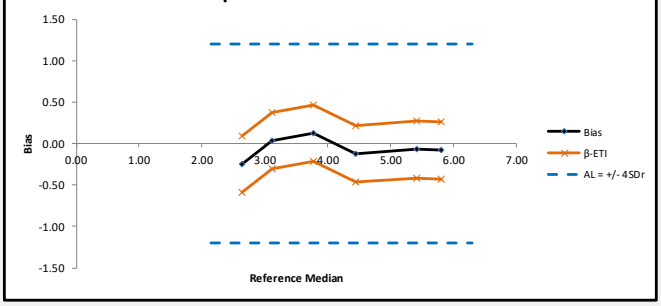
3.2.2 Calculations and interpretation of accuracy profile study

The raw data are provided in an excel spread sheet: 2019LR87 Accuracy profile and the summary tables (in log CFU/g) in Annex D. The statistical results and the accuracy profiles are provided 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

Figure 8 – Accuracy profile: Dairy Products

(Food) Category	Dairy
(Food) Type	panna cotta and cream

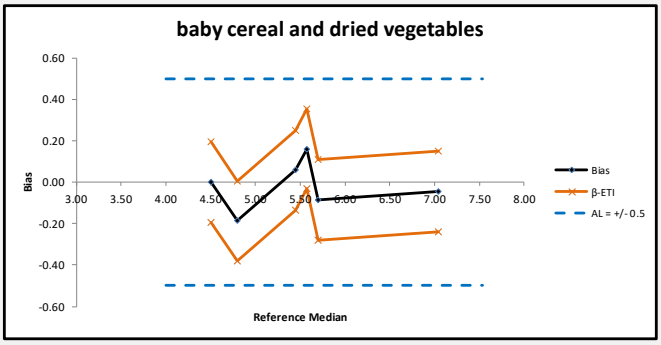


Sample Name	Reference central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
1 a-e	2.64	-0.249	-0.591	0.093	NO	YES
4 a - e	3.11	0.032	-0.310	0.374	YES	YES
2 a- e	3.78	0.125	-0.217	0.467	YES	YES
5 a - e	4.45	-0.125	-0.467	0.217	YES	YES
3 a-e	5.41	-0.073	-0.415	0.270	YES	YES
6 a - e	5.80	-0.083	-0.425	0.259	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.300	0.237	NO	+/- 1.200

Figure 9 – Accuracy profile: Dried cereals, fruits, nuts seeds and vegetables

(Food) Category	Dried products
(Food) Type	baby cereal and dried vegetables

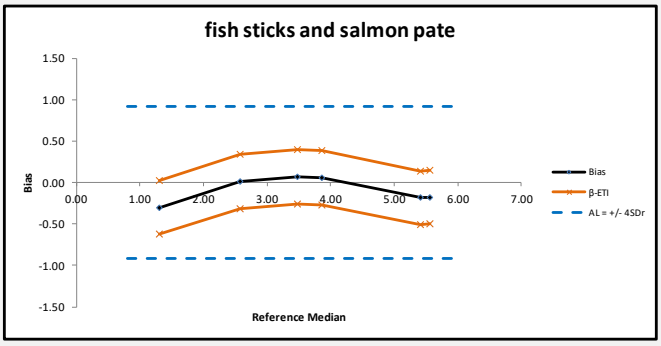


Sample Name	Reference central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
10 a - e	4.51	0.000	-0.193	0.193	YES	YES
7 a-e	4.80	-0.187	-0.380	0.007	YES	YES
9 a-e	5.45	0.058	-0.135	0.251	YES	YES
8 a- e	5.58	0.161	-0.033	0.354	YES	YES
11 a - e	5.70	-0.086	-0.280	0.107	YES	YES
12 a - e	7.04	-0.046	-0.239	0.148	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.285	0.134	NO	+/- 0.500

