

NF VALIDATION
Validation of alternative analytical methods
Application in food microbiology

Summary report

Validation study according to the EN ISO 16140-2:2016

CHROMID® Coli (COLI ID-F)

(Certificate number: BIO 12/20 - 12/06)

for the enumeration of coliforms in a broad range of foods

Quantitative method

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This report consists of 55 pages, including 8 appendices.

Only copies including the totality of this report are authorised.

Competencies of the laboratory are certified by COFRAC accreditation for the analyses marked with the symbol♦.

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Quality Assurance documents related to this study can be consulted upon request from **bioMérieux**.

Measurement uncertainty on the reference method results is not taken into account to provide the conclusion in this report; this measurement uncertainty is however available.

The technical protocol and the result interpretation were realised according to the EN ISO 16140-2:2016 and the AFNOR technical rules (PR Revision 7).

Validation protocols	<ul style="list-style-type: none"> ▪ ISO 16140-1 (2016): Microbiology of the food chain - Method validation — <i>Part 1: Vocabulary</i> ▪ ISO 16140-2 (2016): Microbiology of the food chain - Method validation — <i>Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method</i> ▪ AFNOR technical rules (PR Revision 7).
Reference method*	ISO 4832 (2006): Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coliforms — Colony-count technique
Alternative method	CHROMID® Coli (COLI ID-F) for the enumeration of coliforms
Scope	Broad range of foods
Certification organization	AFNOR Certification (http://nf-validation.afnor.org/)

* Analyses performed according to the COFRAC accreditation

1 INTRODUCTION

The CHROMID® Coli (COLI ID-F) for the enumeration of coliforms was validated in 2006 according to the EN ISO 16140:2003 protocol and the AFNOR technical rules (Certificate number: BIO 12/20 - 12/06).

Table 1 summarizes the different steps of the validation that occurred since the initial validation.

Table 1 - Steps of the AFNOR Certification validation

Date	Study
14/12/2006	Initial Validation for a broad range of food according to ISO 16140 (2003)
2010	Renewal study
27/11/2014	Renewal study
December 2018	Renewal study according to the EN ISO 16140-2:2016.
October 2022	Renewal study

2 METHODS DESCRIPTION

2.1 Alternative method

2.1.1 Principle

The CHROMID® Coli (Coli ID) is a chromogenic medium which allows the enumeration of coliforms and *Escherichia coli*. This medium contains 2 chromogenic substrates. The coliforms (different from *Escherichia coli*) appear as blue to grey colonies. The *Escherichia coli* appear as pink to violet due to the presence of β -glucuronidase. Both aspects of colonies need to be considered for coliforms enumeration.

2.1.2 Protocol

The protocol is described in **Appendix 1**.

2.1.3 Restrictions

There is no restriction for use.

2.2 Reference method♦

The reference method is the NF ISO 4832 (2006): Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of coliforms - Colony-count technique. The flow diagram is provided in **Appendix 2**.

2.3 Protocol applied during the initial validation and the renewal study

The plates were incubated for 22 h at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

3 INITIAL VALIDATION, EXTENSION/RENEWAL STUDIES: RESULTS

3.1 Method comparison study

The method comparison study is a study performed by the expert laboratory to compare the alternative method with the reference method.

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.

For any comment on the alternative method, please contact AFNOR Certification at <http://nf-validation.afnor.org/contact-2/>.

3.1.1 Relative trueness

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.

3.1.1.1 Number and nature of the samples

Five food categories were tested with a minimum of 15 interpretable results per category and 5 interpretable results per type.

96 samples were tested for the initial validation study, 51 for a study run for bioMérieux in 2011 and 30 samples for the last renewal study (2018).

The repartition per tested category and type is provided in **Table 2**. Combining all the studies 177 samples were tested, leading to 135 exploitable results.

Table 2 – Repartition per tested category and type

Category	Type	Number of samples tested				Number of samples with interpretable results by both methods			
		2006	2011	2018	Total	2006	2011	2018	Total
1 Meat and meat products	a Raw (unseasoned)	6	11	0	17	5	10	0	15
	b Raw and cooked delicatessen	9	15	0	24	6	14	0	19
	c RTE, RTRH	3	6	0	9	1	6	0	7
	Total	18	32	0	50	12	30	0	41
2 Milk and dairy products	a Raw milk	4	0	3	7	3	0	3	6
	b Cream, desserts and cheeses	10	0	4	14	4	0	2	6
	c Milk powder	3	0	5	8	3	0	2	5
	Total	17	0	12	29	10	0	7	17
3 Seafood products	a Raw fish	6	0	9	15	4	0	5	9
	b Smoked and marinated	4	1	0	5	4	1	0	5
	c RTE, RTRH	16	2	3	21	16	2	1	19
	Total	26	3	12	41	24	3	6	33
4 Vegetables	a Raw vegetables	10	0	0	10	5	0	0	5
	b Frozen	5	3	0	8	2	3	0	5
	c RTE, RTRH	6	10	0	16	5	9	0	14
	Total	21	13	0	34	12	12	0	24
5 Eggs and egg-based products	a Eggs	6	0	0	6	6	0	0	6
	b Egg-based products	4	0	3	7	3	0	2	5
	c Pastries	4	3	3	10	4	3	2	9
	Total	14	3	6	23	13	3	4	20
All categories		96	51	30	177	71	48	17	135

3.1.1.2 Artificial and natural contamination of the samples

50 samples were artificially contaminated using seeding protocols. The inoculated strains and the injury protocols applied are provided in **Appendix 3**.

43 samples gave interpretable results by both methods.

93 samples giving interpretable results by both methods were naturally contaminated.

68.4 % of the samples were naturally contaminated.

3.1.1.3 Raw data

The raw data are provided in **Appendix 4**.

The samples were analyzed 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 three 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 with “*” in the data) in order to have a more precise result. These results were 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} unit less than the observed value. Similarly, any value greater than the upper limit should be amended by adding \log_{10} unit more. These results are not included in the calculations but also appear on the graphs.

Table 3 - Classification of the data

Category	Type	Number of samples tested	Number of interpretable results by both methods	Number of samples with <4 CFU/plate	Number of samples with results below or above the quantification limit
1	Meat and meat products	a Raw (unseasoned)	17	15	2
		b Raw and cooked delicatessen	24	19	2
		c RTE, RTRH	9	7	0
		Total	50	41	3
2	Milk and dairy products	a Raw milk	7	6	0
		b Cream, desserts and cheeses	14	6	1
		c Milk powder	8	5	2
		Total	29	17	10
3	Seafood products	a Raw fish	15	9	1
		b Smoked and marinated	5	5	0
		c RTE, RTRH	21	19	2
		Total	41	33	7
4	Vegetables	a Raw vegetables	10	5	0
		b Frozen	8	5	1
		c RTE, RTRH	16	14	1
		Total	34	24	8
5	Eggs and egg-based products	a Eggs	6	6	0
		b Egg-based products	7	5	1
		c Pastries	10	9	1
		Total	23	20	2
All categories		177	135	11	30

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	ISO 4832♦ (log CFU/g)	CHROMID® (COLI ID-F) for the enumeration of coliforms	Category	Type
594	Raw pork meat	1,65	<1,00	1	a
1867	Columbo turkey skewer	>4,18	>4,18	1	a
563	Ready to cook veal meal	1,30*	1,00*	1	b
564	Ready to cook veal meal	1,00*	1,00*	1	b
592	Sausage meat	2,11	1,48*	1	b
1865	Frozen sausages	2,18	<1,00	1	b
589	Tartar	1,81	1,48*	1	c
642	Ready to heat meal	2,98	ND	1	c
842	Raw milk	>5,18	3,92	2	a
847	Goat cheese	>6,18	>6,18	2	b
848	Goat cheese	>5,18	>5,18	2	b
1146	Cheese (Raclette)	2,11	<2,00	2	b
1273	Strawberries ice cream	<1,00	1,30*	2	b
1274	Pear ice cream	<1,00	<1,00	2	b
1145	Cheese	2,00	<2,00	2	b
6283	Raw milk cheese (Roquefort)	<1,00	<1,00	2	b
6284	Raw milk cheese (Selles sur Cher)	>5,18	>5,18	2	b
6297	Skimmed milk powder	<1,00	<1,00	2	c
6298	Skimmed milk powder	1,48*	1,30*	2	c
6299	Milk powder	1,30*	<1,00	2	c
1652	Pilchards	1,18*	<1,00	3	a
1653	Mackerel	<1,00	0,70*	3	a
6402	Lobster	<1,00	<1,00	3	a
6723	Fish fillet	>4,18	>4,18	3	a
6724	Fish fillet	>5,18	4,23	3	a
6280	Ready to reheat salmon	<1,00	<1,00	3	c
627	Brussels sprouts	1,70	<1,00	4	a
629	Green beans	1,40*	<1,00	4	a
630	Courgette	<1,00	<1,00	4	a
1275	Green beans	4,36	>5,18	4	a
1277	Green pepper	4,67	>5,18	4	a
631	Frozen vegetables mix	<1,00	<1,00	4	b
1276	Frozen vegetables mix	>5,18	>5,18	4	b
1348	Sliced courgette	2,70	2,00*	4	b
1279	Deli salad (leeks)	>5,18	>5,18	4	c
2123	Oriental tabouleh	2,18	1,00*	4	c
1137	Liquid egg product	4,18	4,18	5	a
1144	Egg based dessert	2,18	2,00*	5	b
6281	Pastry	1,85	<1,00	5	c

