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ISO 16140-2:2016 validation study of TEMPO® BC for enumeration of *Bacillus cereus* in food and environmental samples

MicroVal study number	2014LR47
Method/Kit name	**TEMPO® BC**
Protocol version	**Summary Report -Version 1** 15 July 2019
MicroVal Expert Laboratory	**ADRIA Développement** ZA Creac'h Gwen F-29196 QUIMPER Cedex (France)

This report consists of 50 pages, including 2 appendices.
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Standardized report - Quantitative methods

MICROVAL® NEN

Foreword

This protocol is prepared in accordance with ISO 16140-2:2016 and MicroVal technical rules v.1.0.

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Expert Laboratory	**ADRIA Développement** ZA Creac'h Gwen F-29196 QUIMPER Cedex (France)
Method/Kit name	**TEMPO® BC**
Validation protocol	☒ **ISO 16140-1 (2016)**: Microbiology of the food chain — Method validation — *Part 1: Vocabulary* ☒ **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): horizontal method for the enumeration of presumptive *Bacillus cereus* - Colony-count technique at 30°C
Scope of validation	☒ **Food products** ☒ **Environmental samples**
Certification organization	Lloyd's Register

♦ Analyses performed according to the COFRAC accreditation

LIST OF ABBREVIATIONS

-	No typical colonies but presence of background microflora
(x)	Number of colonies in the plate
*	1/2 dilution
1/2	50% level of target analyte
AL	Acceptability Limit
BPW	Buffered Peptone Water
CFU	Colony Forming Units
d	Doubtful result
$\overline{D}$	Average difference
g	Gram
h	Hour
ILS	Inter-laboratory Study
MCS	Method Comparison Study
min	minute
ml	millilitre
MR	(MicroVal) Method Reviewer
MVTC	MicroVal Technical Committee
RT	Relative Trueness
RTC	Ready to cook
RTE	Ready to eat
RTRH	Ready to reheat

Bold typing: artificially inoculated samples

1 INTRODUCTION

A MicroVal renewal validation study, based on ISO 16140-2:2016, of the TEMPO® BC (*B. cereus*) method for the enumeration of *Bacillus cereus* in food and environmental samples was carried out by ADRIA Développement as the MicroVal Expert Laboratory.

The method was validated on the 30th of December 2015 for all food categories and environmental samples (certificate number: 2014LR47) according to ISO /FIS 16140(2014). *The method was renewed in 2019 according to ISO 16140-2 (2016).*

The alternative method used is an enumeration of *Bacillus cereus* with TEMPO® BC from sample primarily diluted in peptone-salt.

The reference method♦ used is ISO 7932 (2004): horizontal method for the enumeration of presumptive *Bacillus cereus* - Colony-count technique at 30°C.

The categories included are the following:

- Meat products, fish products and seafood,
- Dairy products,
- Egg products,
- Fruits and vegetables,
- Baby food,
- Environmental samples.

The criteria to be evaluated during the study are:

- Method Comparison Study (MCS)
  - Relative Trueness study
  - Accuracy profile study
  - Limit of Quantification study(LOQ)
  - Inclusivity and exclusivity study

- Inter-laboratory Study (ILS).

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2 METHOD PROTOCOLS

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

2.1 Reference method♦

The reference method used was the 7932 (2004): horizontal method for the enumeration of presumptive *Bacillus cereus* - Colony-count technique at 30°C. See the flow diagram in **Annex 1.**

Sample preparations were done according to ISO 6887-series (1 to 5).

2.2 Alternative method

See the flow diagram of the alternative method in **Annex 2**.

- ***Principle***

The TEMPO® BC test consists of a vial of culture medium and a card which are specific to this test.

The culture medium is inoculated with the sample to be tested. The inoculated medium is transferred by the TEMPO® Filler instrument into the card containing 48 wells of three different volumes. The card contains 3 sets of 16 wells (small, medium and large wells) with a one log difference in volume for each set of wells. The card is designed to simulate the Most Probable Number (MPN) method (3, 4). The card is then hermetically sealed in order to avoid any risk of contamination during subsequent handling.

The target microorganisms present in the card reduce the substrate in the culture medium during incubation and cause a fluorescent signal to appear, which is detected by the TEMPO® Reader instrument. Depending on the number and type of the positive wells, the TEMPO® system calculates the number of microorganisms present in the original sample according to a calculation based on the MPN method.

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Protocol:

The protocol is the following:

- Preparation of primary dilution in appropriate diluent according the ISO 6887 recommendations in a TEMPO® bag filter;
- Preparation of subsequent dilutions if necessary;
- Reconstitution of the culture medium (TEMPO® BC culture medium) by dispensing 3 ml of secondary diluent sterile distilled water per vial;
- Transfer of 1 ml of primary dilution into the vial containing the reconstituted culture medium (dilution 1/40);
- Fill and seal the TEMPO® cards using the TEMPO® Filler;
- Incubation of the cards for 22 – 27 h at 30°C ± 1°C;It is possible to store the cards for 48h at 5°C ± 3°C prior reading.
- Read the cards using the TEMPO® Reader.

2.3 Study design

This is a paired study design as the same diluent is used for primary dilution of the alternative method and the reference method.

3 METHOD COMPARISON STUDY

The study was carried out on a diversity of samples and strains representative of agri-food products. This does not constitute an exhaustive list of the different matrices included in the scope.

3.1 Sample preparation

The Method Comparison Study was carried out using 10 gram test portions of the sample. The samples were prepared for analysis and diluted in accordance with ISO 6887 (parts 1 to 5) unless specified differently in the alternative method.
The dilutions usually applied for the alternative method are given in Table 1 but it is also possible to select another dilution which could be more appropriate to the contamination level. The interpretation has been done using the usual dilution.

Table 1 – Dilutions usually applied

Products	Dilution
Baby food	1/40
Milk powder	
Egg powder	
Dusts from infant formula and baby food production units	
Infant formula	
Spices, inhibitory products (aromatic herbs...) Strongly coloured products	1/200, 1/400
All other products	1/200

3.2 Relative trueness study

The relative trueness is the degree of correspondence between the response obtained by the reference method and the response obtained by the alternative method on identical samples.

3.2.1 Number and nature of the samples

The selected types per category are listed in Table 2.

A total of 5 food categories and environmental samples were included in this validation study. Three types were evaluated within each category. A

minimum of 15 items for each category was tested by both the reference method and the alternative method in the relative trueness study, with a minimum of 15 interpretable results per category. The cards were read after 22h incubation time at 30°C and after storage for 48h at 5°C ± 3°C.

Table 2 - List of Categories, types, and examples of Items to be tested within the relative trueness study

Category		Type		Number of samples tested	Number of samples with interpretable results by both methods	
					22h at 30°C	22h at 30°C + 48h at 5°C
1	Meat products, fish products and seafood	a	RTE foods	11	6	6
		b	RTE composite foods	7	5	5
		c	RTH and RTRC foods	7	5	5
	Total			25	16	16
2	Dairy products	a	Milk powders	7	6	6
		b	Cream cheeses, cheese preparations	8	5	5
		c	Dairy desserts	7	5	5
	Total			22	16	16
3	Egg products	a	Pasteurized liquid eggs	7	5	5
		b	Egg powders	8	5	5
		c	Pastries, creams, desserts	7	7	7
	Total			22	17	17
4	Fruits and vegetables	a	Flours, spices, dehydrated vegetables	9	6	6
		b	Pasta, rice	13	7	7
		c	Deli salads, vegetables preparations	12	5	5
	Total			34	18	18
5	Baby food	a	Infant formula and cereals, raw materials without probiotics	5	5	5
		b	Infant formula and cereals, raw materials with probiotics	5	5	5
		c	Ready to reheat foods	5	5	5
	Total			15	15	15
6	Environmental samples	a	Process waters	18	6	6
		b	Wipes, sponges, swabs	22	7	7
		c	Dusts	6	6	6
	Total			46	19	19
All categories				**164**	**101**	**101**

164 samples were tested, leading to 101 interpretable results by both methods.

3.2.2 Test sample preparation

Naturally contaminated samples were preferentially used but artificial contaminations were also realised using spiking or seeding protocols. Injury efficiency was evaluated by enumerating the pure culture on selective and non-selective agars. The observed injury measurements vary from 0.20 to more than 2.65 log CFU/ml difference.

32 samples were naturally contaminated and 84 samples were artificially contaminated; 69 gave interpretable results.

3.2.3 Protocols applied during the validation study

- ***Incubation time***

For the reference method, the plates were incubated for 18 to 48 h.

For the TEMPO® BC method, the cards were incubated during the minimum incubation time: 22 h at 30°C ± 1°C.

- ***TEMPO® card storage***

The TEMPO® BC cards were stored for 48 h at 5°C ± 3 and read again.

3.2.4 Test results

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

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

- Interpretable results with the reference and the alternative methods;

- Results with less than 4 colonies per plate with the reference and/or the alternative method (indicated in orange cells in the data) in order to have a more precise result. These results are not included in the calculation.

- Results below or above the quantification limit: according to the ISO 16140-2:2016, if any result (either reference or alternative method) is below the quantification limit, the data should be plotted using a substituted value of 1 $\log_{10}$ units less than the observed value in case of a lower than value. Similarly, any value greater than the upper limit should be amended by adding 1 log unit. These results are not included in the calculations but also appear on the graphs.

- Samples with no result (ND): too much colonies on MYP plates to provide a result.

Table 3 - Classification of the data

Category			Type	Number of samples tested	Number of samples with no result (ND)	22 h at 30°C			22 h at 30°C + 48h at 5°C ± 3°C		
						Number of interpretable results by both methods	Number of samples with less than 4 colonies / plate	Number of samples below or above the quantification limit	Number of interpretable results by both methods	Number of samples with less than 4 colonies /plate	Number of samples below or above the quantification limit
1	Meat products, fish products and seafood	a	RTE foods	11	1	6	0	4	6	0	4
		b	RTE composite foods	7	1	5	0	1	5	0	1
		c	RTH and RTRC foods	7	0	5	1	1	5	1	1
	Total			25	2	16	1	6	16	1	6
2	Dairy products	a	Milk powders	7	0	6	1	0	6	1	0
		b	Cream cheeses, cheese preparations	8	0	5	1	2	5	1	2
		c	Dairy desserts	7	0	5	1	1	5	1	1
	Total			22	0	16	3	3	16	3	3
3	Egg products	a	Pasteurized liquid eggs	7	0	5	1	1	5	1	1
		b	Egg powders	8	0	5	0	3	5	0	3
		c	Pastries, creams, desserts	7	0	7	0	0	7	0	0
	Total			22	0	17	1	4	17	1	4
4	Fruits and vegetables	a	Flours, spices, dehydrated vegetables	9	1	6	0	2	6	1	1
		b	Pasta, rice	13	1	7	0	5	7	0	5
		c	Deli salads, vegetables preparations	12	1	5	1	5	5	1	7
	Total			34	3	18	1	12	18	2	13
5	Baby food	a	Infant formula and cereals, raw materials without probiotics	5	0	5	0	0	5	0	0
		b	Infant formula and cereals, raw materials with probiotics	5	0	5	0	0	5	0	0
		c	Ready to reheat foods	5	0	5	0	0	5	0	0
	Total			15	0	15	0	0	15	0	0
6	Environmental samples	a	Process waters	18	0	6	0	12	6	0	12
		b	Wipes, sponges, swabs	22	13	7	0	2	7	0	2
		c	Dusts	6	0	6	0	0	6	0	0
	Total			46	13	19	0	14	19	0	14
All categories				**164**	**18**	**101**	**6**	**39**	**101**	**7**	**40**

The samples, which were not used in the calculations, are provided in Table 4.