Figure 10 – Accuracy profile: RTE Fishery products

(Food) Category	Fishery products
(Food) Type	fish sticks and salmon pate

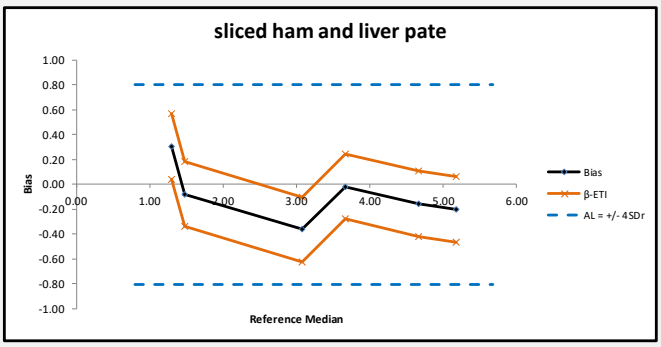


Sample Name	Reference central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
13 a - e	1.30	-0.301	-0.627	0.025	NO	YES
16 a-e	2.58	0.011	-0.315	0.338	YES	YES
17 a- e	3.48	0.067	-0.259	0.393	YES	YES
14 a-e	3.87	0.057	-0.269	0.384	YES	YES
15 a - e	5.41	-0.185	-0.511	0.142	NO	YES
18 a - e	5.56	-0.176	-0.502	0.150	NO	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.229	0.226	NO	+/- 0.916

Figure 11– Accuracy profile: RTE meat and poultry products

(Food) Category	RTE meat and poultry
(Food) Type	sliced ham and liver pate

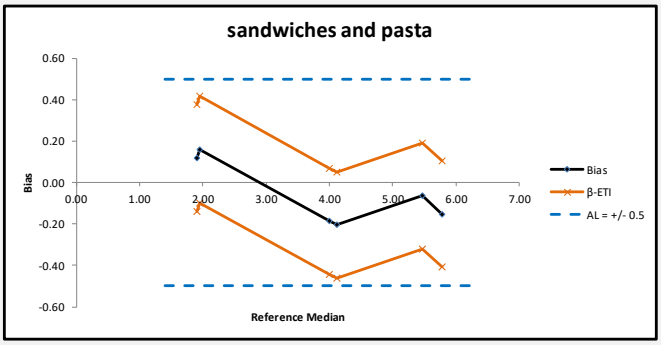


Sample Name	Reference central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
22 a-e	1.30	0.301	0.038	0.564	NO	YES
19 a - e	1.48	-0.079	-0.342	0.184	YES	YES
23 a- e	3.08	-0.365	-0.628	-0.102	NO	YES
20 a-e	3.67	-0.019	-0.282	0.244	YES	YES
24 a - e	4.66	-0.158	-0.420	0.105	YES	YES
21 a - e	5.18	-0.203	-0.466	0.060	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.201	0.182	NO	+/- 0.804

Figure 12 – Accuracy profile: Multi component foods

(Food) Category	Multi component foods
(Food) Type	sandwiches and pasta



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
28 a - e	1.90	0.118	-0.139	0.375	YES	YES
25 a-e	1.95	0.160	-0.097	0.417	YES	YES
29 a- e	4.00	-0.187	-0.444	0.070	YES	YES
26 a - e	4.11	-0.205	-0.462	0.052	YES	YES
30 a-e	5.46	-0.064	-0.321	0.193	YES	YES
27 a - e	5.79	-0.152	-0.409	0.105	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.256	0.178	NO	+/- 0.500

Comments

In this study the following categories met the AL of 0.5log : Dried cereals, fruits, nuts seeds and vegetables and Multi component foods

The following categories required the new AL to be calculated; Dairy, RTE fishery products and RTE meat and poultry products. All of these categories met the new AL values shown below.

For the Dairy category, 1 of the 12 ß-ETI values exceeded the 0.5log AL. This was for low level pana cotta. All categories met the newly calculated AL of 1.2logs. Whilst this is quite a large AL, this seems to be influenced by the repeatability of the reference method which has a SD repeatability value of 0.300 as compared to a lower level of 0.237 for the alternative method.

For the RTE Fishery products category, 3 of the 12 ß-ETI values exceeded the 0.5log AL. This was for low level fish, high level fish and high level salmon pate where the Lower ß-ETI was outside of the 0.5log AL . All categories met the newly calculated AL of 0.96 logs. The SD repeatability was similar for both methods at 0.226- 0.229.