*: Results with less than 4 colonies per plate with the reference and/or the alternative method

♦ Analyses performed according to the COFRAC accreditation

3.1.1.4 Statistical interpretation

The calculations are provided in **Appendix 5**.

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

The **Figures 1 to 5** show the data plotted for each individual category.

The **Figure 6** shows the data plotted for all the products.

Figure 1 - Data plotted for the Meat and meat products

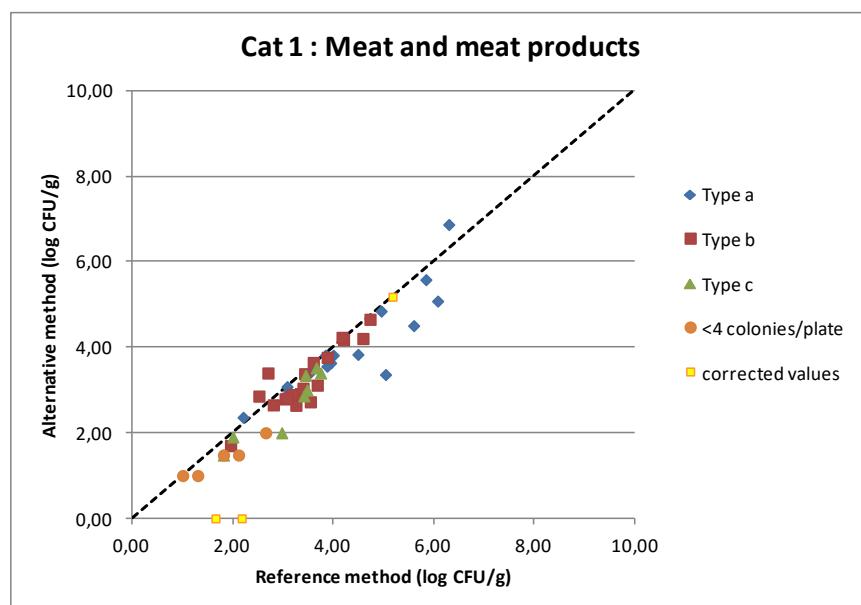


Figure 2 - Data plotted for Milk and dairy products

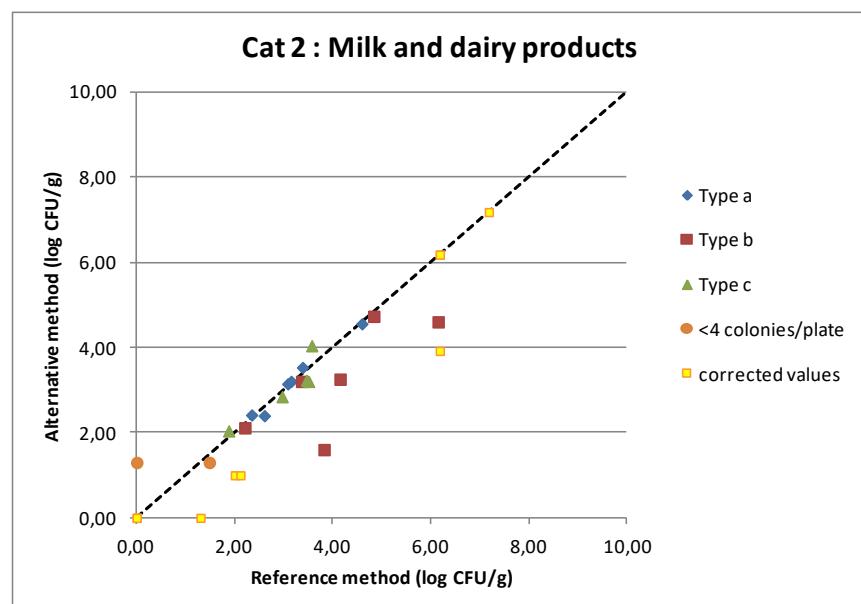


Figure 3 - Data plotted for Seafood products

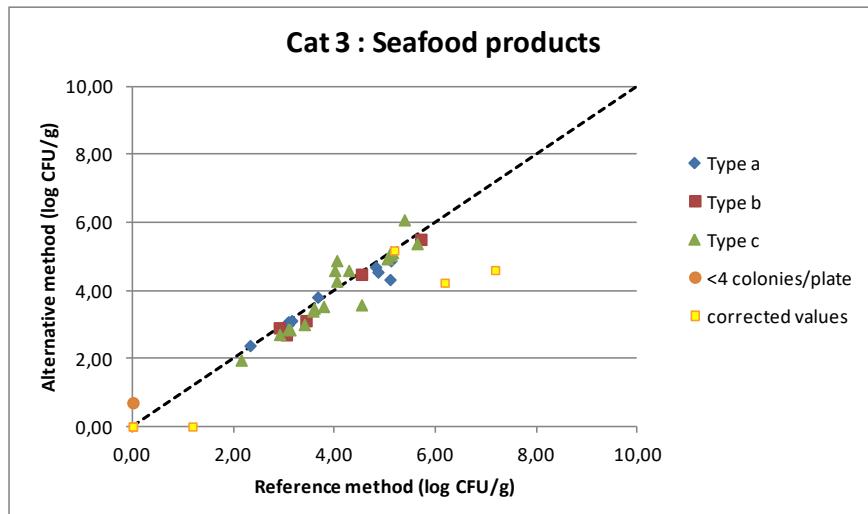


Figure 4 - Data plotted for Vegetables

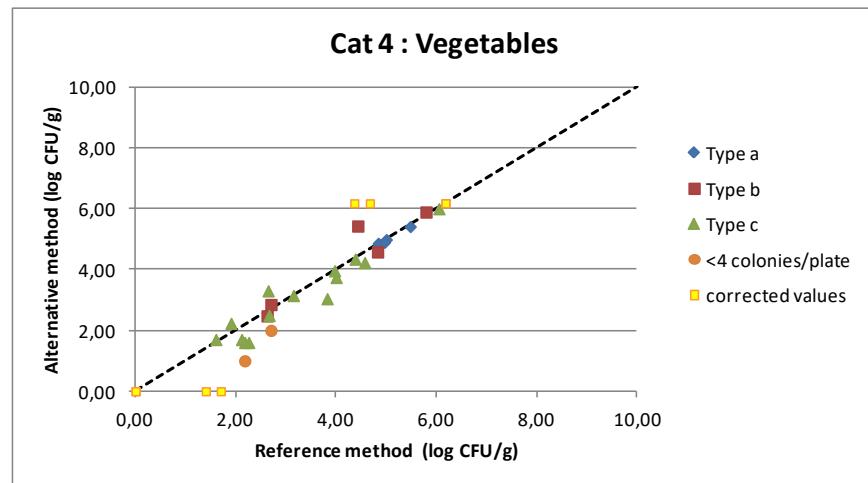


Figure 5 - Data plotted for Eggs and egg-based products

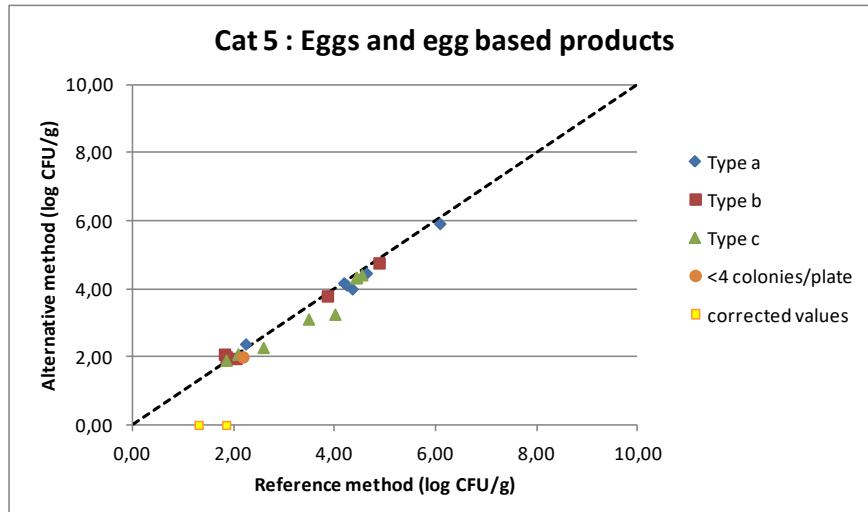
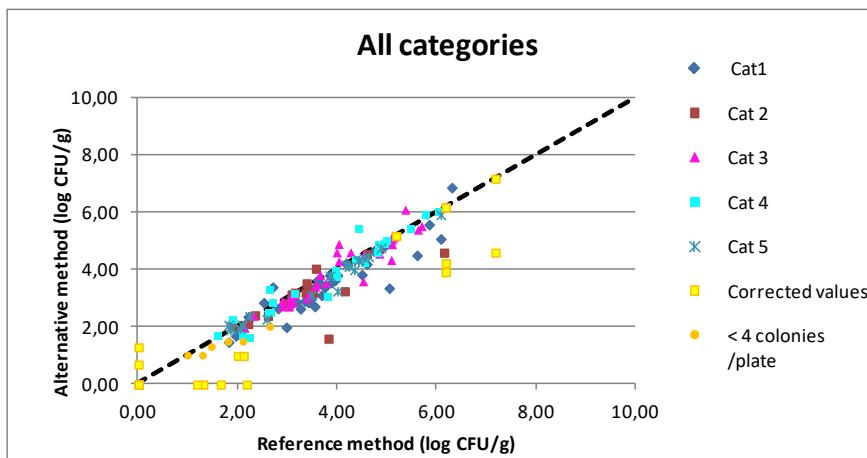


Figure 6 - Data plotted for all the products



The calculated values for Average difference and Standard deviation differences per category are provided in **Table 5**.

Table 5 - Calculated values

Category		Number of samples with interpretable results	\bar{D}	SD	Lower limit (95%)	Upper limit (95%)
1	Meat and meat products	41	-0.28	0.43	-1.16	0.60
2	Milk and dairy products	17	-0.29	0.67	-1.75	1.18
3	Seafood products	33	-0.08	0.35	-0.81	0.64
4	Vegetables	24	-0.06	0.38	-0.87	0.74
5	Eggs and egg-based products	20	-0.11	0.22	-0.58	0.36
All categories		135	-0.17	0.42	-1.01	0.67

\bar{D} : Average difference

SD: Standard deviation of differences

The average differences vary from - 0.29 log (dairy products) to - 0.06 log (vegetables).

The bias between both methods for all categories combined is -0.17 log CFU.

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

Figure 7 – Bland-Altman difference plot for all the samples

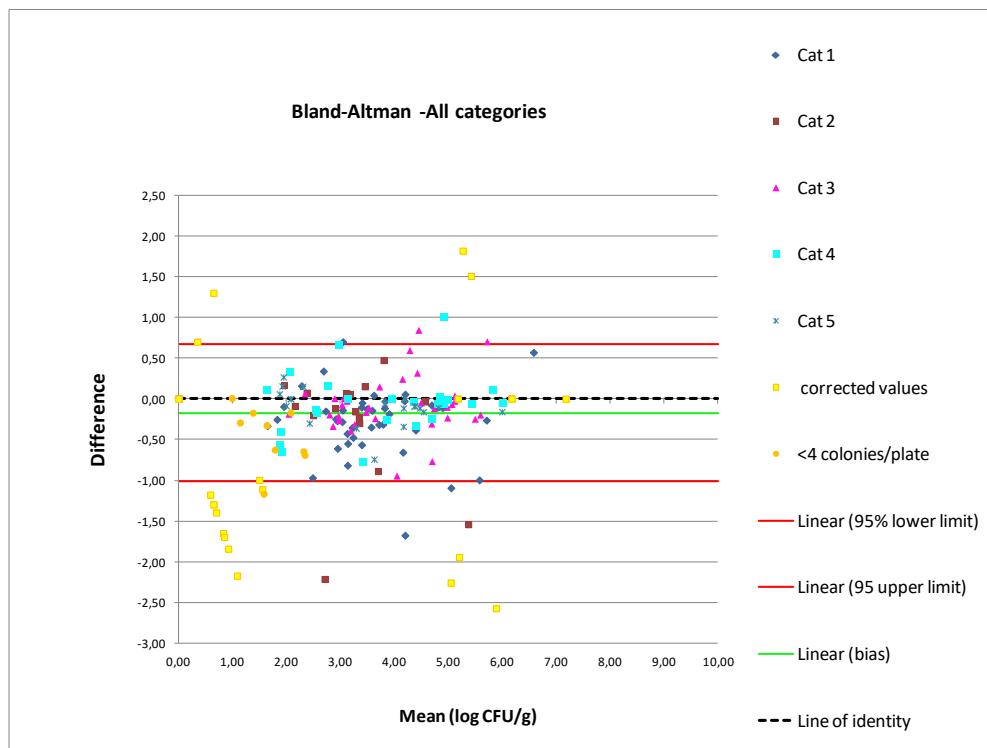


Table 6 - Analysis of the data out of the confidence limits

Classification of the data	CHROMID® (COLI ID-F) for the enumeration of coliforms									