Table 4 - Samples which were not used in the calculations

Sample N°	Product	Reference method : ISO 7932♦ (log CFU/g)	Alternative method: TEMPO® BC		Category	Type
			22h at 30°C (usual dilutions CFU/g)	22h at 30°C + 48h at 5°C ± 3°C (usual dilutions CFU/g)		
84	Pork meat	ND	<1,70	<1,70	1	a
422	Ready to reheat pork	<1,00	<1,70	<1,70	1	a
426	Chicken meat	<2,00	<1,70	<1,70	1	a
556	Octopus	<1,00	<1,70	<1,70	1	a
1111	**Ready to reheat Fish preparation**	>4,18	4,36	4,36	1	a
555	Accras	ND	1,32	<1,70	1	b
1363	Deli salad	<1,00	1,00	1,00	1	b
551	Ready to reheat fish	<1,00	2,04	2,04	1	c
1194	**Ready to reheat Fish preparation**	1,30	1,72	1,72	1	c
76	Milk powder	1,30	1,00	1,00	2	a
1036	**Cream cheese**	1,48	1,00	1,00	2	b
1037	**Cream cheese**	<1,00	<1,00	<1,00	2	b
1038	**Cream cheese**	<1,00	<1,00	<1,00	2	b
1041	**Dairy dessert**	1,48	1,32	1,32	2	c
1043	**Semolina pudding**	<1,00	<1,00	<1,00	2	c
1095	**Pasteurized liquid egg yolk sweet (50%)**	<1,00	1,72	1,72	3	a
1097	**Pasteurized liquid egg white**	1,48	1,72	1,72	3	a
1109	**Whole egg powder**	<1,00	<1,00	<1,00	3	b
5679	Egg powder	<1,00	<1,00	<1,00	3	b
5680	Egg powder	<1,00	<1,00	<1,00	3	b
5759	Dehydrated mushrooms	ND	5,30	>5,69	4	a
73	Dehydrated mushrooms	3,62	>5,69	>5,69	4	a
75	Dehydrated onions	1,30	<1,70	1,72	4	a
5682	Cooked rice	<1,00	<1,70	<1,00	4	b
420	Rice and vegetables	<3,00	2,00	3,94	4	b
421	Cooked pasta	1,00	<1,70	<1,70	4	b
425	Rice pasta	<2,00	<1,70	2,40	4	b
548	Cooked pasta	<1,00	<1,70	<1,00	4	b
554	Cooked pasta	ND	>5,40	>5,40	4	b
5757	Ready to reheat potatoes	1,60	<1,70	<1,70	4	c
85	Vegetables soup	1,00	<1,70	<1,70	4	c
430	Cooked rice with vegetables	<1,00	<1,70	<1,70	4	c
549	Broccolis purée	1,30	<1,70	<1,70	4	c

♦ Analyses performed according to the COFRAC accreditation

Sample N°	Product	Reference method : ISO 7932♦ (log CFU/g)	Alternative method: TEMPO® BC		Category	Type
			22h at 30°C (usual dilutions CFU/g)	22h at 30°C + 48h at 5°C ± 3°C (usual dilutions CFU/g)		
552	Ready to reheat potatoes	1,00	<1,70	<1,70	4	c
1962	Vegetables for couscous	ND	<1,70	<1,70	4	c
1963	Vegetables for "pot au feu"	1,48	1,72	1,72	4	c
1459	Process water	<1,00	<1,70	<1,70	6	a
1460	Process water	<1,00	<1,70	<1,70	6	a
1461	Process water	<1,00	<1,70	<1,70	6	a
1965	Process water dairy industry	<1,00	<1,70	<1,70	6	a
1966	Process water dairy industry	<1,00	<1,70	<1,70	6	a
1967	Process water dairy industry	<1,00	<1,70	<1,70	6	a
1968	Process water dairy industry	<1,00	<1,70	<1,70	6	a
2206	**Rinsing water dairy industry**	<1,00	<1,70	<1,70	6	a
2207	**Rinsing water dairy industry**	1,48	<1,70	<1,70	6	a
2208	**Rinsing water dairy industry**	<1,00	<1,70	<1,70	6	a
2209	**Process water vegetables industry**	1,00	<1,70	<1,70	6	a
2210	**Process water ready-to-eat meal**	1,30	<1,70	<1,70	6	a
1462	Wipe	ND	<1,70	ND	6	b
1463	Wipe	ND	<1,70	4,93	6	b
1464	Wipe	ND	<1,70	<1,70	6	b
1465	Wipe	ND	<1,70	<1,70	6	b
1466	Wipe	ND	5,40	>5,40	6	b
1467	Wipe	ND	<1,70	>4,69	6	b
1468	Wipe	ND	4,04	>4,69	6	b
1469	Wipe	ND	>4,69	>5,40	6	b
1470	Wipe	ND	<1,70	>4,69	6	b
1471	Wipe	ND	>4,69	>5,40	6	b
1989	Wipe vegetables industry	ND	<1,70	<1,70	6	b
1990	Wipe vegetables industry	ND	1,72	2,20	6	b
1991	Wipe vegetables industry	ND	<1,70	<1,70	6	b
1992	Swab vegetables industry	<1,00	1,72	1,72	6	b
1993	Swab vegetables industry	<1,00	<1,70	<1,70	6	b

Results calculated using enumeration lower than 4 CFU/plate

3.2.5 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).

The Figures 1 to 6 show the data plotted for each individual category.

The Figure 7 shows the data plotted for all the products.