For the RTE meat and poultry products category, 2 of the 12 ß-ETI values exceeded the 0.5log AL. This was for low level pate which exceeded the upper ß-ETI and medium level pate where the Lower ß-ETI was outside of the 0.5log AL . All categories met the newly calculated AL of 0.804 logs. The SD repeatability was similar for both methods at 0.201 for the reference and 0.182 for the alternative.

The accuracy of the Alternative method is satisfied as the all categories met the 0.5log AL or the re-calculated AL. Although the recalculated AL was large for the Dairy category this was influenced more by the SD repeatability of the reference method than the alternative method.

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

Fifty pure cultures of the target microorganisms were tested. Strains chosen represented *B.cereus* and the wider *B.cereus* group strains.

Each test was performed once with the alternative method, the reference method and a non-selective agar. Each strain was grown overnight in a non-selective broth and diluted so that the inoculum level was at least 100 times greater than the minimum level for quantification of the alternative method being validated.

- Exclusivity

A minimum of 30 pure cultures of (non-target) microorganisms were tested. Each test was performed once with the alternative, the reference method and a non-selective agar.

The inoculum level was similar to the greatest level of contamination expected to occur in any of the categories being used. The pure culture was grown in a suitable non-selective broth under optimal conditions of growth for at least 24 h and diluted to an appropriate level before testing.

3.3.2 Results
All raw data are given in excel spread: 2019LR87 Inclusivity

- Inclusivity

The results from the inclusivity study are summarised in Table 5. Any unexpected results are highlighted in yellow. Forty seven of these strains showed a positive result. Three strains showed a negative result.

No.34 *B.cytotoxicus* (DSM 22905) and No 35 *B.mycoides* (CRA 16597) did not grow on MYP or CD BC. A further strain No 40 *B.pseudomycoides* CRA 16382 did not grow on CD BC but did grow on MYP.

Table 5. Summarised Inclusivity data

No.	Organism	Code	Source (if known)	Reaction on CD BC	Reaction on MYP
1	*B.cereus*	84	Meat loaf	+	+
2	*B.cereus*	193	Environmental	+	+
3	*B.cereus*	1549	Dried milk	+	+
4	*B.cereus*	1731	Chocolate ice-	+	+
5	*B.cereus*	1740	Cream cake	+	+
6	*B.cereus*	1741	Flour	+	+
7	*B.cereus*	1749	Cream cake	+	+
8	*B.cereus*	1764	Milk/cream	+	+
9	*B.cereus*	4110	Contaminated flask	+	+
10	*B.cereus*	6295	Flavouring	+	+
11	*B.cereus*	6452	Flour	+	+
12	*B.cereus*	7616	Dairy	+	+
13	*B.cereus*	8711	Infant formula	+	+
14	*B.cereus*	16100	Flavour	+	+
15	*B.cereus*	16101	Flavour	+	+
16	*B.cereus*	16381	Environmental	+	+
17	*B.cereus*	16439	Environmental	+	+
18	*B.cereus*	16563	Unknown	+	+
19	*B.cereus*	16564	Food poisoning	+	+

No.	Organism	Code	Source (if known)	Reaction on CD BC	Reaction on MYP
20	*B.cereus*	16565	Pharmaceutical	+	+
21	*B.cereus*	16566	Unknown	+	+
22	*B.cereus*	16569	Meat loaf	+	+
23	*B.cereus*	16570	Food poisoning	+	+
24	*B.cereus*	16571	Unknown	+	+
25	*B.cereus*	16579	Industrial isolate	+	+
26	*B.cereus*	16580	Industrial isolate	+	+
27	*B.cereus*	16582	Environmental	+	+
28	*B.cereus*	16583	Industrial isolate	+	+
29	*B.cereus*	16662	Dried potato	+	+
30	*B.cereus*	17010	Mangoes	+	+
31	*B.cereus*	17011	Water	+	+
32	*B.cereus*	17012	Milk	+	+
33	*B.cereus*	17013	Soil	+	+
34	*Bacillus cytotoxicus*	DSM 22905	Vegetable puree	-	+
35	*Bacillus mycoides*	16597	UHT Custard	-	-
36	*Bacillus mycoides*	1522	Dried milk	+	+
37	*Bacillus mycoides*	16646	Soft drinks factory	+	+
38	*Bacillus mycoides*	1510	Dried milk	+	+
39	*Bacillus mycoides*	8504	Food environment	+	+
40	*Bacillus pseudomycoides*	16382	Soil	-	+
41	*Bacillus thuringiensis kurstaki*	17032	Insecticide	+	+
42	*Bacillus thuringiensis aizawai*	17033	Insecticide	+	+
43	*Bacillus thuringiensis isrealensis*	17034	Insecticide	+	+
44	*Bacillus thuringiensis*	16616	Broccoli	+	+
45	*Bacillus thuringiensis*	16314	Flour moth	+	+
46	*Bacillus thuringiensis*	1744	Flour	+	+
47	*Bacillus thuringiensis*	16619	Broccoli	+	+
48	*Bacillus weihenstephanensis*	16578	Pasteurised milk	+	+
49	*Bacillus weihenstephanensis*	DSM 104135	Soil	+	+
50	*Bacillus weihenstephanensis*	DSM104109	Soil	+	+