	Category	Type	Sample N°	Product	Reference method	Alternative method	Values before correction (reference and/or alternative)	Mean	Difference	CLs
Interpretable results	1	a	1913	Ground turkey meat	5.04	3.36	/	4.20	-1.68	-1.01 / 0.67
	1	a	1917	Chicken mechanically deboned meat	5.60	4.51	/	5.05	-1.10	
	2	b	6419	Raw milk cheese	6.15	4.60	/	5.37	-1.54	
	2	b	6420	Dairy dessert	3.82	1.60	/	2.71	-2.22	
	1	b	635	Sausage	2.70	3.40	/	3.05	0.70	
	4	c	1280	Deli salad	5.38	6.08	/	5.73	0.70	
	5	c	1281	Salmon cubes	4.04	4.88	/	4.46	0.84	
	4	b	2367	Frozen tomato and pepper pie	4.43	5.43	/	4.93	1.00	
<4 CFU/plate	4	c	2123	Oriental tabouleh	2.18	1.00	/	1.59	-1.18	
< or >	1	a	594	Raw pork meat	1.65	0.00	1.00	0.83	-1.65	-1.01 / 0.67
	1	b	1865	Frozen sausages	2.18	0.00	1.00	1.09	-2.18	
	2	a	842	Raw milk	6.18	3.92	5.18	5.05	-2.26	
	2	b	1145	Cheese	2.00	1.00	2.00	1.50	-1.00	
	2	b	1146	Cheese (Raclette)	2.11	1.00	2.00	1.56	-1.11	
	2	c	6299	Milk powder	1.30	0.00	1.00	0.65	-1.30	
	3	a	1652	Pilchards	1.18	0.00	1.00	0.59	-1.18	
	3	a	6724	Fish fillet	6.18	4.23	5.18	5.21	-1.95	
	3	a	6725	Fish	7.18	4.60	6.18	5.89	-2.58	
	4	a	627	Brussels sprouts	1.70	0.00	1.00	0.85	-1.70	
	4	a	629	Green beans	1.40	0.00	1.00	0.70	-1.40	
	4	b	6278	Quiche lorraine	1.30	0.00	1.00	0.65	-1.30	
	5	c	6281	Pastry	1.85	0.00	1.00	0.92	-1.85	
	2	b	1273	Strawberries ice cream	0.00	1.30	1.00	0.65	1.30	
	3	a	1653	Mackerel	0.00	0.70	1.00	0.35	0.70	
	4	a	1275	Green beans	4.36	6.18	5.18	5.27	1.82	
	4	a	1277	Green pepper	4.67	6.18	5.18	5.43	1.51	

Values in green: differences in favour of the alternative method

Values in red: differences in favour of the reference method

Values in black: equivalent enumeration observed for the two methods



Corrected value



Results calculated using plates with less than 4 colonies

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

- 8 samples with interpretable results by both method (4 samples below the LCL and 4 samples above the UCL)
- 1 sample with less than 4 colonies on the plate (CHROMID Coli);
- 17 samples below or above the quantification limits (5 for the reference method, 12 for the alternative method).