Figure 1 - Data plotted for the Meat products, fish and seafood

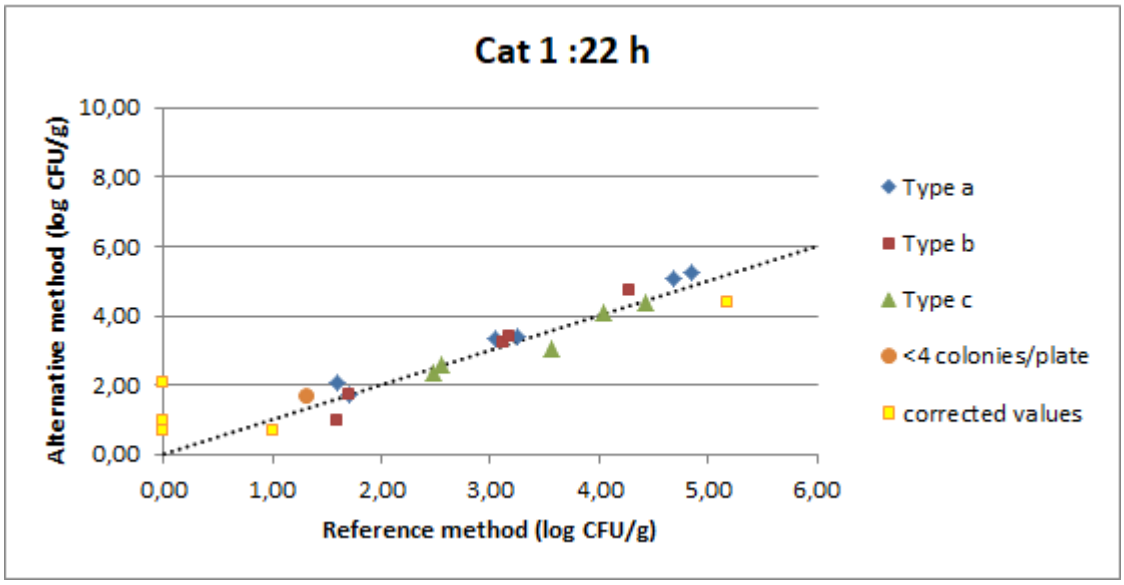


Figure 2- Data plotted for Dairy products

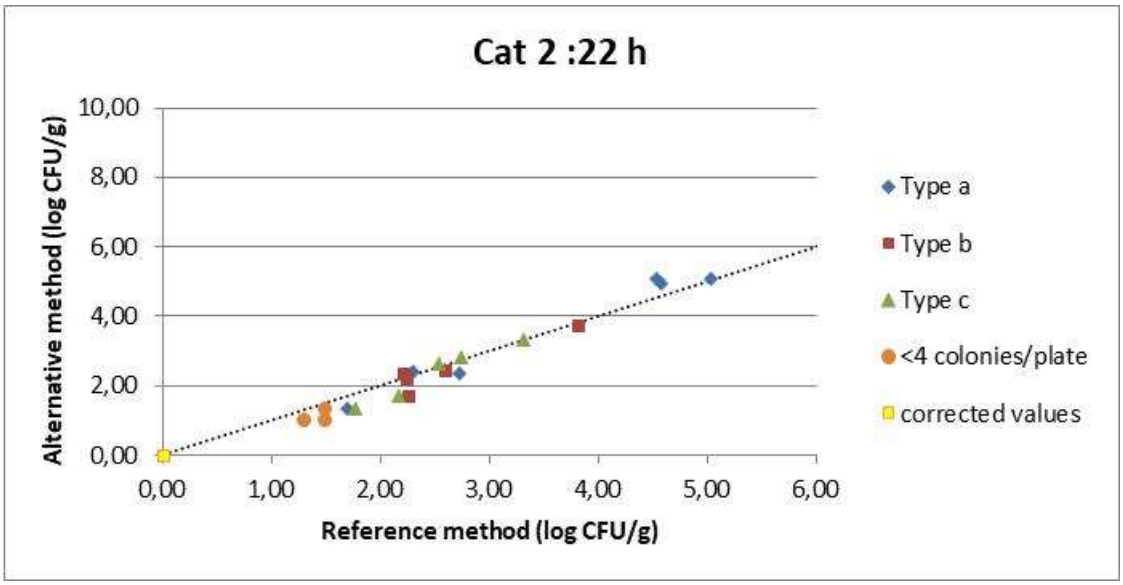


Figure 3- Data plotted for Egg products

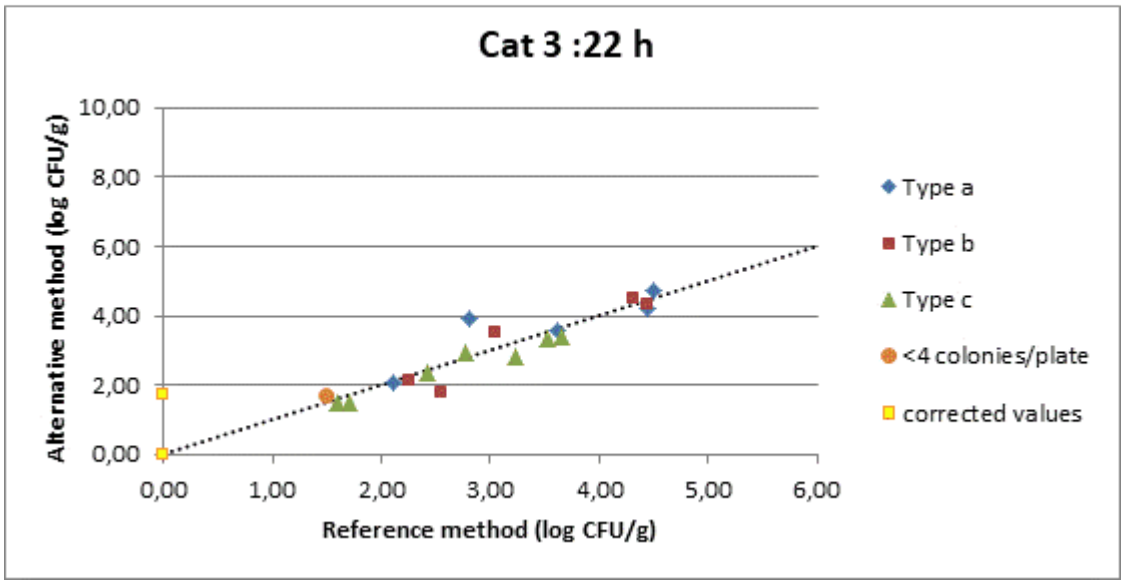


Figure 4- Data plotted for Fruits and vegetables

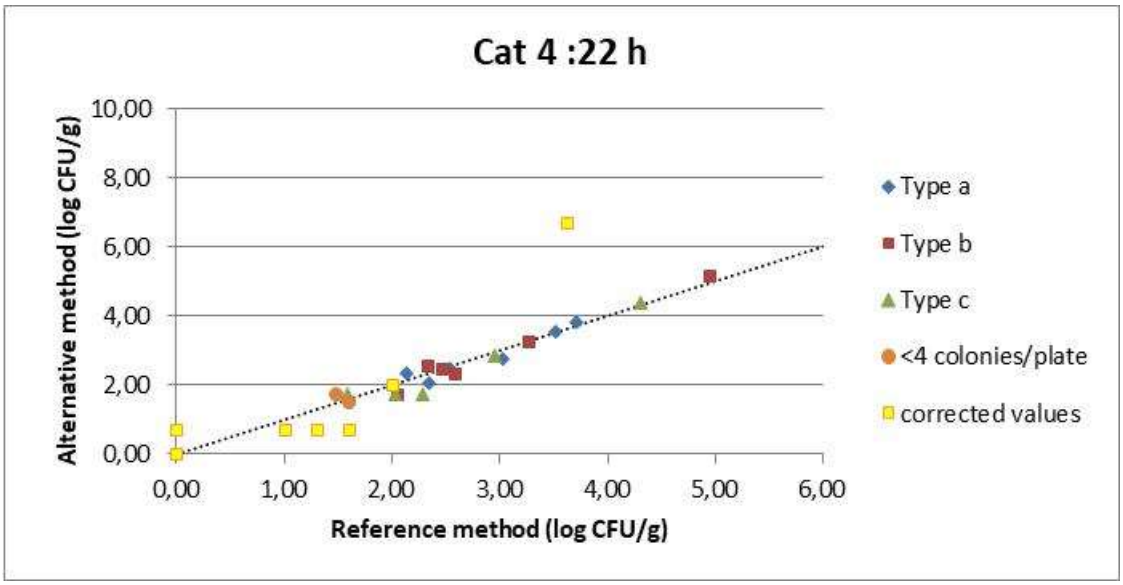


Figure 5- Data plotted for Baby foods

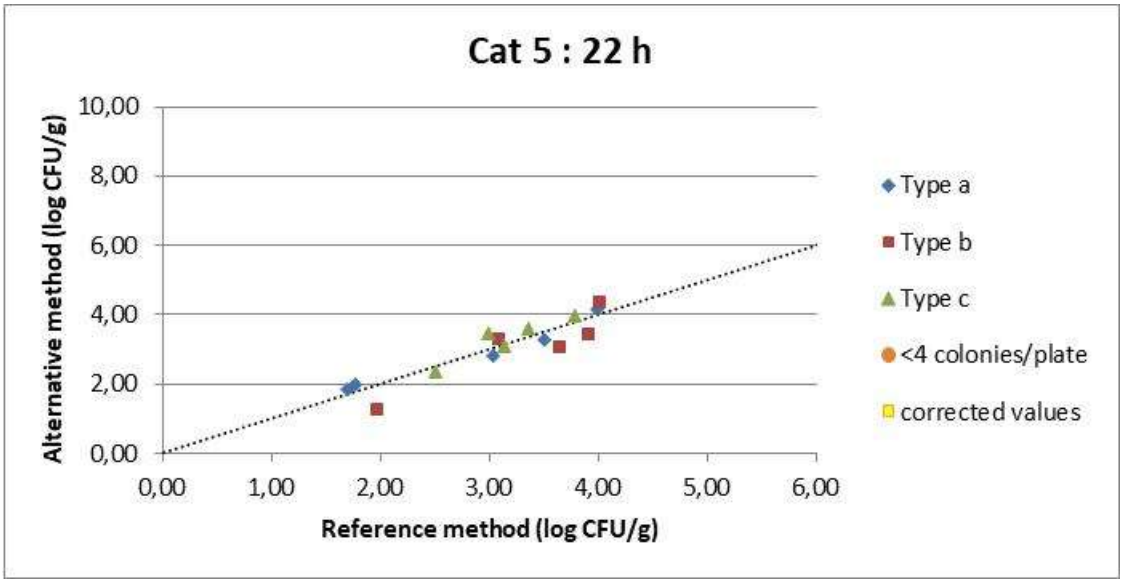


Figure 6- Data plotted for Environmental samples

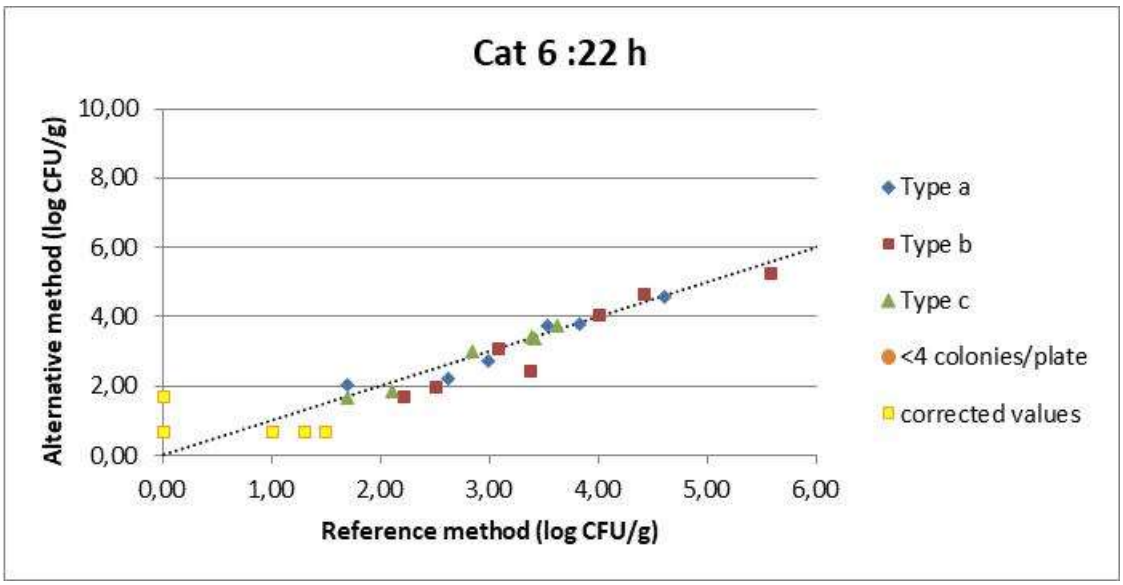
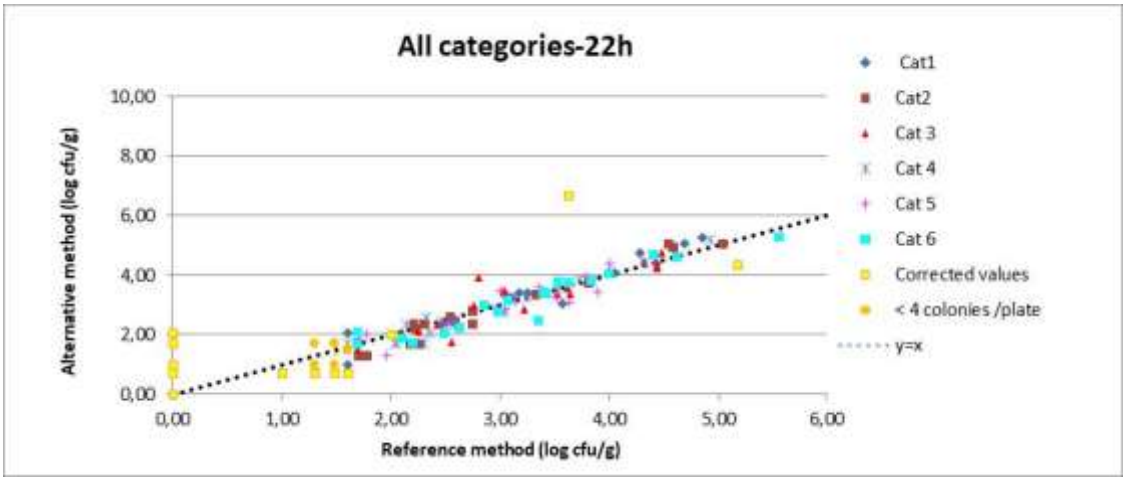


Figure 7 - Data plotted for all the products



The calculated values for Average difference, Standard deviation differences per category, the lower and upper limits at 95 % are provided in Table 5.

Table 5 - Calculated values

Incubation time	Category		n	$\bar{D}$	SD	95% lower limit	95% upper limit
22h	1	Meat products, fish products and seafood	16	0,08	0,31	-0,60	0,76
	2	Dairy products	16	-0,07	0,31	-0,74	0,60
	3	Egg products	17	-0,03	0,40	-0,91	0,84
	4	Fruits and vegetables	18	-0,08	0,23	-0,57	0,42
	5	Baby food	15	-0,02	0,35	-0,79	0,75
	6	Environmental samples	19	-0,12	0,31	-0,78	0,54
	All categories		**101**	**-0,04**	**0,32**	**-0,67**	**0,59**

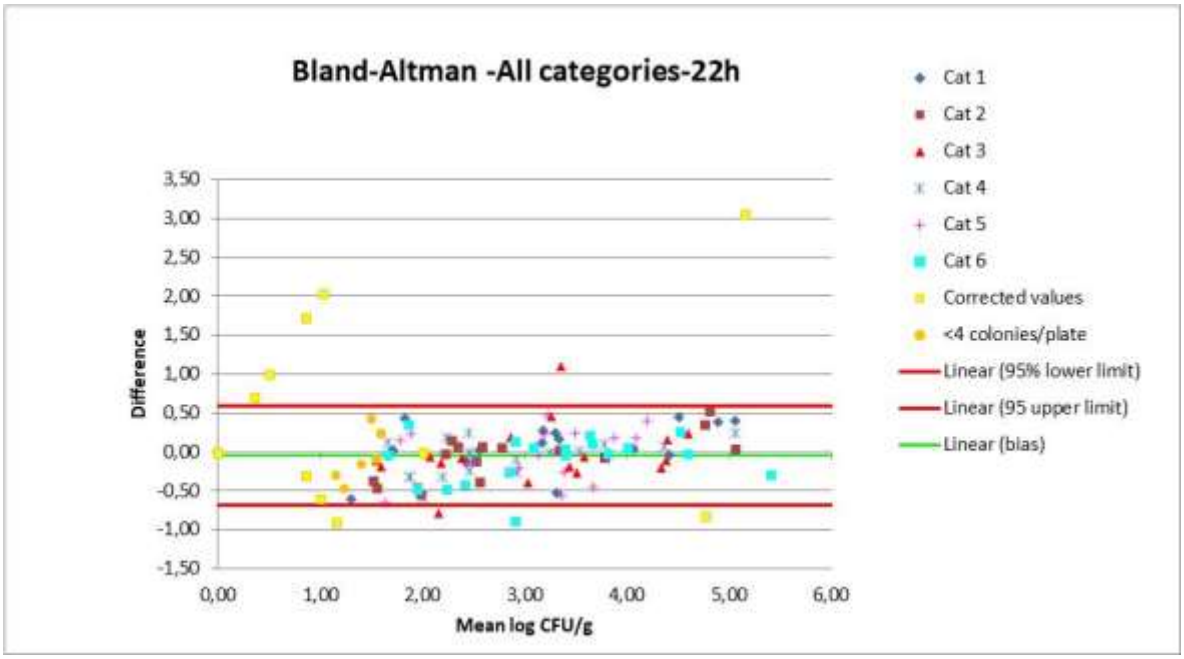
$\bar{D}$: Average difference SD: Standard deviation of differences

The bias (Average Difference) varies from -0.12 log (environmental samples) to 0.08 log (meat products, fish products and seafood). The bias for all combined categories is closed to 0 (-0.04 Log).

The LCL (lower confidence limit) varies from -0.91 to -0.57 log and the UCL (upper confidence limit) from 0.42 to 0.84 log. For all products, the LCL is -0.67 log and the UCL 0.59 log.

The Bland-Altman difference plot for all the samples is given Figure 8.

Figure 8 – 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 confidence limits are listed in Table 6.