- Exclusivity

Table 6 : Summarised Inclusivity data

No	Organism	Code	Source	Reaction on CD BC	Reaction on MYP
1	*Allicyclobacillus acidoterrestris*	5331	Apple juice	-	-
2	*Alicyclobacillus cycloheptanicus*	16823	Soil		
3	*Alicyclobacillus fastidiosus*	16831	Apple juice	-	-
4	*Alicyclobacillus pomorum*	16830	Fruit juice	-	-
5	*Aneurinibacillus aneurinolyticus*	7751	Flavour	-	-
6	*Anoxybacillus flavithermus*	17047	Food isolate	-	-
7	*Bacillus amyloliquefaciens*	6317	crumpets	-	-*
8	*Bacillus circulans*	16584	Cream	-	-
9	*Bacillus coagulans*	10205	Evaporated milk	-*	-*
9 repeat	*Bacillus coagulans repeat test*	10205	Evaporated milk	+	+
10	*Bacillus fusiformis*	16652	Soft drinks	-	-
11	*Bacillus laterosporus*	1523	Dried milk	-*	-*
11repeat	*Bacillus laterosporus repeat test*	1523	Dried milk	+	+
12	*Bacillus licheniformis*	6335	Pesto	-	-
13	*Bacillus megaterium*	16512	Soil	-	-
14	*Bacillus oceanisediminis*	17220	Food isolate	-	-
15	*Bacillus pumilus*	16594	Industrial isolate	-	-
16	*Bacillus psychrodurans*	16694	Soil		
17	*Bacillus smithii*	7240	Pineapple	-	-
18	*Bacillus sonorensis*	17231	Food isolate	-	-
19	*Bacillus sphaericus*	7950	Flavouring	-	-
20	*Bacillus subtilis*	14161	Milk shake	-	-*
21	*Brevibacillus brevis*	7748	Flavour	-*	-*
21repeat	*Brevibacillus brevis repeat test*	7748	Flavour	+	+
22	*Brevibacillus parabrevis*	7757	Flavour	-	-
23	*Leuconostoc mesenteroides*	16022	Soft ham	-	-
24	*Listeria ivanovii*	1123	Soft cheese	-	-
25	*Lysinibacillus sphaericus*	7746	Unknown	-	-*
26	*Paenibacillus amylolyticus*	16606	Barley	-	-
27	*Paenibacillus macerans*	16488	DSM 357	-	-
28	*Paenibacillus pabuli*	16605	Barley	-	-
29	*Paenibacillus polymyxa*	7747	Food isolate	-*	-*
29repeat	*Paenibacillus polymyxa repeat test*	7747	Food isolate	+	+
30	*Staphylococcus aureus*	1224	Margarine	-	-
31	*B.laterosporus*	1515	Dried milk	+	+
32	*Paenibacillus polymyxa*	16386	ATCC 43865	-	-
33	*B.coagulans*	17185	Industrial isolate	-	-

-* strains showed typical growth on MYP and CD BC but the colonies did not show characteristic halos on Blood agar so were ultimately deemed to be negative

A total of 30 strains were originally tested for exclusivity numbered 1-30.