3.1.1.5 Discordant results

The number of samples below or above the CLs is given **Table 7**.

Table 7 – Number of samples outside the CLs

	Number of samples	
Interpretable results by both methods	< LCL	4
	> UCL	4
	Total	8
<4 CFU/plate	< LCL	1
	> UCL	0
	Total	1
< or > the quantification limit	< LCL	13
	> UCL	4
	Total	17
Total < LCL		18
Total >UCL		8
TOTAL		26

For samples giving interpretable results by both methods, the number of samples with higher enumeration with the reference method is the same than the number of samples with higher enumeration using the alternative method (4 samples)

For the samples giving results below or above the quantification limit, more samples are below the LCL (13 vs 4) but note that for a majority of these samples, the difference is linked to the fact that a substituted value was used for the calculation.

For 11 samples outside of the confidence interval limits, the results can be considered equivalent.

The bias between the tested methods is low (-0.17log CFU).

3.1.1.6 Conclusion

**The relative trueness of the alternative method is satisfying.
The alternative method is equivalent to the reference method.**

3.1.2 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.

3.1.2.1 Matrices

Five matrices were tested. A minimum of one type per category, and 2 different batches, was selected, using 6 samples per type. 2 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. The tested categories, types, matrix and inoculated strains are provided in **Table 8**.

Table 8 - Categories, types and matrices

Category		Type	Matrix	Inoculated strain	Origin	Inoculation level (CFU/g)
1	Meat products and meat	a Raw meat	Ground beef	<i>Klebsiella oxytoca</i> 42	Food product	300 50000 100000
2	Milk and dairy products	b Dessert	Vanilla dairy-based dessert	<i>Enterobacter agglomerans</i> 74	Cheese	
3	Seafood products	a Raw fish	Raw fish fillet	<i>Enterobacter cloacae</i> Ad230	Tuna	
4	Vegetables	c RTE, RTRH	Grated carrots	<i>Escherichia coli</i> 19	Grated carrots	
5	Eggs and egg based products	a Eggs	Liquid egg product	<i>Cronobacter sakazakii</i> Ad890	Liquid egg product	

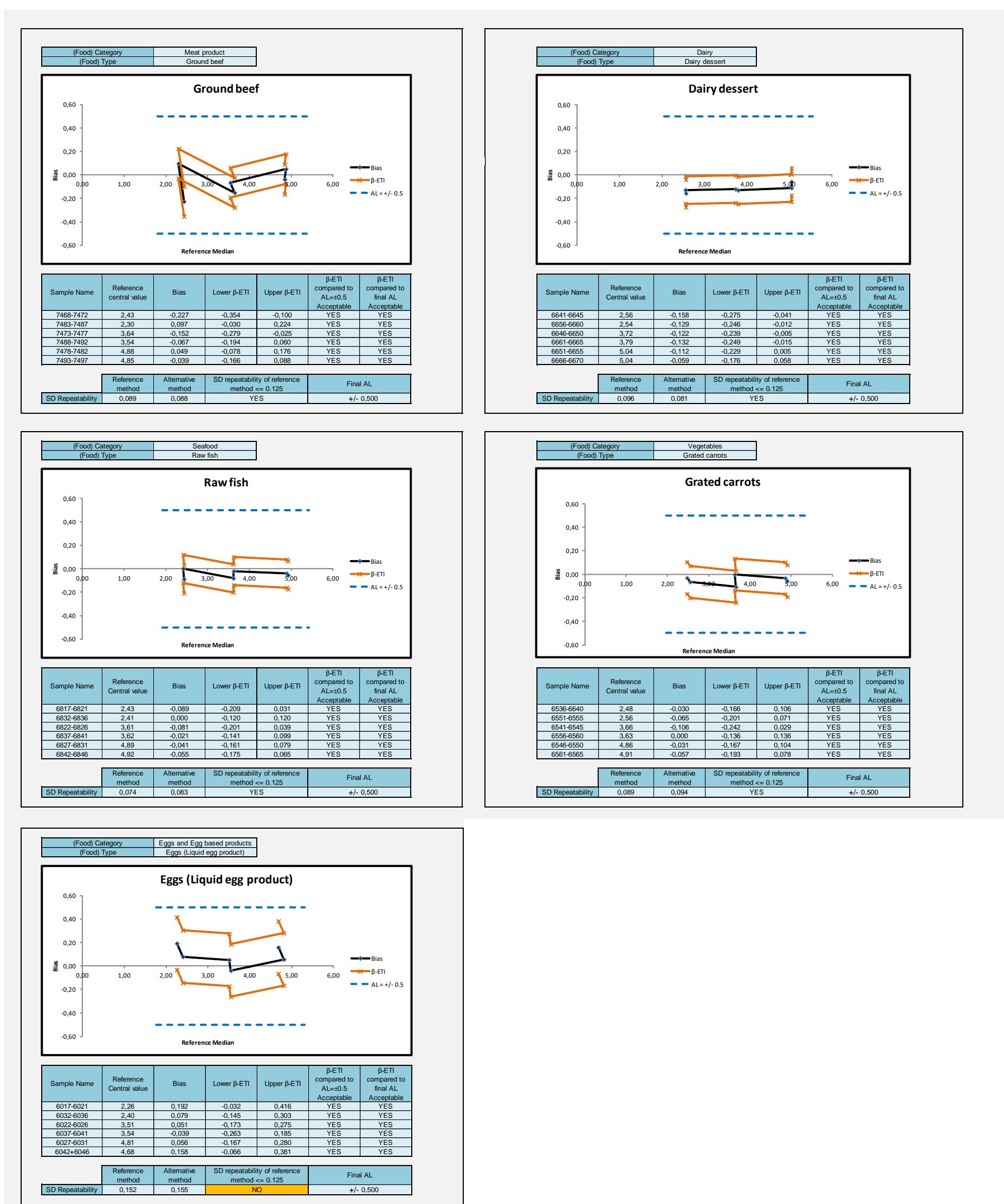
3.1.2.2 Calculation and interpretation

The raw data are provided in **Appendix 6**. The summary tables (in log CFU/g) and calculations are provided in **Appendix 7**. The statistical results and the accuracy profiles are provided **Figure 8**.

The calculations were done using the AP Calculation Tool MCS (Clause 6-1-3-3 calculation and interpretation of accuracy profile study) ver 31-07-2018 available on <http://standards.iso.org/iso/16140>

The accuracy profiles are comprised within the Acceptability Limits for all the tested matrices.

Figure 8 – Accuracy profile



3.1.2.3 Conclusion

For the five matrices tested the observed profiles are comprised within the AL fixed at $\pm 0.5 \log$. All the accuracy profiles fulfil the performance criteria.

3.1.3 Inclusivity and exclusivity studies

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.

For the initial validation study (2006), 30 target and 20 non-target strains were tested in duplicate on PCA, VRBL and CHROMID Coli (37°C).

20 additional target strains and 10 non-target strains were tested in 2017, once on PCA, VRBL and CHROMID Coli (37°C).

The raw data are provided in **Appendix 8**.

> **Inclusivity**

Among the 50 target strains tested 5 gave non-typical colonies using the CHROMID Coli:

- *Escherichia fergusonii* Ad 1381
- *Serratia liquefaciens* Ad2601
- *Serratia proteomaculans* Ad 1698
- *Serratia marcescens* Ad 2604
- *Serratia fonticola* Ad 1696

> **Exclusivity**

30 non target strains were tested, 5 strains gave typical colonies on CHROMID Coli plates and VRBL plates

- *Kluyvera ascorbata* Ad 229
- *Leclercia adecarboxylata* Ad 707
- *Lelliottia amnigena* Ad 1379
- *Pantoea agglomerans* A00L065
- *Raoultella terrigena* Ad 1370

Two additional strains gave typical colonies only on VRBL plates:

- *Buttiauxella agrestis* Ad 1328
- *Yersinia enterocolitica* Ad 1028

The CHROMID Coli method is as specific and selective, as the reference method.