Table 6 - Analysis of the data out of the confidence limits

Values in green: differences in favour of the alternative method
Values in red: differences in favour of the reference method

Corrected value
Results calculated using enumeration lower than 4 CFU/plate

	TEMPO® BC for 22h at 30°C									
Classification of the data	**Category**	**Type**	**N°Sample**	**Product**	**Reference method**	**Alternative method**	**Values before correction (Reference or/and alternative method)**	**Mean**	**Difference**	**LCL / UCL**
Interpretable results by both methods	3	b	**1959**	**White egg powder**	2,56	1,76	/	2,16	-0,80	
	6	b	**2212**	**Wipe ready to eat meal**	3,36	2,46	/	2,91	-0,90	
	3	a	**1093**	**Pasteurized liquid whole egg**	2,81	3,90	/	3,35	1,10	
< or > quantification limit	1	a	**1111**	**Ready to reheat fish preparation**	5,18	4,36	4,18	4,77	-0,82	
	4	c	5757	Ready to reheat potatoes	1,60	0,70	1,70	1,15	-0,90	
	6	a	**2207**	**Rinsing water dairy industry**	1,48	0,70	1,70	1,09	-0,78	
	1	a	422	Ready to reheat pork	0,00	0,70	1,00 / 1,70	0,35	0,70	
	1	a	556	Octopus	0,00	0,70	1,00 / 1,70	0,35	0,70	-0,67 / 0,59
	1	b	1363	Deli salad	0,00	1,00	1,00	0,50	1,00	
	1	c	551	Ready to reheat fish	0,00	2,04	1,00	1,02	2,04	
	3	a	**1095**	**Pasteurized liquid egg yolk sweet (50%)**	0,00	1,72	1,00	0,86	1,72	
	4	a	73	Dehydrated mushrooms	3,62	6,69	5,69	5,16	3,07	
	4	b	548	Cooked pasta	0,00	0,70	1,00 / 1,70	0,35	0,70	
	4	c	430	Cooked rice with vegetables	0,00	0,70	1,00 / 1,70	0,35	0,70	
	6	a	1459	Process water	0,00	0,70	1,00 / 1,70	0,35	0,70	

	TEMPO® BC for 22h at 30°C									
Classification of the data	**Category**	**Type**	**N°Sample**	**Product**	**Reference method**	**Alternative method**	**Values before correction (Reference or/and alternative method)**	**Mean**	**Difference**	**LCL / UCL**
< or > quantification limit	6	a	1460	Process water	0,00	0,70	1,00 / 1,70	0,35	0,70	-0,67 / 0,59
	6	a	1461	Process water	0,00	0,70	1,00 / 1,70	0,35	0,70	
	6	a	1965	Process water dairy industry	0,00	0,70	1,00 / 1,70	0,35	0,70	
	6	a	1966	Process water dairy industry	0,00	0,70	1,00 / 1,70	0,35	0,70	
	6	a	1967	Process water dairy industry	0,00	0,70	1,00 / 1,70	0,35	0,70	
	6	a	1968	Process water dairy industry	0,00	0,70	1,00 / 1,70	0,35	0,70	
	6	a	**2206**	**Rinsing water dairy industry**	0,00	0,70	1,00 / 1,70	0,35	0,70	
	6	a	**2208**	**Rinsing water dairy industry**	0,00	0,70	1,00 / 1,70	0,35	0,70	
	6	b	1992	Swab vegetables industry	0,00	1,72	1,00	0,86	1,72	
	6	b	1993	Swab vegetables industry	0,00	0,70	1,00 / 1,70	0,35	0,70	

The values outside of the confidence limits at 95% concern:

- 3 samples with interpretable results by both methods;
- 22 samples below or above the quantification limits.

Their repartition is given in Table 7.

Table 7 – Repartition of the samples outside of the 95 % confidence limits

		Number of samples
		22h at 30°C
Interpretable results by both methods	< LCL	2
	> UCL	1
	Total	3
<4 CFU/plate	< LCL	0
	> UCL	0
	Total	0
< or > the quantification limit	< LCL	3
	> UCL	19
	Total	22
Total < LCL		5
Total >UCL		20
Total		25

Negative biases were observed for 5 samples; the enumeration was higher using the MYP plates. Three of them were artificially contaminated using vegetative cells, one using spores and one was naturally contaminated.

Positive bias were observed for 3 samples (1093, 551 and 73). For these samples, the TEMPO® broths contained in the TEMPO® cards were streaked onto MYP plate and confirmation tests were performed on characteristics colonies. The presence of *Bacillus cereus* was confirmed for these samples.

For the other samples showing bias below or upper the confidence limits, the enumerations were below or above the quantification limit for one or both tested methods.

3.2.6 TEMPO® cards storage for 48 h at 5°C ± 3°C

All the cards were stored for 48 h at 5°C ± 3°C before proceeding to a second reading. The scatter splots are presented Figures 9 to 14 per category and Figure 15 for all combined categories.