Twenty six of these strains showed a negative result on CD BC whilst four of the strains gave a positive result on CD BC. The four *Bacillus* species which gave a positive reaction on the alternative method were No 9 *Bacillus coagulans* (CRA10205); No 11 *Bacillus laterosporus* (CRA1523); No 21 *Brevibacillus brevis* (*CRA* 7748) and No 29 *Paenibacillus polymyxa* (CRA 7747). All strains showed a negative reaction on SBA and so were deemed negative

Seven strains (Nos 7, 9, 11, 20, 21, 24, 29) showed positive colonies on MYP but did not give typical halos on Blood Agar and so ultimately gave the true negative result.

In order to check these results and to see whether the results were specific to these 4 particular strains, a further 3 strains were tested (31-33) and the tests with the four original strains were repeated.

The results from the repeat test showed that the four *Bacillus* species were positive on both the alternative method and the reference method when repeated. One of the three additional strains No 31 *Bacillus laterosporus* (CRA1515) was also positive on both the reference method and the alternative method. These data are all highlighted in Table 6.

3.3.3 Conclusion
The alternative Compact Dry BC enumeration method is selective and specific for *B.cereus* and the wider *B.cereus* group. There are some minor differences between the reference method and the alternative method and the use of a confirmation procedure on SBA according to ISO 7932.

3.4 Conclusion (MCS)

Overall, the conclusions for the Method Comparison are:

- The alternative method Compact Dry BC for enumeration of *Bacillus cereus* shows satisfactory results for relative trueness;
- The alternative Compact Dry BC for enumeration of *Bacillus cereus* shows satisfactory results for accuracy profile;
- The alternative Compact Dry BC for enumeration of *Bacillus cereus* 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 9 laboratories with a single collaborator per laboratory. (See Annex E).

4.1.2 Matrix and strain used
Liver pate was inoculated with *B.cereus* CRA16569 isolated from meat loaf.

4.1.3 Sample preparation
Samples were prepared and inoculated on Tuesday 21st January 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. A set of samples was also prepared for the EL although the data from these was not used in the data analysis. Following inoculation the sampes were frozen at -18°C prior to dispatch.

The target levels and codes are shown below (Table 7)

Table 7: Contamination levels

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

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 analysed.

Samples were shipped on Thursday 23rd January in a frozen state so that they should be received by Monday 27th January 2020. Samples were to be set up Tuesday 28th January. The temperature conditions were intended to stay lower or equal to 8°C during transport, and between 0°C – 8°C in the labs. Stability trials were carried out under the intended storage condionts to demonstrate they did not allow any evolution of target organisms.

4.1.5 Analysis of Samples
Collaborative study laboratories and the expert laboratory carried out the analyses on 28/01/2020 with the alternative and reference methods. The analyses by the reference method and the alternative method were performed on the same day.

4.2 Experimental parameters controls

4.2.1 Strain stability during transport

Three samples inoculated at a low level were tested for enumeration of *Bacillus cereus* after 24 h and 48 h storage at 5°C ± 3°C. (Table 8)

Table 8 Bacillus cereus stability in the matrix

	Time (h) at chill storage after thawing									
	0h	24h	48h	72h	96h	0h	24h	48h	72h	96h
	Compact Dry BC					MYP				
Low A	1.60	0.70	0.70	0.70	0.70	1.30	1.00	1.00	1.00	1.00
Low B	1.60	0.70	1.18	0.70	0.70	1.60	1.30	0.00	0.00	1.00
Low C	1.00	0.70	1.00	1.18	1.30	1.70	0.00	1.00	1.00	1.30
Medium A	3.40	3.52	2.70	2.79	2.65	3.56	3.48	2.89	2.74	2.98
Medium B	3.57	3.32	2.69	2.56	2.69	3.76	3.46	2.87	2.60	2.78
Medium C	3.51	3.34	2.80	2.67	2.56	3.69	3.41	2.80	2.65	2.95
High A	5.28	5.93	4.54	4.41	4.18	5.51	4.82	4.78	4.45	4.23
High B	5.40	5.88	4.63	4.53	4.26	5.51	4.93	4.75	4.36	4.38
High C	5.26	5.97	4.82	4.61	4.54	5.43	5.04	4.97	4.59	4.61

No growth was observed during storage at 5°C ± 3°C and there was a slight decrease in levels of inoculated organisms during storage.