3.1.4 Practicability

The alternative method practicability was evaluated according to the AFNOR criteria relative to method comparison study.

Storage conditions, shelf-life and modalities of utilisation after first use	The bottles are stored in their boxes at 2°C -8°C until the expiry date and kept away from light The agar cannot be melted more than twice		
Time to result	Steps	Reference method	Alternative method
	Sampling analysis	D0	D0
Common step with the reference method	Preparation of initial suspension		

The enumeration of coliforms is available in one day for both methods.

3.2 Inter-laboratory study organisation and results

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 study was run in 2006.

Pasteurised half-skimmed milk inoculated with *Escherichia coli* 94 and *Enterobacter cloacae* Fb2 was used for the study.

14 laboratories participated in the study.

The results of the inter-laboratory study run in 2006 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).

3.2.1 Experimental parameters controls

3.2.1.1 Sample stability

Strain stability during transport

Two samples inoculated per inoculation level were tested for enumeration after 24 h and 48 h storage at $3^{\circ}\text{C} \pm 2^{\circ}\text{C}$ (See **Table 9**)

Table 9 – *Enterobacter cloacae* Fb2 and *Escherichia coli* 94 stability in the matrix

Level 1		Level 2		Level 3	
	Replicate 1		Replicate 1		Replicate 1
Day 0	71	87	720	760	8 100
Day 1	72	78	810	640	9 300
Day 2	63	55	880	770	7 500
					6 600

No evolution was observed during storage for 48 h at $3^{\circ}\text{C} \pm 2^{\circ}\text{C}$.

3.2.2 Logistic conditions

The temperatures measured at reception by the Labs, the temperatures registered by the thermo-probe, and the receipt dates are given in **Table 10**.

Table 10 - Sample temperatures at receipt

Collaborator	Temperature measured at receipt (°C)	Date and hour receipt of the samples	Temperature measured by the probe (°C)
A	7.5	Day 2 14h00	0.5
B	2.0	Day 1 11h15	0.0
C	4.0	Day 1 11h30	Probe not received
D	3.0	Day 1 08h30	0.0
E	2.5	Day 2 09h10	0.0
F	0.3	Day 1 11h20	- 2.5 ¹
G	4.6	Day 1 10h00	0.00
H	0.2	Day 1 08h15	0.00
I	0.4	Day 1 09h30	0.00
J	1.0	Day 1 11h30	- 1.00 ¹
K	0.6	Day 1 09h20	0.00
L	0.3	Day 1 13h15	0.00
M	0.0	Day 1 11h00	0.00
N	0.3	Day 1 08h45	0.00

All the packages were declined at Day 1 except for two labs (A and E) which received their sample at Day 2. All the temperature measured at receipt were correct or below 8.4°C. No problem was encountered during transport.

3.2.3 Result analysis

3.2.3.1 Results obtained by the expert Lab.

The results obtained by the expert Lab. are the following (See **Table 11**).

Table 11 – Results obtained by the expert Lab.

Level (log CFU/g)	Reference method		Alternative method	
	Replicate 1	Replicate 2	Replicate 1	Replicate 2
< 1	< 1	< 1	< 1	< 1
1 to 2	1,97	1,92	1,92	1,85
2 to 3	2,90	2,75	2,90	2,82
3 to 4	3,98	3,89	3,89	3,92

¹ Some temperatures below 0°C were noticed but the samples were not frozen.

3.2.3.2 Results obtained by the collaborators

Samples were sent to 14 collaborators.

Mesophilic aerobic microflora

The mesophilic aerobic microflora was done on the matrix with ISO 4833 method.

The results varied from 22 000 to 480 000 CFU/ml.

Coliforms enumeration

A summary of the test results is given in **Table 12** (CFU/g) and **Table 13** (log CFU/g).

Table 12 - Summary of data (CFU/g)

Collaborator	Level 0				Level 1				Level 2				Level 3			
	Reference method		Alternative method		Reference method		Alternative method		Reference method		Alternative method		Reference method		Alternative method	
	<10	<10	<10	<10	75	45	80	50	660	750	750	640	5800	5900	6600	8200
A	<10	<10	<10	<10	75	45	80	50	660	750	750	640	5800	5900	6600	8200
B	<1	<1	<1	<1	79	90	94	81	960	1000	1000	730	12000	9400	11000	9400
C	<10	<10	<10	<10	86	100	80	50	760	740	530	720	8700	8700	7800	7100
D	<10	<10	<10	<10	40	50	80	90	820	660	790	660	6300	8000	7400	5700
E	<1	<1	<1	<1	75	76	93	63	990	730	920	840	9500	8500	6800	9500
F	<1	<1	<1	<1	80	82	85	76	1200	820	1000	750	12000	13000	7100	8800
G	<10	<10	<10	<10	45	73	50	110	760	670	550	580	7100	8000	5800	6900
H	<1	<1	<1	<1	70	76	61	60	700	750	810	750	8200	8000	8000	8500
I	<1	<1	<1	<1	73	82	73	85	980	1000	900	810	10000	11000	9700	8500
J	<1	<1	<1	<1	70	68	65	69	860	730	770	800	7300	8100	8300	8100
K	<10	<10	<10	<10	110	80	80	50	780	750	710	750	8400	7100	8400	7800
L	<10	<10	<10	<10	130	77	110	80	900	630	630	790	8500	7400	7500	8700
M	<1	<1	<1	<1	72	66	71	66	830	710	820	780	8300	9100	9600	8500
N	<1	<1	<1	<1	72	76	73	65	890	780	820	930	9700	9500	9000	9000

Table 13 - Summary of data (log CFU/g)

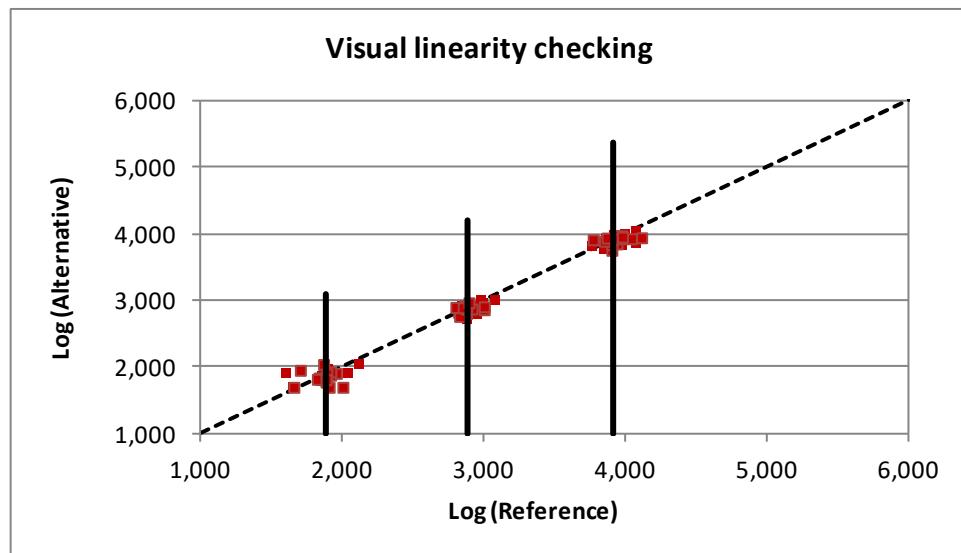
Collabo-rator	Level 0				Level 1				Level 2				Level 3			
	Reference method		Alternative method		Reference method		Alternative method		Reference method		Alternative method		Reference method		Alternative method	
A	<1,00	<1,00	<1,00	<1,00	1,875	1,653	1,903	1,699	2,820	2,875	2,875	2,806	3,763	3,771	3,820	3,914
B	<0,00	<0,00	<0,00	<0,00	1,898	1,954	1,973	1,908	2,982	3,000	3,000	2,863	4,079	3,973	4,041	3,973
C	<1,00	<1,00	<1,00	<1,00	1,934	2,000	1,903	1,699	2,881	2,869	2,724	2,857	3,940	3,940	3,892	3,851
D	<1,00	<1,00	<1,00	<1,00	1,602	1,699	1,903	1,954	2,914	2,820	2,898	2,820	3,799	3,903	3,869	3,756
E	<0,00	<0,00	<0,00	<0,00	1,875	1,881	1,968	1,799	2,996	2,863	2,964	2,924	3,978	3,929	3,833	3,978
F	<0,00	<0,00	<0,00	<0,00	1,903	1,914	1,929	1,881	3,079	2,914	3,000	2,875	4,079	4,114	3,851	3,944
G	<1,00	<1,00	<1,00	<1,00	1,653	1,863	1,699	2,041	2,881	2,826	2,740	2,763	3,851	3,903	3,763	3,839
H	<0,00	<0,00	<0,00	<0,00	1,845	1,881	1,785	1,778	2,845	2,875	2,908	2,875	3,914	3,903	3,903	3,929
I	<0,00	<0,00	<0,00	<0,00	1,863	1,914	1,863	1,929	2,991	3,000	2,954	2,908	4,000	4,041	3,987	3,929
J	<0,00	<0,00	<0,00	<0,00	1,845	1,833	1,813	1,839	2,934	2,863	2,886	2,903	3,863	3,908	3,919	3,908
K	<1,00	<1,00	<1,00	<1,00	2,041	1,903	1,903	1,699	2,892	2,875	2,851	2,875	3,924	3,851	3,924	3,892
L	<1,00	<1,00	<1,00	<1,00	2,114	1,886	2,041	1,903	2,954	2,799	2,799	2,898	3,929	3,869	3,875	3,940
M	<0,00	<0,00	<0,00	<0,00	1,857	1,820	1,851	1,820	2,919	2,851	2,914	2,892	3,919	3,959	3,982	3,929
N	<0,00	<0,00	<0,00	<0,00	1,857	1,881	1,863	1,813	2,949	2,892	2,914	2,968	3,987	3,978	3,954	3,954