Figure 9 - Data plotted for the Meat products, fish and seafood

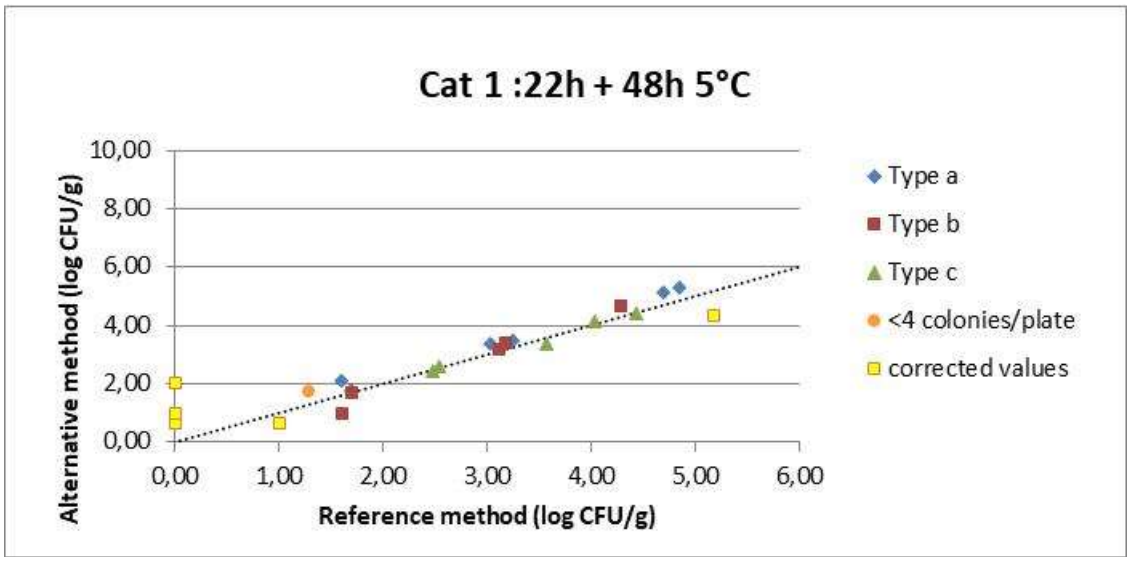


Figure 10- Data plotted for Dairy products

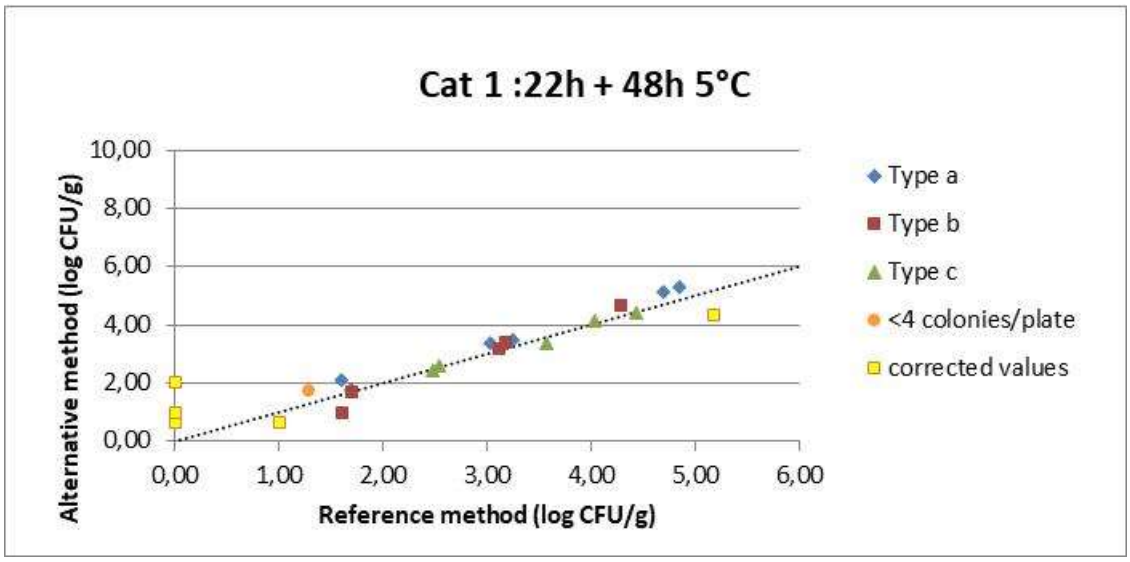


Figure 11- Data plotted for Egg products

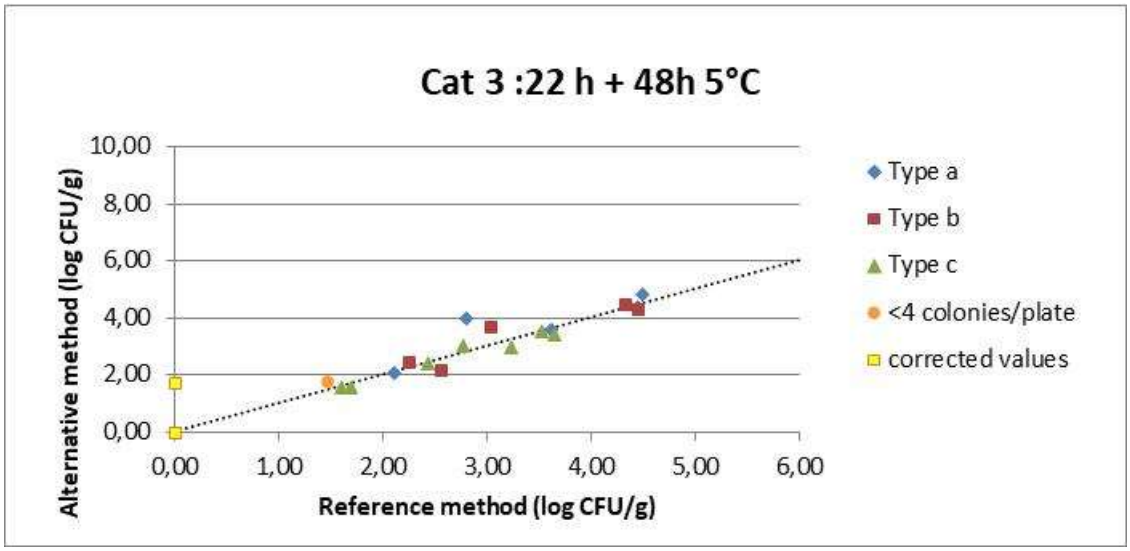


Figure 12- Data plotted for Fruits and vegetables

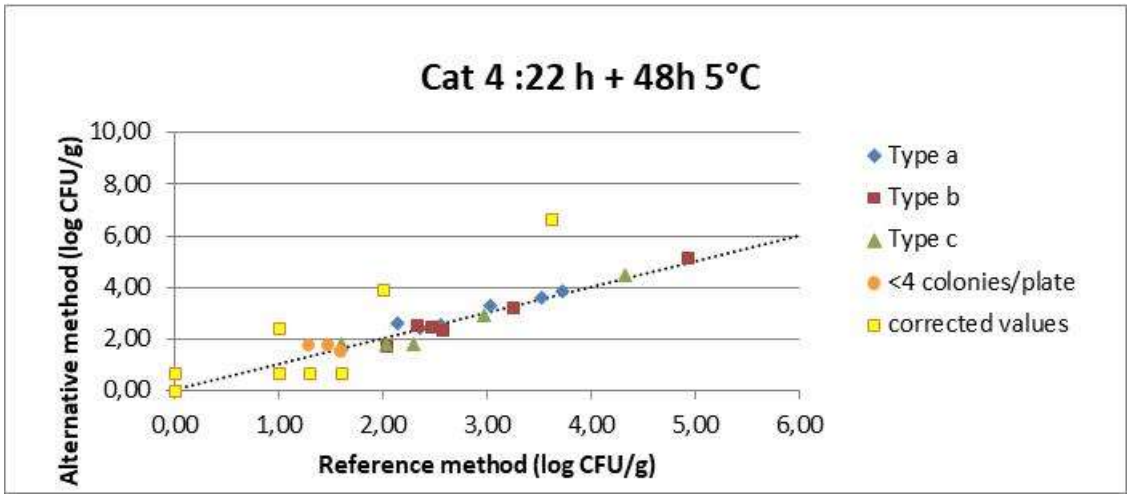


Figure 13- Data plotted for Baby foods

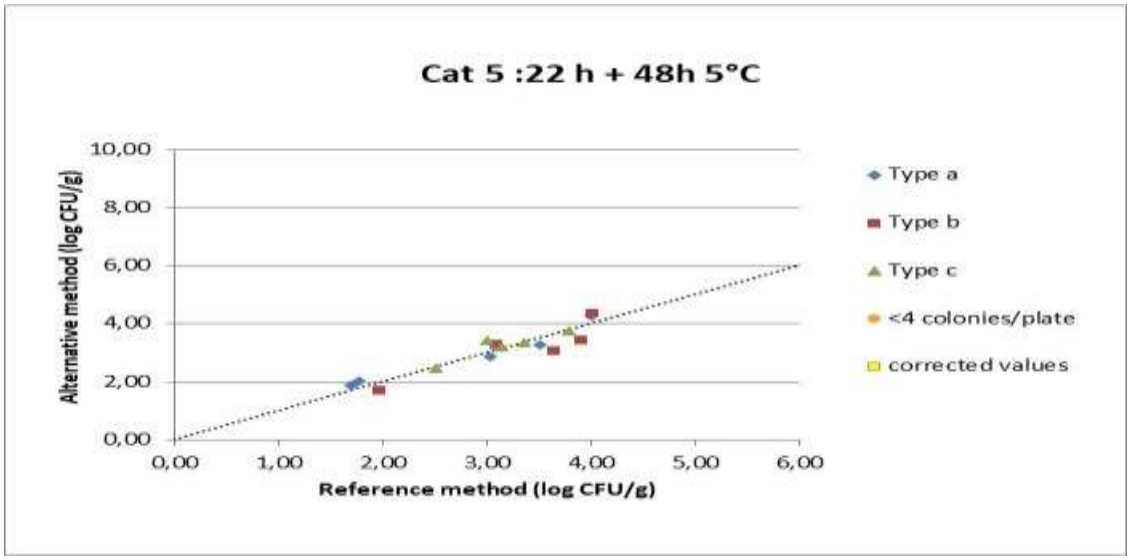


Figure 14- Data plotted for Environmental samples

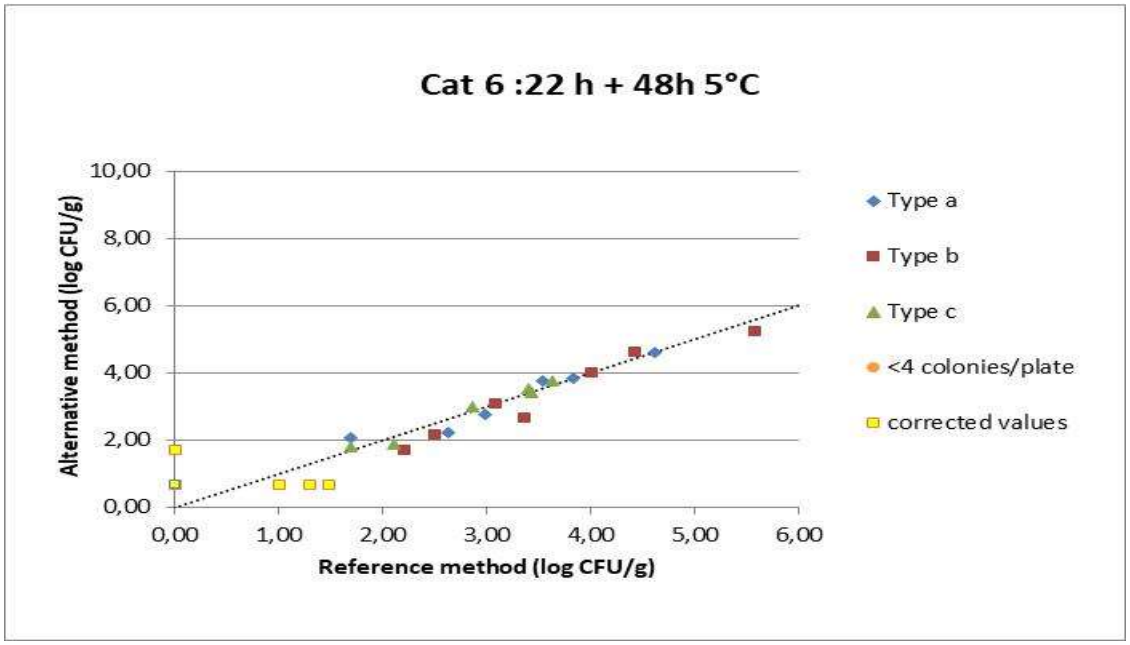
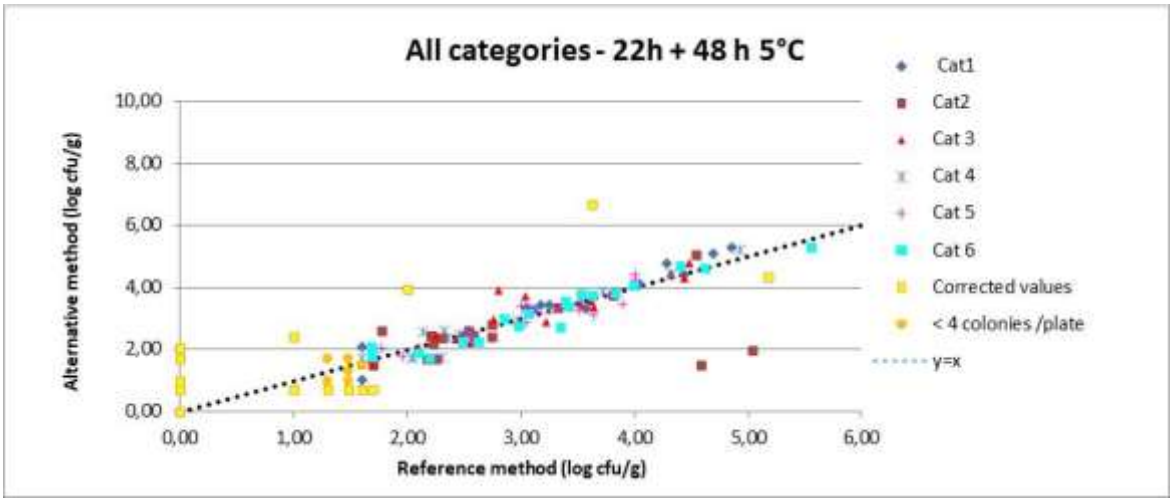


Figure 15 - Data plotted for all the products



The calculated values for Average difference, Standard deviation differences per category, the lower and upper limits at 95 % are provided in Table 8.

Table 8 - Calculated values

Incubation time	Category		n	$\bar{D}$	SD	95% lower limit	95% upper limit
22h +48h at 5°C ±3°C	1	Meat products, fish products and seafood	16	0,10	0,28	-0,52	0,71
	2	Dairy products	16	-0,37	1,11	-2,80	2,06
	3	Egg products	17	0,05	0,37	-0,77	0,86
	4	Fruits and vegetables	18	-0,02	0,24	-0,54	0,49
	5	Baby food	15	-0,02	0,29	-0,65	0,62
	6	Environmental samples	19	-0,08	0,27	-0,66	0,49
	All categories		**101**	**-0,06**	**0,52**	**-1,10**	**0,98**

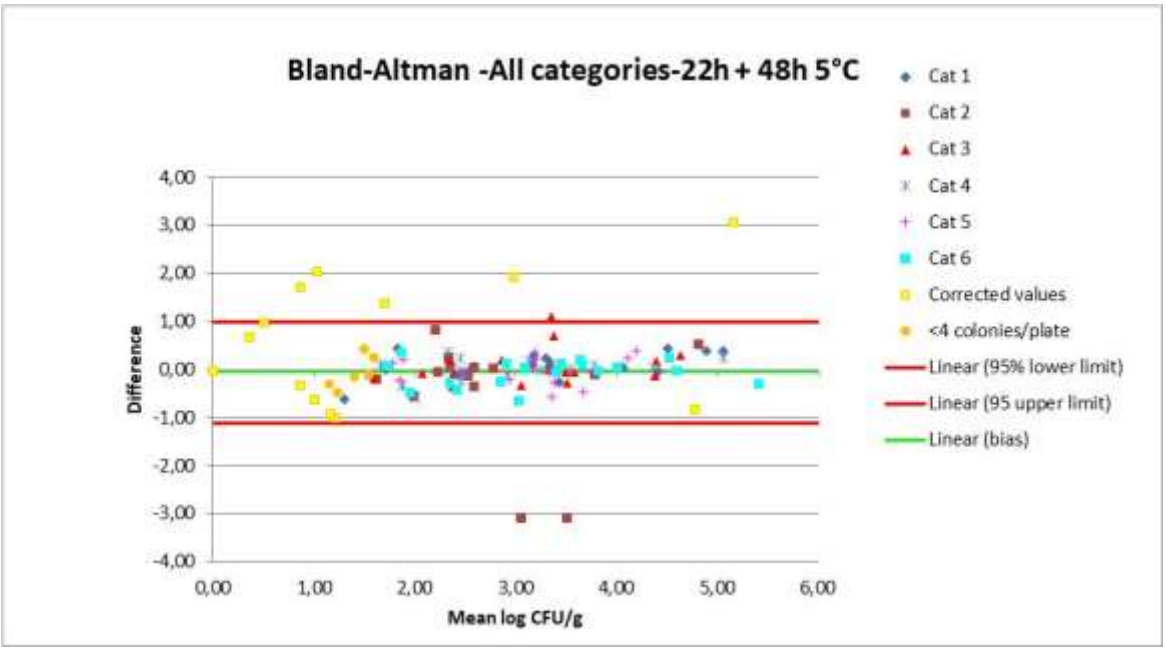
$\bar{D}$: Average difference SD: Standard deviation of differences

The bias (Average Difference) varies from -0.37 log (dairy products) to 0.10 log (meat products, fish products and seafood). The bias for all combined categories is closed to 0 (-0.06 Log).

The LCL (lower confidence limit) varies from -2.80 to -0.52 log and the UCL (upper confidence limit) from 0.49 to 2.06 log. For all products, the LCL is -1.10 log and the UCL 0.98 log.

The Bland-Altman difference plot for all the samples is given Figure 16.

Figure 16 – 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 confidence limits are listed in Table 9.

Table 9 - Analysis of the data out of the confidence limits

Values in green: differences in favour of the alternative method
Values in red: differences in favour of the reference method

Corrected value
Results calculated using enumeration lower than 4 CFU/plate

	TEMPO BC for 22h 30°C + 48h at 5°C ± 3°C									
Classification of the data	Category	Type	N° Sample	Product	Reference method	Alternative method	Values before correction (Reference or/and alternative method)	Mean	Difference	LCL / UCL
Interpretable results by both methods	2	a	**1104**	**Milk powder**	4,58	1,52	/	3,05	-3,06	-1,10 / 0,98
	2	a	**1105**	**Milk powder**	5,04	1,96	/	3,50	-3,08	
	3		**1093**	**Pasteurized liquid whole egg**	2,81	3,90	/	3,35	1,10	
< or > quantification limit	1	b	1363	Deli salad	0,00	1,00	1,00	0,50	1,00	
	1	c	551	Ready to reheat fish	0,00	2,04	1,00	1,02	2,04	
	3	a	**1095**	**Pasteurized liquid egg yolk sweet (50%)**	0,00	1,72	1,00	0,86	1,72	
	4	a	73	Dehydrated mushrooms	3,62	6,69	5,69	5,16	3,07	
	4	b	420	Rice and vegetables	2,00	3,94	3,00	2,97	1,94	
	4	b	425	Rice pasta	1,00	2,40	2,00	1,70	1,40	
	6	b	1992	Swab vegetables industry	0,00	1,72	1,00	0,86	1,72	

The values outside of the confidence limits at 95% concern:

- 3 samples with interpretable results by both methods;
- 7 samples below or above the quantification limits.

Their repartition is given in Table 10.

Table 10 – Repartition of the samples outside of the 95 % confidence limits

		Number of samples
		22h at 30°C + 48h at 5°C ± 3°C
Interpretable results by both methods	< LCL	2
	> UCL	1
	Total	3
<4 CFU/plate	< LCL	0
	> UCL	0
	Total	0
< or > the quantification limit	< LCL	0
	> UCL	7
	Total	7
Total < LCL		2
Total >UCL		8
Total		10

Similar results were observed before and after card storage except in two cases. For two samples, the enumeration decreased. This concerns samples 1104 and 1105 (milk powder artificially contaminated with *Bacillus cereus* Ad848).

Additional testing was performed in order to explain these results:

- The strain (*Bacillus cereus* Ad848) was inoculated in another matrix;
- The matrix was inoculated with another strain (*Bacillus cereus* Ad840);
- The strain was tested using the inclusivity protocol;
- The matrix was tested without any inoculation.