4.2.2 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 Tempera measured by the probe (°C)	Temperature measured at receipt (°C)	Receipt date and time	Analysis date
1	Not returned	5.2	24/01/2020	28/01/2020
3	4.6	13.2	27/01/2020	28/01/2020
4	2.4	5.3	24/01/2020	28/01/2020
5	2.5	3.2	24/01/2020	28/01/2020
6	1.2	4.6	24/01/2020	28/01/2020

Collaborator	Average Tempera measured by the probe (°C)	Temperature measured at receipt (°C)	Receipt date and time	Analysis date
7	3.1	8.7	28/01/2020	28/01/2020
8	2.9	5.3	24/01/2020	28/01/2020
9	2.4	4.6	24/01/2020	28/01/2020

No data was received from Lab 2 and the samples were not tested. They have been removed from further discussion.

No problem was encountered during the transport or at receipt for 8 of the collaborators. All the samples were delivered on time and in appropriate conditions. Temperatures during shipment and at receipt were all correct. Lab 3 recorded a high temperature from the water vial but the temperature probe data showed that the temperature was satisfactory during transport and storage.

The temperature curves are given in Annex F.

4.3 Calculation and summary of data

The raw data are given in Annex G.

4.3.1 MicroVal Expert laboratory results

The results obtained by the expert laboratory are given in Table 10.

Table 10 – Results obtained by the expert lab Log 10 cfu/g

Level	Reference method	Alternative method
Blank	<10	<10
Low	<1.00	1.00
Low	1.30	<1.00
Medium	2.94	2.87
Medium	2.93	2.86
High	5.56	5.49
High	5.38	5.40

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 11.

The low inoculum level was slightly lower than anticipated and in 3 cases the level observed was <10cfu/g. In order to allow the calculation to be done on all the data sets a value of LOD/sqrt(2) was substituted (7 cfu/g) was substituted for the <10 values.

The statistical analysis was done twice, once with the substituted values included and once by removing the three low level data sets with the <10 results from the analysis.

The accuracy profile plot is shown in Figure 13 a and b and the statistical analysis of the data shown in Table 12a and b.

Table 11: Summary of the results of the interlaboratory study per analyte level (k

Collaborator	**Level**	**Reference method (Log cfu/g)**		**Alternative method (Log cfu/g)**	
		Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
01	low	1.30	0.85*	1.00	1.48
03	low	0.85*	1.00	1.00	1.30
04	low	1.00	1.30	1.00	1.30
05	low	1.78	1.30	1.48	1.60
06	low	1.00	0.85*	1.00	1.00
07	low	1.48	1.60	1.18	1.18
08	low	1.00	1.00	1.30	1.00
09	low	1.60	1.48	1.60	1.48
01	medium	2.66	2.93	2.93	3.13
03	medium	3.05	2.98	2.92	2.87
04	medium	2.99	3.33	3.08	3.42
05	medium	2.89	3.06	3.01	3.01
06	medium	2.97	3.01	2.79	3.06
07	medium	2.54	2.90	2.97	3.00
08	medium	3.17	3.07	3.23	3.15
09	medium	2.95	3.05	3.04	3.19
01	high	5.58	5.70	5.42	5.65
03	high	5.46	5.34	5.57	5.38
04	high	5.42	5.40	5.65	5.53
05	high	5.46	5.36	5.53	5.34
06	high	5.48	5.64	5.60	5.49
07	high	5.59	5.40	5.54	5.52
08	high	5.62	5.60	5.62	5.54
09	high	5.36	5.63	5.43	5.73
01	blank	<10		<10	
03	blank	<10		<10	
04	blank	<10		<10	
05	blank	<10		<10	
06	blank	<10		<10	
07	blank	<10		<10	
08	blank	<10		<10	
09	blank	<10		<10	

* actual counts were <10 so a value of LOD/sqrt(2) was substituted

Figure 13a. Accuracy profile of Compact Dry from the ILS using substituted values for 3 <10cfu/g data points