Some labs enumerated the dilution -1, -2, -3 instead of the dilution 0, -1, -2, -3 this explains the results obtained for level 0 (< 1 or < 10 CFU/ml).

3.2.4 Calculation and interpretation

3.2.4.1 Visual linearity checking

The **Figure 9** 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 9 - Visual linearity checking

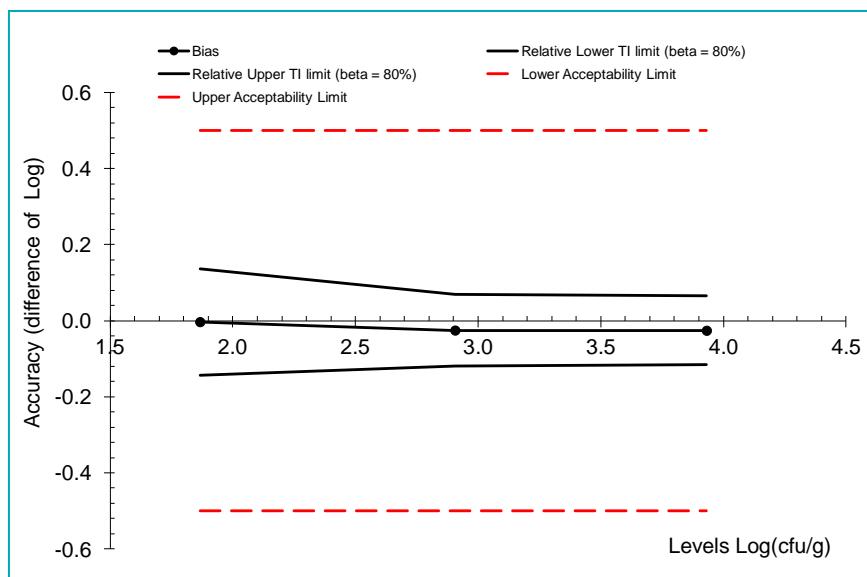
3.2.4.2 Accuracy profile calculation

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 14**.

Table 14 - Summary of statistical tests

Accuracy profile	0,5		
Study Name	CHROMID Coli		
Date	2006		
Coordinator	ADRIA Développement		
Tolerance probability (beta)	80%	80%	80%
Acceptability limit in log (lambda)	0,50	0,50	0,50
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			
Levels	Low	Medium	High
Target value	1,866	2,906	3,931
Number of participants (K)	14	14	14
Average for alternative method	1,863	2,881	3,905
Repeatability standard deviation (sr)	0,105	0,054	0,052
Between-labs standard deviation (sL)	0,000	0,045	0,042
Reproducibility standard deviation (sR)	0,105	0,070	0,067
Corrected number of dof	26,963	22,584	22,749
Coverage factor	1,337	1,353	1,352
Interpolated Student t	1,314	1,320	1,320
Tolerance interval standard deviation	0,1067	0,0719	0,0684
Lower TI limit	1,723	2,786	3,815
Upper TI limit	2,003	2,976	3,996
Bias	-0,003	-0,025	-0,026
Relative Lower TI limit (beta = 80%)	-0,143	-0,120	-0,116
Relative Upper TI limit (beta = 80%)	0,137	0,070	0,065
Lower Acceptability Limit	-0,50	-0,50	-0,50
Upper Acceptability Limit	0,50	0,50	0,50
New acceptability limits may be based on reference method pooled variance			
Pooled repro standard dev of reference	0,090		

These values are collected in a graphical representation together with the acceptability limits (AL). This representation is given **Figure 10**.

Figure 10 - 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 results obtained with the alternative method are not statically different than those obtained with the reference method.

3.2.4.3 Conclusion

The alternative method is equivalent to the reference method.

4 CONCLUSION

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

- 177 samples were tested in the relative trueness study, providing 135 interpretable results by both methods which clearly satisfied the required criteria for quantitative method comparison per ISO 16140-2.
- The observed profiles are comprised within the AL set at ± 0.5 Log CFU/g in the EN ISO 16140-2:2016.
- The inclusivity and exclusivity testing shows satisfying results.
- The quality assurance parameters were verified (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 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 alternative method is considered equivalent to the reference method.