The results were the following (See Table 11).

Table 11 – Additional testing results

Strain	Matrix	TEMPO® BC	
		22 h	22 h + 48 h at 5°C ± 3°C
Bacillus cereus Ad848	/	910	910
Bacillus cereus Ad848	Milk powder (1104)	< 10	< 10
Bacillus cereus Ad848	Milk powder (1105)	< 10	< 10
Bacillus cereus Ad840	Milk powder (1104)	25 000	25 000
	Milk powder (1105)	18 000	18 000
Bacillus cereus Ad848	Cream cheese	39 000	39 000
	Milk powder	33 000	33 000

The same results were observed before and after storage of the TEMPO® cards. It was impossible to repeat the results observed during the relative trueness study.

3.2.7 Conclusion

The relative trueness study of the alternative method is satisfying when the TEMPO cards are incubated for 22 h at 30°C and when they are stored for 48 h at 5°C ± 3°C before reading: the alternative and the reference methods can be considered equivalent.

3.3 Accuracy profile study

The accuracy profile is a graphical representation of the capacity of measurement of the quantitative method, obtained by combining acceptability intervals and β-expectation tolerance intervals, both reported to different levels of the reference value.

The accuracy profile study is a comparative study between the results obtained by the reference method and the results of the alternative method. According to ISO 16140-2:2016, this study was conducted using artificially contaminated samples. One type per category was tested with 1 item per type and 2 batches per item.

A minimum of one type per category, and therefore 2 different batches, is selected, using 6 samples per type. 2 samples are contaminated at a low level, 2 at intermediate level, 2 at a high level. For each sample, 5 replicates (5 different test portions) are tested.

3.3.1 Categories, sample types and strains

6 accuracy profiles were performed using 9 items with three contamination levels and five test portions per level. The tested categories, types, items and inoculated strains are provided in Table 12.

Table 12 - Categories, types, items, strains and inoculation levels for accuracy profile study

Category	Type	Item	Strain	Target inoculation level (CFU/g)	Sample dilution in the TEMPO® BC sample preparation
Meat products, fish products and seafood	❒ RTE foods	- Pâté	*Bacillus cereus* Ad20 (group IV)	100 - 500 - 1 000 – 5 000 - 10 000 – 50 000	1/200
Dairy products	❒ Dairy desserts	- Panna cotta	Bacillus cereus Ad1467 (group II)	100 - 500 - 1 000 – 5 000 - 10 000 – 50 000	1/200
Egg products	❒ Egg products	- Whole egg product	*Bacillus weihenstephanensis* Ad730 (group VI)	500 – 5000 - 50 000	1/200
		- Liquid egg yolk	*Bacillus cereus* Ad797 (group II)	100 - 1 000 - 10 000	1/200
Fruits and vegetables	❒ Deli salads, vegetables preparations	- Deli salad (tabbouleh)	*Bacillus cytotoxicus* Ad2031 (group VII)	500 – 5 000 - 50 000	1/200
		- Purée	*Bacillus cereus* Ad754 (group V)	1 000 – 5 000 - 50 000	1/200
Baby foods	❒ Infant formula and cereals, raw materials with probiotics	- Infant formula with probiotics	*Bacillus cereus* AER400 (group IV) spores	100 – 100 – 1000	1/40
		- Infant cereals with probiotics	*Bacillus cereus* Ad495 (group III) spores	50 – 500 – 5000	1/40
Environmental samples	❒ Dusts	- Dusts from dairy industry	*Bacillus thuringiensis* Ad777 (group VI) (spores)	100 - 500 - 1 000 – 5 000 - 10 000 – 50 000	1/200

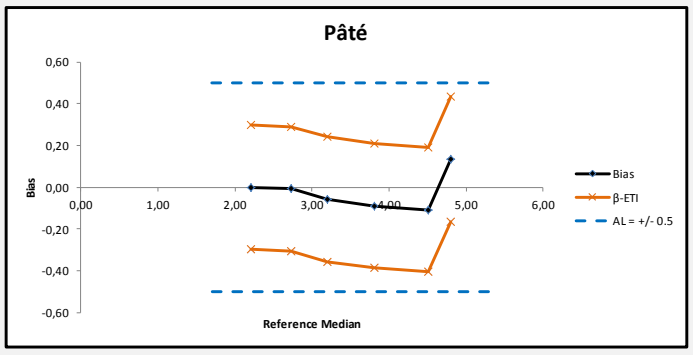
3.3.2 Calculation and interpretation

The statistical results and the accuracy profiles are provided Figure 17.

The calculations were done using the AP Calculation Tool MCS (Clause 6-1-3-3 calculation and interpretation of accuracy profile study) ver 2027-01-2015 available on http://standards.iso.org/iso/16140

Figure 17 – Accuracy profile

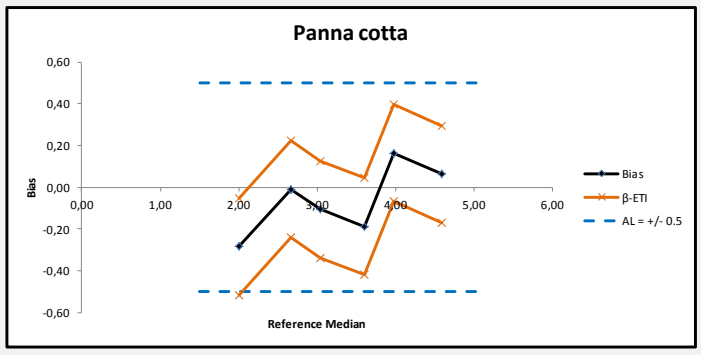
(Food) Category	Meat, fish, seafood products
(Food) Type	Pâté



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
939-943	2,20	0,000	-0,299	0,299	YES	YES
1236-1240	2,73	-0,008	-0,307	0,291	YES	YES
944-948	3,20	-0,058	-0,357	0,241	YES	YES
1241-1245	3,81	-0,089	-0,387	0,210	YES	YES
761-764	4,51	-0,107	-0,406	0,192	YES	YES
1246-1250	4,81	0,133	-0,165	0,432	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0,104	0,207	YES	+/- 0,500

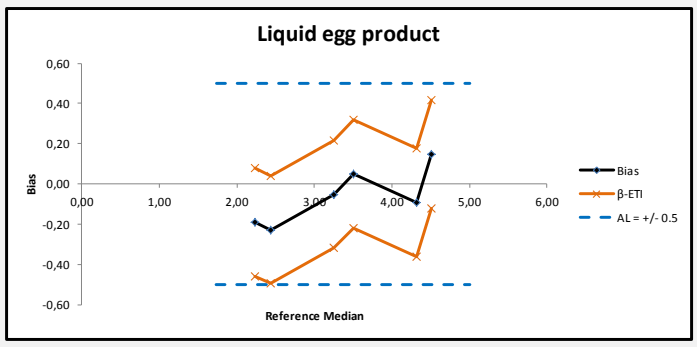
(Food) Category	Dairy products
(Food) Type	Panna cotta



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
1046-1050	2,00	-0,284	-0,516	-0,052	NO	NO
534-538	2,66	-0,010	-0,242	0,223	YES	YES
1051-1055	3,04	-0,107	-0,339	0,126	YES	YES
539-543	3,60	-0,187	-0,420	0,045	YES	YES
1056-1060	3,98	0,164	-0,069	0,396	YES	YES
544-548	4,59	0,062	-0,170	0,295	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0,082	0,161	YES	+/- 0,500

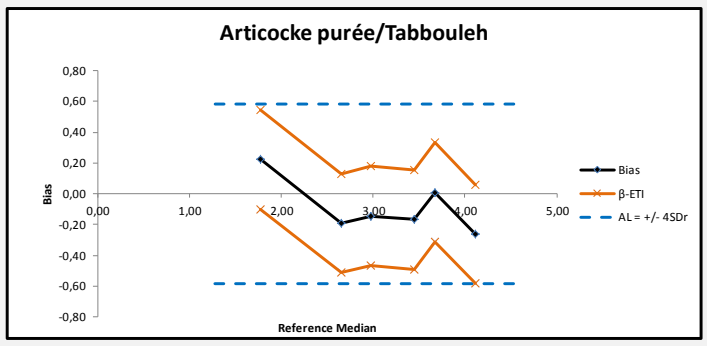
(Food) Category	Egg products
(Food) Type	Liquid egg product



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
1152-1156	2,23	-0,189	-0,458	0,079	YES	YES
1137-1141	2,43	-0,227	-0,496	0,041	YES	YES
1157-1161	3,26	-0,051	-0,320	0,217	YES	YES
1142-1146	3,51	0,051	-0,217	0,320	YES	YES
1162-1166	4,32	-0,092	-0,360	0,177	YES	YES
1147-1151	4,51	0,148	-0,120	0,417	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0,110	0,186	YES	+/- 0,500

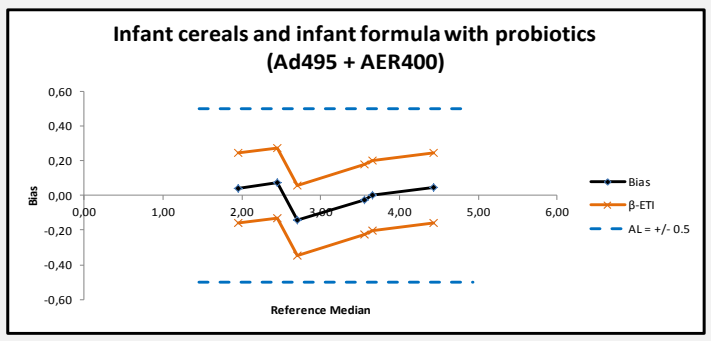
(Food) Category	Fruits and vegetables
(Food) Type	Articocke purée/Tabbouleh



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
5896-5900	1,78	0,222	-0,100	0,544	NO	YES
5901-5905	2,65	-0,191	-0,513	0,131	NO	YES
2766-2770	2,98	-0,145	-0,467	0,177	YES	YES
1219-1223	3,45	-0,168	-0,490	0,154	YES	YES
1239-1243	3,67	0,009	-0,313	0,331	YES	YES
1224-1228	4,11	-0,263	-0,585	0,059	NO	NO

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0,146	0,223	NO	+/- 0,584

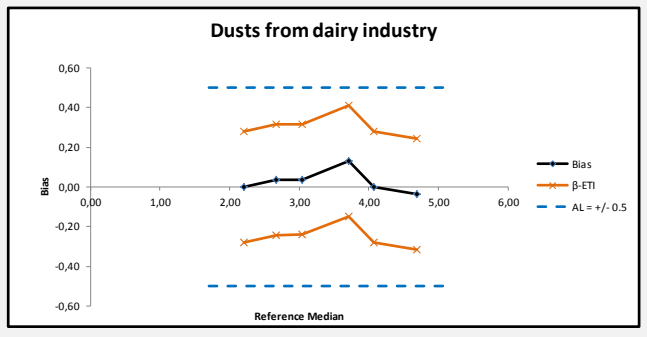
(Food) Category	Baby food
(Food) Type	Infant cereals and infant formula



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
295-299	1,95	0,041	-0,161	0,243	YES	YES
2593-2597	2,45	0,071	-0,131	0,273	YES	YES
300-304	2,70	-0,143	-0,345	0,059	YES	YES
2588-2592	3,56	-0,025	-0,227	0,177	YES	YES
310-314	3,65	0,000	-0,202	0,202	YES	YES
2583-2587	4,43	0,046	-0,156	0,248	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0,096	0,140	YES	+/- 0,500

(Food) Category	Environment
(Food) Type	Dusts from dairy industry



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
1912-1926	2,20	0,000	-0,279	0,279	YES	YES
3621-3625	2,67	0,035	-0,243	0,314	YES	YES
1917-1921	3,04	0,038	-0,241	0,316	YES	YES
3626-3630	3,71	0,131	-0,147	0,410	YES	YES
1922-1926	4,08	0,000	-0,279	0,279	YES	YES
3631-3635	4,69	-0,037	-0,316	0,242	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0,055	0,193	YES	+/- 0,500

The accuracy profiles are within the Acceptability Limits (AL) fixed at ± 0.5 log for four matrices:

- Pâté,
- Liquid egg product,
- Infant cereals and infant cereals with probiotics,
- Dusts from dairy products.