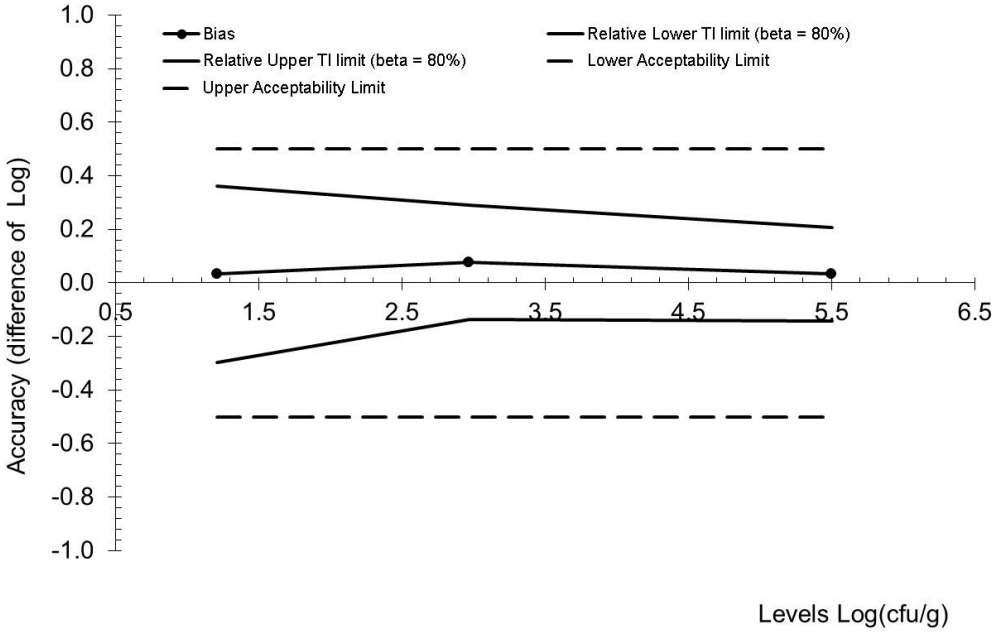


Figure 13b. Accuracy profile of Compact Dry from the ILS removing low level 3 data sets with <10cfu/g values

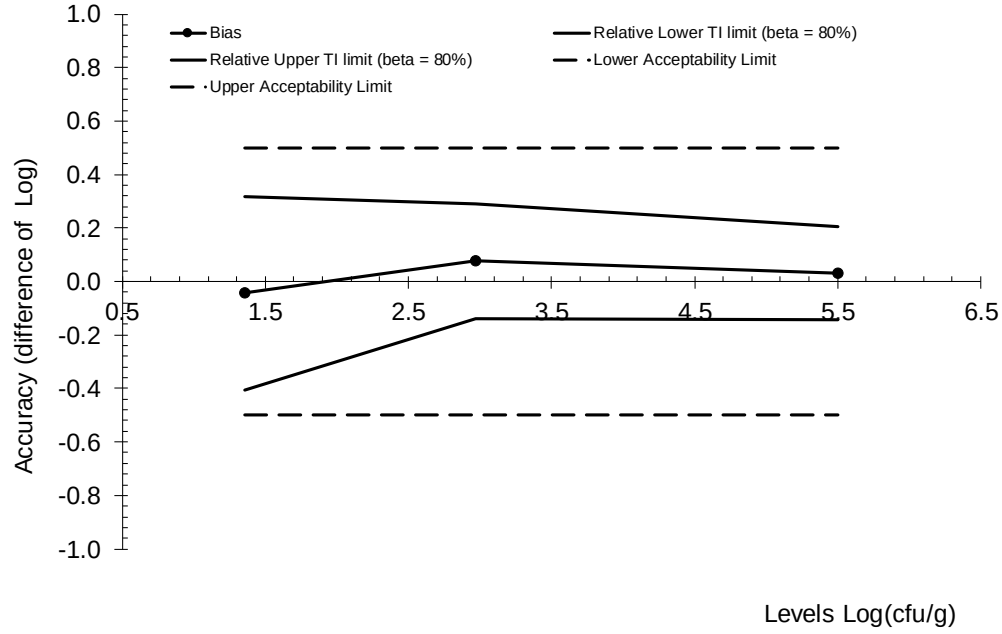


Table 12a. Statistical analysis of the ILS data according to the ISO spreadsheet using substituted values for 3 <10cfu/g data points

Accuracy profile	0.5		
Study Name	Compact Dry BC		
Date	calculated 06/03/2020		
Coordinator	Campden BRI		
Tolerance probability (beta)	80%	80%	80%
Acceptability limit in log (lambda)	0.50	0.50	0.50

FALSE

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.