Quimper, 03 November 2022

Maryse RANNOU

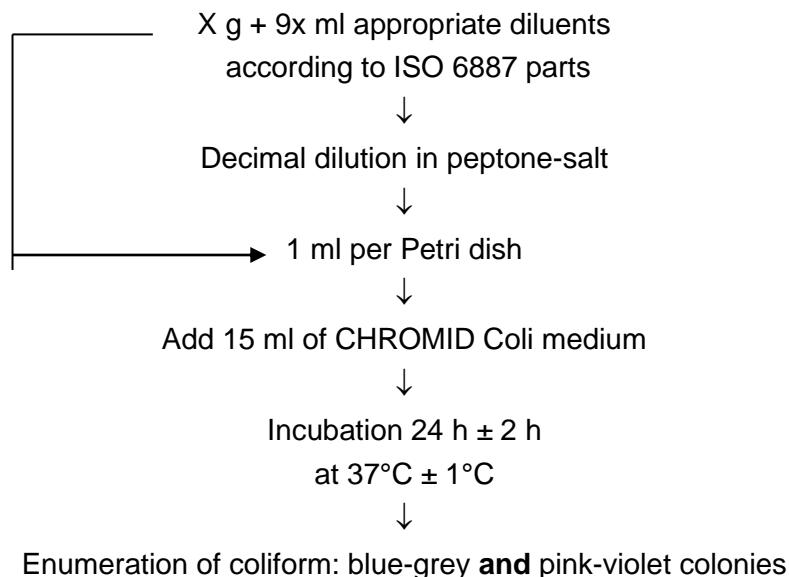
Project Manager

Validation of Alternative methods

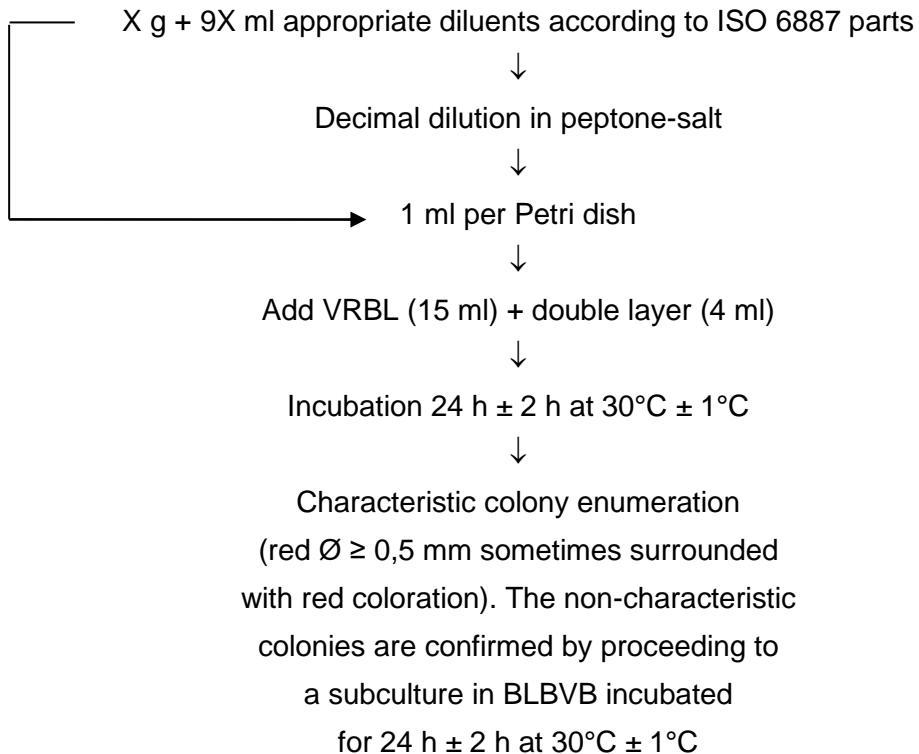


I hereby attest to the validation of the verification of the conformity of the report (opinion and interpretation).

**Appendix 1 - Flow diagram of the alternative method:
CHROMID Coli (Coli ID-F) for the enumeration of coliforms**



**Appendix 2 – Flow diagram of the reference method:
NF ISO 4832 (2006): Microbiology of food and animal feeding stuffs —
Horizontal method for the enumeration of coliforms — Colony-count technique**



Appendix 3 – Artificial contaminations of samples

Analysis date	Sample N°	Product (French name)	Product	Artificial contamination			Category	Type
				Strain	Origin	Injury		
2011	2370	Escalope de dinde	Turkey escalope	<i>Escherichia coli</i> Ad 218	Poultry	Seeding 7 days at 4°C	1	a
2011	2474	Blanc de poulet	White chicken meat	<i>Escherichia coli</i> Ad 218	Poultry	Seeding heat treatment 10 min 56°C	1	a
2011	2152	Saucisses fumées bio	Smoked sausages	<i>Escherichia coli</i> 21	Cured breast	Seeding heat treatment 10 min 56°C	1	b
2011	2153	Saucisses de Montbéliard	Montbéliard sausage	<i>Escherichia coli</i> 21	Cured breast	Seeding heat treatment 10 min 56°C	1	b
2011	2185	Saucisses natures	Sausages	<i>Escherichia coli</i> 6	Sausage	Seeding 6 days at 4°C	1	b
2011	2471	Chorizo	Chorizo	<i>Escherichia coli</i> 1	Pork	Seeding heat treatment 10 min 56°C	1	b
2011	2472	Chorizo	Chorizo	<i>Escherichia coli</i> 1	Pork	Seeding heat treatment 10 min 56°C	1	b
2011	2473	Saucisson à l'ail	Garlic sausage	<i>Escherichia coli</i> 1	Pork	Seeding heat treatment 10 min 56°C	1	b
2011	2477	Filets de bacon fumé	Smoked bacon	<i>Escherichia coli</i> 21	Pork	Seeding 7 days at 4°C	1	b
2011	2478	Poitrine fumée tranches fines	Sliced bacon	<i>Escherichia coli</i> 1	Pork	Seeding 7 days at 4°C	1	b
2011	2479	Jambon cru	Raw ham	<i>Escherichia coli</i> 21	Pork	Seeding 7 days at 4°C	1	b
2011	2059	Chili con carné	Chilli con carne	<i>Escherichia coli</i> 144	Paella	Seeding heat treatment 15 min 56°C	1	c
2011	2065	Nems au porc	Pork nem	<i>Escherichia coli</i> 108	Bouchées à la reine	Seeding heat treatment 10 min 56°C	1	c
2011	2066	Ravioli chinois au porc	Chinese ready to eat food	<i>Escherichia coli</i> 108	Ready to cook meal	Seeding heat treatment 15 min 56°C	1	c
2011	2151	Sandwich jambon œuf crudités	Sandwich (ham and vegetables)	<i>Escherichia coli</i> Ad 222	Egg product	Seeding 4°C 48 h	1	c
2011	2368	Sandwich jambon œuf tomate	Ham, tomato and egg sandwich	<i>Escherichia coli</i> 101	Pork	Seeding heat treatment 10 min 56°C	1	c
2011	2369	Jambon cuit à la broche	Cooked ham	<i>Escherichia coli</i> 101	Pork	Seeding heat treatment 10 min 56°C	1	c

Analysis date	Sample N°	Product (French name)	Product	Artificial contamination			Category	Type
				Strain	Origin	Injury		
2018	6297	Lait en poudre écrémé	Skimmed milk powder	<i>Escherichia coli</i> Ad1816	Dairy product	Seeding lyophilized strain 2 weeks at room temperature	2	c
2018	6298	Lait en poudre écrémé	Skimmed milk powder	<i>Escherichia coli</i> 118	Dairy product	Seeding lyophilized strain 2 weeks at room temperature	2	c
2018	6299	Lait en poudre entier	Milk powder	<i>Escherichia coli</i> Ad1816	Dairy product	Seeding lyophilized strain 2 weeks at room temperature	2	c
2018	6300	Lait en poudre entier	Milk powder	<i>Escherichia coli</i> 118	Dairy product	Seeding lyophilized strain 2 weeks at room temperature	2	c
2018	6301	Lait en poudre écrémé	Skimmed milk powder	<i>Escherichia coli</i> 118	Dairy product	Seeding lyophilized strain 2 weeks at room temperature	2	c
2018	6723	Filet de julienne	Fish fillet	<i>Escherichia coli</i> Ad1384	Sea water	Seeding 48 h 3 ± 2°C	3	a
2018	6724	Filet de Merlan	Fish fillet	<i>Escherichia coli</i> Ad1384	Sea water	Seeding 48 h 3 ± 2°C	3	a
2018	6725	Bar	Fish	<i>Escherichia coli</i> Ad1384	Sea water	Seeding 48 h 3 ± 2°C	3	a
2018	6726	Lamelles d'encornet géant	Squids	<i>Escherichia coli</i> Ad1385	Sea water	Seeding 48 h 3 ± 2°C	3	a
2018	6727	Encornet criée	Squids	<i>Escherichia coli</i> Ad1385	Sea water	Seeding 48 h 3 ± 2°C	3	a
2018	6728	Crevettes crues décortiquées	Raw shrimps	<i>Escherichia coli</i> Ad1385	Sea water	Seeding 48 h 3 ± 2°C	3	a
2018	6729	Noix de saint jacques	Scallops	<i>Escherichia coli</i> Ad1385	Sea water	Seeding 48 h 3 ± 2°C	3	a
2011	2122	Sandwich saumon fumé ciboulette	Sandwich (smoked salmon and chives)	<i>Escherichia coli</i> 93	Ready to cook cod	Seeding 4°C 8 days	3	b
2011	2061	Nems au crabe	Crab nem	<i>Escherichia coli</i> 93	Ready to cook cod	Seeding heat treatment 15 min 56°C	3	c
2011	2150	Salade de pâtes saumon mayonnaise	Pasta salad (salmon and mayonnaise)	<i>Escherichia coli</i> Ad 222	Egg product	Seeding heat treatment 10 min 56°C	3	c
2011	2120	Ratatouille surgelée	Frozen ratatouille	<i>Escherichia coli</i> 19	Sliced carrots	Seeding -20°C 8 days	4	b
2011	2183	Quiche aux poireaux surgelée	Frozen links quiche	<i>Escherichia coli</i> 142	Egg product	Seeding 6 days at -20°C	4	b
2011	2367	Tarte tomate poivron surgelée	Frozen tomato and pepper pie	<i>Escherichia coli</i> 142	Egg product	Seeding 7 days -20°C	4	b