For Panna cotta, lower β.ETI (-0.516) is just above the AL fixed at 0.5 log for the lowest inoculation level.

For the Artichoke purée/Tabbouleh, as the SD repeatability of the reference method is higher than 0.125, new acceptability limits were recalculated (ALs = 4 x Sref = ± 0.584 log). The lower β.ETI (-0.585) for the high inoculation level was just above the AL fixed at -0.584 log.

3.3.3 Conclusion

The accuracy profiles are comprised within the Acceptability Limits (AL) for 4 matrices (pâté, liquid egg product, infant cereals with probiotics and dusts from dairy industry) and just above or below the AL for 2 matrices (panna cotta and artichoke/tabbouleh). This was considered acceptable by the MTVC for the initial validation study.

3.4 Quantification limits (LOQ)

The limit of Quantification (LOQ) is the lowest analyte concentration that can be quantified with an acceptable level of precision and trueness under the conditions of the test.

As TEMPO® BC is not based counting visible colonies target microorganism, the LOQ was determined. One food matrix already tested in the accuracy profiles was selected by tested category. Blank samples were tested per tested matrix. 10 test portions from the same sample were analyzed by the alternative method.

The threshold standard deviation s_0 was calculated as followed:

$$s_0 = \frac{1}{n-1}\sum_{j=1}^{n}(y_j - \bar{y})^2$$

where

n is the total number of test portions used;

y_j is the $\log_{10}$ transformed result of test portion j;

$\bar{y}$ *is* the average $\log_{10}$ transformed result of all test portions.

The limit of quantification is calculated as $LOQ = 10\ s_0$.

The results are summarized in Table 13.

Table 13 - Quantification limits per tested matrix

Matrix	So	LOQ
Salmon terrine	0	0
Infant formula with probiotics	0	0
Artichoke purée	0	0
Whole egg	0	0
Dairy dessert	0	0
Dusts from egg industry	0	0

Whatever the tested matrix, the quantification limit is 0.

3.5 Inclusivity and exclusivity study

The inclusivity study is a study involving pure target strains to be detected or enumerated by the alternative method.
The exclusivity study is a study involving pure non-target strains, which can be potentially cross-reactive, but are not expected to be detected or enumerated by the alternative method.

3.5.1 Protocol

After growth according to appropriate conditions, decimal dilutions were done and each strain was enumerated in duplicate by the TEMPO® BC method and ISO 7932 standard. Target strains and non-target strains dilutions were enumerated in parallel with a non-selective agar (TSA).

50 target strains from different species, genotypes and origins, as well as 31 non target strains were tested.

3.5.2 Results

50 target strains were tested; all were enumerated by the TEMPO® *Bacillus cereus* method. One of them (*Bacillus mycoïdes* Ad746) showed lowest enumeration data than the reference method. The *Bacillus weihenstephanensis* Ad854 strain shows as well slightly lower enumeration results with the TEMPO® BC method than with MYP plates.

Bacillus cytotoxicus strains were not tested in the inclusivity study but gave satisfying results in the trueness study and accuracy profile study.

Among the 31 non target tested strains, one was enumerated by the alternative method giving a very low result (2 log less than the control media), i.e. *Lysinibacillus fusiformis* Ad842. For this strain, the test was repeated and an enumeration was obtained only for one replicate (3 log less than the control media). Note that two other *Lysinibacillus fusiformis* strains were tested (Ad841 and Ad828) and gave negative results as expected.

4 INTER-LABORATORY 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.

The results of the inter-laboratory study run in 2015 were interpreted according to the EN ISO 16140-2:2016 standard using the Excel spread sheet available at http://standards.iso.org/iso/16140 (AP Calculation tool ILS (clause 6.2.3 Calculation summary and interpretations of data) ver 14.03.2016

4.1 Study organisation

4.1.3 Collaborators

Samples were sent to 18 laboratories.

4.1.4 Matrix used.

The study was carried out on a **scallop terrine** contaminated with spores of *Bacillus cereus* Ad 825.

4.1.5 Sample preparation

For each collaborator, a set of samples was prepared containing two samples contaminated at a low level, two samples at a medium level, two samples at a high level, and two uninoculated samples.

Samples were inoculated on Monday, September 7th 2015; each collaborator received:

- 8 blind coded samples (10 g) in TEMPO Stomacher bags for enumeration of *Bacillus cereus* with the reference ISO 7932 and alternative method TEMPO® BC,

- 1 sample (10 g labelled “Sample for Total Count enumeration”) in stomacher bag for aerobic mesophilic flora enumeration by ISO 4833-1 method,
- 1 water flask labelled “Temperature Control” with a temperature probe for temperature control during transport and storage in the laboratory until the beginning of the analyse.

The targeted inoculation levels were the following:

- Level 0: <10 CFU/ml,
- Level 1: 100 – 1 000 CFU/ml,
- Level 2: 1 000 – 10 000 CFU/ml,
- Level 3: 10 000 – 100 000 CFU/ml.

4.1.6 Labelling end shipping

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

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

Samples were shipped in 24 h to 48 h to the involved laboratories. The temperature conditions had to stay lower or equal to 8°C during transport, and between 0°C – 8°C in the labs.

4.1.7 Analysis of samples

Collaborators and ADRIA Développement carried out the analyses with the alternative and reference methods at Day 2 (Wednesday, September 9th 2015). **The 1/200 dilution was run with the TEMPO® BC method, as a RTE food (scallop terrine) was tested.**

The collaborative study instructions were sent to the collaborators on 19th of August 2015.

4.2 Experimental parameters control

4.2.1 Strain stability during transport

In order to evaluate the *Bacillus cereus* strain viability during transport, bacterial count of samples were checked at different times, i.e. inoculation time, and after 48 h of storage at 2 - 8°C.

Six samples (3 contamination levels x 2 samples) were enumerated. The results are reported in Table 14.

Table 14 – *Bacillus cereus* count with reference method (in log CFU/g)

Day of analysis	*Bacillus cereus* enumeration (cfu/g)				Aerobic mesophilic numeration(cfu/g)
	Reference method		Alternative method		ISO 4833-1
	Replicate 1	Replicate 2	Replicate 1	Replicate 2	
D0	530	850	630	740	900
	3 500	2 500	2 200	2 900	
	38 000	36 000	45 000	53 000	
D2	390	480	450	530	1 300
	2 900	3 900	3 600	3 600	
	28 000	26 000	27 000	30 000	

No evolution of the inoculated strain was observed between Day 0 and Day 2.

4.2.2 Logistic conditions

The temperature measured at receipt by the collaborators, the temperatures registered by the temperature probe and the receipt dates and time are given in Table 15.

Table 15 - Sample temperatures at receipt

Laboratories	Temperature measured by the probe (°C) all along the delivery and receipt	Temperature measured at receipt (°C) by the Lab itself	Receipt date and time	
A	2.5	6.6	08/09/2015	09h30
B	3.5	8.3	08/09/2015	11h40
C	2.0	5.5	08/09/2015	11h00
D	3.5	6.2	08/09/2015	11h45
E	2.0	2.6	08/09/2015	08h30
F	2.5	5.5	08/09/2015	08h40
G	2.5	3.9	08/09/2015	12h00
H	2.5	5.6	08/09/2015	14h20
I	1.5	4.4	08/09/2015	11h30
J	1.0	2.2	08/09/2015	09h15
K	2.5	3.0	08/09/2015	09h30
L	3.0	4.5	08/09/2015	09h30
M	2.0	4.5	08/09/2015	09h00
N	2.0	4.0	08/09/2015	13h30
O	3.0	4.0	08/09/2015	13h00
P	2.5	5.2	08/09/2015	12h45
Q	3.5	5.0	08/09/2015	10h45
T	Not received	7.9	08/09/2015	10h00

All the samples were delivered in appropriated conditions. Temperatures during shipment and at receipt were all correct.

4.2.3 Homogeneity of inoculation

Homogeneity tests were conducted according to the ISO/TS 22117.

Ten samples per inoculation level were analysed in duplicate by the reference method. The test concluded that the samples were sufficiently homogeneous for the three contamination levels.

4.3 Calculation and summary of data

4.3.1 MicroVal Expert laboratory results

The results obtained by the Expert laboratory are given in Table 16.

Table 16 – Results obtained by the Expert lab. (in log CFU/g)

***Bacillus cereus* enumeration (cfu/g)**				**Aerobic mesophilic numeration(cfu/g)**
Reference method		**Alternative method**		ISO 4833-1
Replicate 1	Replicate 2	Replicate 1	Replicate 2	
390	480	450	530	1 300
2 900	3 900	3 600	3 600	
28 000	26 000	27 000	30 000	

4.3.2 Results obtained by the collaborative laboratories

As mentioned, the 1/200 dilution was run with the TEMPO® BC method as a RTE food was analysed.

One Lab (P) did not respect the instructions: they tested 1/40 and 1/400 dilution for the TEMPO® method instead of 1/40 and 1/200. The results of this Lab were not taken into account for the interpretation.

A summary of the test results is given Table 17 (CFU/g and log CFU/g).

Table 17 - Summary of data

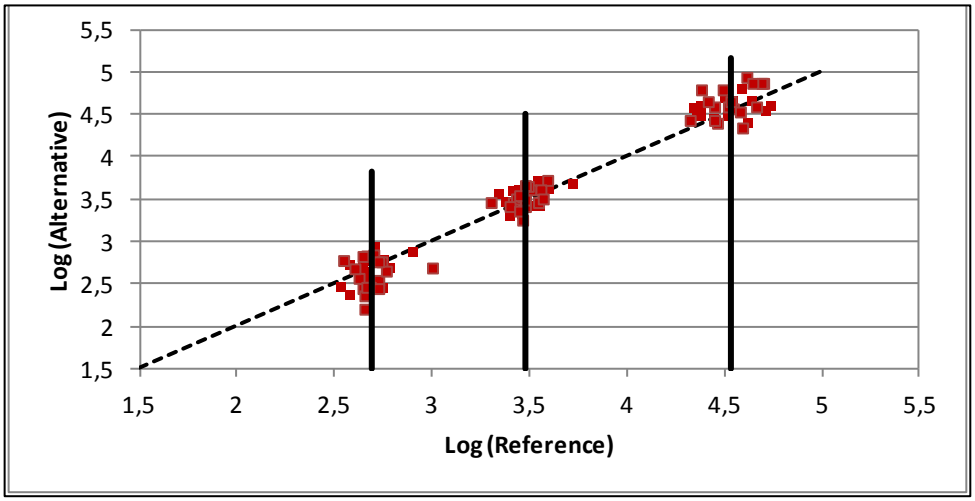
Labo-ratory	Raw data (cfu/g)															
	Reference method		Alternative method		Reference method		Alternative method		Reference method		Alternative method		Reference method		Alternative method	
	Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
	Blank Level				Low Level				Medium Level				High Level			
Lab A	<10	<10	<50	<50	530	420	600	370	2800	2600	4000	2900	48000	24000	74000	62000
Lab B	<10	<10	<50	<50	440	460	530	680	2600	3900	3900	5300	28000	33000	28000	39000
Lab C	<10	<10	<50	<50	450	440	580	280	2700	2900	2200	1800	23000	21000	34000	27000
Lab D	<10	<10	<50	<50	380	400	530	480	2200	2700	3600	3200	22000	28000	37000	39000
Lab E	<10	<10	<50	<50	510	530	820	350	4000	2500	4600	2600	44000	41000	45000	87000
Lab F	<10	<10	<50	<50	470	480	370	290	3500	3000	5200	2600	52000	50000	34000	74000
Lab G	<10	<10	<50	<50	380	450	230	160	3200	2000	4100	2900	35000	35000	45000	37000
Lab H	<10	<10	<50	<50	430	500	450	700	3300	3200	2600	4100	32000	39000	53000	22000
Lab I	<10	<10	<50	<50	560	550	280	580	2500	2900	2900	3400	35000	38000	39000	34000
Lab J	<10	<10	<50	<50	470	530	370	280	3500	3600	4100	4100	55000	46000	39000	39000
Lab K	<10	<10	<50	<50	340	450	290	230	4000	3500	4100	2900	24000	29000	30000	25000
Lab L	<10	<10	<50	<50	470	350	430	600	2500	2800	2000	2300	24000	28000	39000	27000
Lab M	<10	<10	<50	<50	610	580	490	450	2400	3100	2900	4500	42000	31000	25000	62000
Lab N	<10	<10	<50	<50	570	460	600	290	5300	3000	4800	3100	39000	34000	62000	45000
Lab O	<10	<10	<50	<50	570	440	600	670	3000	3700	2600	3200	45000	49000	74000	74000
Lab Q	<10	<10	<50	<50	800	1000	740	490	3600	3000	2600	4600	33000	44000	30000	74000
Lab T	<10	<10	<50	<50	510	530	860	580	2900	2800	2100	3500	32000	26000	49000	45000