	Alternative method			**Reference method**		
Levels	**Low**	**Medium**	**High**	**Low**	**Medium**	**High**
Target value	**1.211**	**2.972**	**5.503**			
Number of participants (K)	8	8	8	8	8	8
Average for alternative method	1.243	3.048	5.535	1.211	2.972	5.503
Repeatability standard deviation (sr)	0.182	0.126	0.126	0.195	0.152	0.106
Between-labs standard deviation (sL)	0.146	0.086	0.000	0.246	0.101	0.057
Reproducibility standard deviation (sR)	0.233	0.153	0.126	0.314	0.182	0.120
Corrected number of dof	12.393	13.040	14.933	10.216	13.147	13.815
Coverage factor	1.411	1.405	1.382			
Interpolated Student t	1.354	1.350	1.341			
Tolerance interval standard deviation	0.2433	0.1590	0.1295			
Lower TI limit	0.914	2.833	5.362			
Upper TI limit	1.573	3.263	5.709			
Bias	**0.032**	**0.077**	**0.032**			
Relative Lower TI limit (beta = 80%)	**-0.297**	**-0.138**	**-0.142**			
Relative Upper TI limit (beta = 80%)	**0.362**	**0.291**	**0.206**			
Lower Acceptability Limit	**-0.50**	**-0.50**	**-0.50**			
Upper Acceptability Limit	**0.50**	**0.50**	**0.50**			

FALSE
FALSE

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

New acceptability limits may be based on reference method pooled variance

Pooled repro standard dev of reference	0.221

Table 12b. Statistical analysis of the ILS data according to the ISO spreadsheet removing low level 3 data sets with <10cfu/g values

Accuracy profile	0.5		
Study Name	Compact Dry BC		
Date	calculated 06/03/2020		
Coordinator	Campden BRI		
Tolerance probability (beta)	80%	80%	80%
Acceptability limit in log (lambda)	0.50	0.50	0.50

FALSE

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.

	Alternative method			**Reference method**		
Levels	**Low**	**Medium**	**High**	**Low**	**Medium**	**High**
Target value	**1.354**	**2.972**	**5.503**			
Number of participants (K)	5	8	8	5	8	8
Average for alternative method	1.311	3.048	5.535	1.354	2.972	5.503
Repeatability standard deviation (sr)	0.146	0.126	0.126	0.187	0.152	0.106
Between-labs standard deviation (sL)	0.181	0.086	0.000	0.224	0.101	0.057
Reproducibility standard deviation (sR)	0.233	0.153	0.126	0.292	0.182	0.120
Corrected number of dof	5.908	13.040	14.933	6.016	13.147	13.815
Coverage factor	1.555	1.405	1.382			
Interpolated Student t	1.443	1.350	1.341			
Tolerance interval standard deviation	0.2508	0.1590	0.1295			
Lower TI limit	0.949	2.833	5.362			
Upper TI limit	1.673	3.263	5.709			
Bias	**-0.043**	**0.077**	**0.032**			
Relative Lower TI limit (beta = 80%)	**-0.404**	**-0.138**	**-0.142**			
Relative Upper TI limit (beta = 80%)	**0.319**	**0.291**	**0.206**			
Lower Acceptability Limit	**-0.50**	**-0.50**	**-0.50**			
Upper Acceptability Limit	**0.50**	**0.50**	**0.50**			

FALSE
FALSE

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

New acceptability limits may be based on reference method pooled variance

Pooled repro standard dev of reference	0.210

5 Overall conclusions of the validation study

- The alternative method Compact Dry BC for enumeration of *Bacillus cereus* shows satisfactory results for relative trueness
- The alternative Compact Dry BC for enumeration of *Bacillus cereus* shows satisfactory results for accuracy profile;
- The alternative Compact Dry BC for enumeration of *Bacillus cereus* is selective and specific.
- The alternative Compact Dry BC for enumeration of *Bacillus cereus* shows satisfactory performance in the ILS
- The alternative Compact Dry BC for enumeration of *Bacillus cereus* shows comparable performance to the reference method ISO 7932:2004 Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of presumptive *Bacillus cereus* – Colony count technique at 30ºC.

Date: 7th April 2020

Signature