Analysis date	Sample N°	Product (French name)	Product	Artificial contamination			Category	Type
				Strain	Origin	Injury		
2011	2058	Ratatouille	Ratatouille	<i>Escherichia coli</i> 19	Grated carrots	Seeding heat treatment 15 min 56°C	4	c
2011	2060	Carottes en lamelles cuites	Cooked sliced carrots	<i>Escherichia coli</i> 144	Paella	Seeding heat treatment 10 min 56°C	4	c
2011	2121	Salade boulghour légumes du soleil	Salad (bulghur and vegetables)	<i>Escherichia coli</i> 19	Sliced carrots	Seeding 4°C 8 days	4	c
2011	2123	Taboulé à l'orientale	Oriental tabbouleh	<i>Escherichia coli</i> 108	Ready to cook meal	Seeding 4°C 8 days	4	c
2011	2148	Riz à la provençale thon basilic	Cooked rice (tuna and basil)	<i>Escherichia coli</i> 93	Ready to cook cod	Seeding heat treatment 10 min 56°C	4	c
2011	2149	Quiche aux légumes	Vegetables quiche	<i>Escherichia coli</i> 93	Ready to cook cod	Seeding heat treatment 10 min 56°C	4	c
2011	2363	Salade carotte céleri	Carrots celery salad	<i>Escherichia coli</i> 19	Sausage	Seeding 7 days at 4°C	4	c
2011	2365	Macédoine de légumes	Mixed vegetables	<i>Escherichia coli</i> 144	Paella	Seeding heat treatment 10 min 56°C	4	c
2011	2366	Macédoine de légumes	Mixed vegetables	<i>Escherichia coli</i> 142	Egg product	Seeding heat treatment 10 min 56°C	4	c
2011	2483	Piémontaise au jambon	Salad (Piémontaise)	<i>Escherichia coli</i> 108	Ready to eat meal	Seeding heat treatment 10 min 56°C	4	c
2018	6730	Mayonnaise fraîche	Mayonnaise	<i>Escherichia coli</i> 143	Egg product	Seeding 48 h 3 ± 2°C	5	b
2018	6731	Tortilla espagnole aux oignons	Tortilla with onions	<i>Escherichia coli</i> 143	Egg product	Seeding 48 h 3 ± 2°C	5	b
2011	2062	Tartelette cocktail	Cocktail pie	<i>Escherichia coli</i> Ad 222	Egg product	Seeding heat treatment 15 min 56°C	5	c
2011	2063	Tartelette cocktail	Cocktail pie	<i>Escherichia coli</i> 142	Egg product	Seeding heat treatment 10 min 56°C	5	c
2011	2064	Tartelette fraise	Strawberries pie	<i>Escherichia coli</i> Ad 222	Egg product	Seeding heat treatment 10 min 56°C	5	c

Matrix	Strain	Level	Sample N°	ISO 4832*				CHROMID® coli (COLI ID-F) for the enumeration of coliforms			
				Dilution	cfu/plate	cfu/g	log cfu/g	Dilution	cfu/plate	cfu/g	log cfu/g
Liquid egg product Batch 1 Aerobic mesophilic flora: 10 CFU/g	Cronobacter sakazakii Ad890	1	6017	10 100	16 2	160	2,20	10 100	13 2	140	2,15
			6018	10 100	18 1	170	2,23	10 100	30 4	310	2,49
			6019	10 100	16 4	180	2,26	10 100	30 1	280	2,45
			6020	10 100	30 3	300	2,48	10 100	25 6	280	2,45
			6021	10 100	23 4	250	2,40	10 100	32 2	310	2,49
			6022	100 1000	20 2	2000	3,30	100 1000	39 1	3600	3,56
		2	6023	100 1000	25 3	2500	3,40	100 1000	49 1	4500	3,65
			6024	100 1000	31 4	3200	3,51	100 1000	28 2	2700	3,43
			6025	100 1000	41 2	3900	3,59	100 1000	50 2	4700	3,67
			6026	100 1000	39 1	3600	3,56	100 1000	35 5	3600	3,56
			6027	1000 10000	20 1	19000	4,28	1000 10000	20 0	18000	4,26
			6028	1000 10000	67 4	65000	4,81	1000 10000	79 2	74000	4,87
		3	6029	1000 10000	63 7	64000	4,81	1000 10000	54 7	55000	4,74
			6030	1000 10000	76 2	71000	4,85	1000 10000	75 10	77000	4,89
			6031	1000 10000	66 9	68000	4,83	1000 10000	78 9	79000	4,90
			6032	10 100	24 4	260	2,41	10 100	21 2	210	2,32
			6033	10 100	34 4	350	2,54	10 100	30 3	300	2,48
			6034	10 100	26 1	250	2,40	10 100	30 6	330	2,52
		2	6035	10 100	23 4	250	2,40	10 100	33 1	310	2,49
			6036	10 100	23 2	230	2,36	10 100	28 3	280	2,45
			6037	100 1000	15 1	1500	3,18	100 1000	27 2	2600	3,41
			6038	100 1000	28 1	2600	3,41	100 1000	25 2	2500	3,40
			6039	100 1000	38 1	3500	3,54	100 1000	38 7	4100	3,61
			6040	100 1000	37 4	3700	3,57	100 1000	34 1	3200	3,51
		3	6041	100 1000	43 6	4500	3,65	100 1000	35 6	3700	3,57
			6042	1000 10000	31 4	32000	4,51	1000 10000	45 2	43000	4,63
			6043	1000 10000	65 3	62000	4,79	1000 10000	44 3	43000	4,63
			6044	1000 10000	47 6	48000	4,68	1000 10000	90 6	87000	4,94
			6045	1000 10000	50 1	46000	4,66	1000 10000	65 11	69000	4,84
			6046	1000 10000	53 4	52000	4,72	1000 10000	91 7	89000	4,95

* Analyses performed according to the COFRAC accreditation

INCLUSIVITY										
Year of testing	N°	Strain		Reference	Origin	PCA	ISO 4832♦	BLBVB	CHROMID Coli	
						CFU/plate (1 ml -7)	CFU/plate (1ml -7)		CFU/plate (1 ml -7)	Description of the colonies
2006	26	Klebsiella	pneumoniae	Adria28	Food	67	58	/	72	blue
						50	58		60	
	27	Klebsiella	pneumoniae	CIP 8291	Unknown	100	8	/	9	grey-blue
						90	11		0	
	28	Escherichia	coli	A00C70	Poultry meat	24	30	/	10	pink
						32	31		4	
2006	29	Serratia	liquefaciens	Adria 8	Egg product	>300	86	/	55	grey-blue
						>300	96		71	
	30	Klebsiella	pneumoniae	CIP 52145	Sweetbread	49	30	/	41	blue
						53	31		33	
2017	34	Citrobacter	braakii	Ad2701	Squids	14	10	/	12	blue
	35	Citrobacter	koseri	Ad2731	Sprouts	82	78	/	91	blue
	36	Citrobacter	farmeri	Ad1116	Environmental sample (egg industry)	98	103	/	116	blue
	37	Enterobacter	aerogenes	Ad2569	Cheese	47	60	/	53	blue
	38	Enterobacter	hormaechei	Ad1373	Water	133	120	/	119	blue
	39	Enterobacter	kobei	Ad706	Milk powder	68	68	/	79	blue
	40	Enterobacter	helveticus	DSM18396	Unknown	70	27	+/-	22	grey
	41	Escherichia	ergusonii	Ad1381	Water	70	74	-	78	white
	42	Escherichia	hermanii	Ad464	Raw milk	130	128	-	136	pale
	43	Klebsiella	oxytoca	Ad1453	Swimming pool water	22	20	/	18	blue
	44	Klebsiella	pneumoniae	Ad1594	Swimming pool water	20	25	/	19	blue
	45	Klebsiella	pneumoniae	Ad1374	Water	98	107	/	75	blue
	46	Klebsiella	oxytoca	Ad1371	Water	14	11	/	21	blue
	47	Serratia	liquefaciens	Ad2601	Dairy product	25	22	/	29	white
	48	Serratia	proteomaculans	Ad1698	Salmon	67	29	/	118	beige
	49	Serratia	marcescens	Ad2604	Dairy product	52	66	/	53	white
	50	Serratia	fonticola	Ad1696	Salmon	25	23	/	14	beige