Labo-ratory	Raw data (log cfu/g)															
	Reference method		Alternative method		Reference method		Alternative method		Reference method		Alternative method		Reference method		Alternative method	
	Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
	Blank Level				Low Level				Medium Level				High Level			
Lab A	<1.00	<1.00	<1.70	<1.70	2.72	2.62	2.78	2.57	3.45	3.41	3.60	3.46	4.68	4.38	4.87	4.79
Lab B	<1.00	<1.00	<1.70	<1.70	2.64	2.66	2.72	2.83	3.41	3.59	3.59	3.72	4.45	4.52	4.45	4.59
Lab C	<1.00	<1.00	<1.70	<1.70	2.65	2.64	2.76	2.45	3.43	3.46	3.34	3.26	4.36	4.32	4.53	4.43
Lab D	<1.00	<1.00	<1.70	<1.70	2.58	2.60	2.72	2.68	3.34	3.43	3.56	3.51	4.34	4.45	4.57	4.59
Lab E	<1.00	<1.00	<1.70	<1.70	2.71	2.72	2.91	2.54	3.60	3.40	3.66	3.41	4.64	4.61	4.65	4.94
Lab F	<1.00	<1.00	<1.70	<1.70	2.67	2.68	2.57	2.46	3.54	3.48	3.72	3.41	4.72	4.70	4.53	4.87
Lab G	<1.00	<1.00	<1.70	<1.70	2.58	2.65	2.36	2.20	3.51	3.30	3.61	3.46	4.54	4.54	4.65	4.57
Lab H	<1.00	<1.00	<1.70	<1.70	2.63	2.70	2.65	2.85	3.52	3.51	3.41	3.61	4.51	4.59	4.72	4.34
Lab I	<1.00	<1.00	<1.70	<1.70	2.75	2.74	2.45	2.76	3.40	3.46	3.46	3.53	4.54	4.58	4.59	4.53
Lab J	<1.00	<1.00	<1.70	<1.70	2.67	2.72	2.57	2.45	3.54	3.56	3.61	3.61	4.74	4.66	4.59	4.59
Lab K	<1.00	<1.00	<1.70	<1.70	2.53	2.65	2.46	2.36	3.60	3.54	3.61	3.46	4.38	4.46	4.48	4.40
Lab L	<1.00	<1.00	<1.70	<1.70	2.67	2.54	2.63	2.78	3.40	3.45	3.30	3.36	4.38	4.45	4.59	4.43
Lab M	<1.00	<1.00	<1.70	<1.70	2.79	2.76	2.69	2.65	3.38	3.49	3.46	3.65	4.62	4.49	4.40	4.79
Lab N	<1.00	<1.00	<1.70	<1.70	2.76	2.66	2.78	2.46	3.72	3.48	3.68	3.49	4.59	4.53	4.79	4.65
Lab O	<1.00	<1.00	<1.70	<1.70	2.76	2.64	2.78	2.83	3.48	3.57	3.41	3.51	4.65	4.69	4.87	4.87
Lab Q	<1.00	<1.00	<1.70	<1.70	2.90	3.00	2.87	2.69	3.56	3.48	3.41	3.66	4.52	4.64	4.48	4.87
Lab T	<1.00	<1.00	<1.70	<1.70	2.71	2.72	2.93	2.76	3.46	3.45	3.32	3.54	4.51	4.41	4.69	4.65

4.3.3 Visual linearity checking

The Figure 18 shows the data points after log_{10} transformation. The visual inspection shows that the alternative method gives results, which are proportional to those of the reference method. The data are distributed closely to the first bisecting lines with a slope equal to 1.

Figure 18 - Visual linearity checking



4.3.4 Accuracy profile calculation

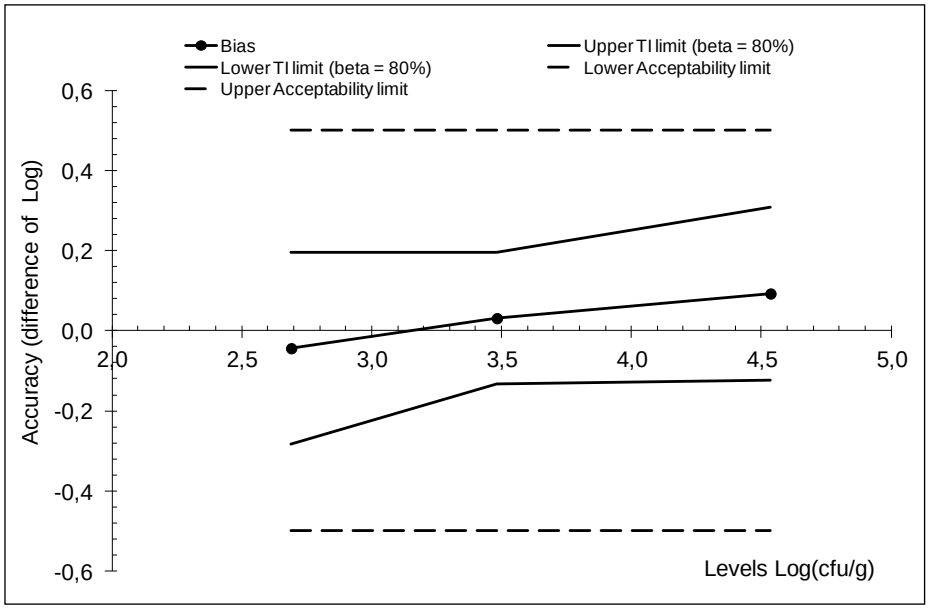
Statistical calculations were done according to the Excel spreadsheet available on http://standards.iso.org/ISO/16140. A summary of the statistical test is provided in Table 18.

Table 18 - Summary of statistical tests

Accuracy profile						
Study Name	TEMPO BC					
Date	07/09/2015					
Coordinator	ADRIA					
Tolerance probability (beta)	80%	80%	80%			
Acceptability limit in log (lambda)	0.5	0.5	0.5			
	Alternative method				**Reference method**	
Levels	**Low**	**Medium**	**High**	**Low**	**Medium**	**High**
Target value	**2.690**	**3.482**	**4.536**			
Number of participants (K)	17	17	17	17	17	17
Average for alternative method	2.646	3.514	4.629	2.690	3.482	4.536
Repeatability standard deviation (sr)	0.141	0.119	0.149	0.050	0.082	0.073
Between-labs standard deviation (sL)	0.110	0.034	0.066	0.077	0.000	0.093
Reproducibility standard deviation (sR)	0.179	0.124	0.163	0.092	0.084	0.119
Corrected number of dof	28.277	32.630	31.767	21.499	32.720	23.184
Coverage factor	1.339	1.329	1.331			
Interpolated Student t	1.312	1.308	1.309			
Tolerance interval standard deviation	0.1826	0.1260	0.1656			
Lower TI limit	2.407	3.349	4.412	Pooled repro standard dev		0.099
Upper TI limit	2.886	3.678	4.845	New acceptability limit (ALs)		0.328
Bias	**-0.044**	**0.031**	**0.093**			
Upper TI limit (beta = 80%)	**-0.284**	**-0.134**	**-0.124**			
Lower TI limit (beta = 80%)	**0.196**	**0.196**	**0.310**			
Lower Acceptability limit	**-0.50**	**-0.50**	**-0.50**			
Upper Acceptability limit	**0.50**	**0.50**	**0.50**			

These values are collected in a graphical representation together with the acceptability limits (AL). This representation is given Figure 19.

Figure 19 - Accuracy profile



It is observed that for all the levels, the tolerance interval limits of the alternative method are within the acceptable limits of 0.5 log.

The values for the β-ETI are within the Acceptability Limits for all levels of contamination. The alternative method is equivalent to the reference method.

5 GENERAL CONCLUSION

<u>The method comparison study conclusions are:</u>

The observed data and interpretation confirm the performances of the alternative method:

- **164 samples were tested in the relative trueness study providing 101 interpretable results by both methods,** which clearly satisfied the required criteria for quantitative method comparison per ISO 16140-2**; this study confirms as well the possibility to store the TEMPO® cards for 48 h at 5°C ± 3°C.**

- **The observed profiles are comprised within the AL actually set at 0.5 log CFU/g in the EN ISO 16140-2:2016 for 4 matrices and are just below the AL for 2 matrices.**

- **The inclusivity and exclusivity testing shows satisfying results.**

The **inter-laboratory study conclusions** are:

- The quality assurance parameters were verified (*i.e.* inoculation homogeneity, targeted levels, strain stability, logistic conditions, analyses), confirming that the inter-laboratory study was conducted in appropriate conditions.

- **The data interpretations were done according to the EN ISO 16140-2:2016**. For the three contamination levels, the values for the β-ETI are within the Acceptability Limits, the alternative method is accepted as equivalent to the reference method.

Based on the results obtained for the method comparison study and the inter-laboratory study, the TEMPO® BC is considered equivalent to the reference method.

Quimper, 15 July 2019

Justine BAGUET	Maryse RANNOU
Technical Study Manager	Project Manager
Validation of Alternative methods	Validation of Alternative methods
Food Safety & Quality	*Food Safety & Quality*
I hereby attest to the validation of the results of the analyses carried out under the COFRAC accreditation.	I hereby attest to the validation of the verification of the conformity of the report (opinion and interpretation).

6 REFERENCES

- ISO 4833-1:2013; Microbiology of the food chain -- Horizontal method for the enumeration of microorganisms -- Part 1: Colony count at 30 degrees C by the pour plate technique

- ISO 6887; Microbiology of the food chain -- Preparation of test samples, initial suspension and decimal dilutions for microbiological examination – All parts.

- ISO 7218; Microbiology of food and animal feeding stuffs -- General requirements and guidance for microbiological examinations.

- 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.

- 2016-028 (GC) Proposed MicroVal Technical committee interpretation of ISO 16140-2 and ISO

- 2017-063 (TC) The MicroVal Process

- ISO 7932 (2004): horizontal method for the enumeration of presumptive *Bacillus cereus* - Colony-count technique at 30°C

Annex 1 - Flow diagram of the ISO 7932 reference method test procedure: Horizontal method for the enumeration of presumptive *Bacillus cereus*: Colony-count technique at 30 °C

Initial suspension according to ISO6887 series
xg sample + 9 x g appropriate diluent
↓
Subsequent dilutions
↓
Surface plating on MYP medium
1 ml on 3 plates (0.4 + 0.4 + 0.2) for low number estimation
or 0.1 ml on 1 plate
↓
Incubation 18 to 24 h at 30°C ± 1°C
If colonies are not visible, incubation until 48 h
↓
Enumeration of characteristic colonies
↓
Streak 5 characteristic colonies on sheep blood agar
↓
Incubation 24 h ± 2 h at 30°C ± 1°C
↓
Haemolysis test result
(*Bacillus cereus*: positive haemolysis test)

Annex 2 - Flow diagram of the alternative method: TEMPO® BC

Sample preparation: 1/10 in TEMPO® Stomacher bag
according to ISO 6887 (parts 1 to 5)

↓

	Dilution		
	1/40	**1/200**	**1/400**
Protocol	3.0 ml sterile distilled water (or equivalent) in a TEMPO® BC vial + 1 ml suspension 1/10	3.9 ml sterile distilled water (or equivalent) in a TEMPO® BC vial + 0.2 ml suspension 1/10	3.9 ml sterile distilled water (or equivalent) in a TEMPO® BC vial + 0.1 ml suspension 1/10

↓

Filling and sealing cards using the TEMPO® Filler

↓

Incubation for 22 h – 27 h at 30°C ± 1°C

↓

Reading using the TEMPO® Reader

The 1/40 dilution allows 10 to 49 000 cfu/g enumeration.
The 1/200 dilution allows 50 to 245 000 cfu/g enumeration.
The 1/400 dilution allows 100 to 490 000 cfu/g enumeration.