Method Comparison Study Report for the ISO 16140-2:2016 extension validation of Assurance® GDS for *Cronobacter* Tq II, for the detection of *Cronobacter* spp. in Infant Formula and Infant Cereals and Environmental Samples

MicroVal study number: 2017LR77, extension

Method/Kit name: Assurance® GDS for *Cronobacter* Tq II

Report version: Summary report

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Foreword

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

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Method/Kit name: Assurance® GDS for *Cronobacter* Tq II

Validation standard: Microbiology of the food chain— Method validation

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

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

and

MicroVal interpretation guidelines for ISO 16140-Part 6 (2019) issued in 2020 for extension study for a new confirmation method from a previously validated qualitative method

Reference method:

ISO 22964:2017 Microbiology of the food chain – Horizontal method for detection of *Cronobacter* spp

Scope of validation: Infant Formula and Infant Cereals and Environmental Samples

Certification organization: Lloyd's Register **List of abbreviations**

List of abbreviations

A(lt)	Alternative method
AL	Acceptability Limit
Art. Cont.	artificial contamination
CFU	Colony Forming Units
EL	Expert Laboratory
FP	False Positive
FPR	False Positive Ratio
g	Gram
h	Hour
ILS	Interlaboratory Study
LOD	Level of Detection
MCS	Method Comparison Study
min	minute
ml	millilitre
MR	(MicroVal) Method Reviewer
MVTC	MicroVal Technical Committee
NA	Negative Agreement
na	not applicable
ND	Negative Deviation
neg (-)	negative/no growth/no reaction/target not detected
NS	Non-Suspect growth
nt	not tested
PA	Positive Agreement
PD	Positive Deviation
pos (+)	positive/growth/target detected
PPNA	Presumptive Positive Negative Argreement (belongs to the False Positive results)
PPND	Presumptive Positive Negative Deviation (belongs to the False Positive results)
R(ef)	Reference method
RLOD	Relative Level of Detection
RT	Relative Trueness
S	Suspect growth
SE	Relative Sensitivity
SP	Relative Specificity
TP	True Positive

And, add for Cronobacter studies:

BPW	Buffered Peptone Water
BGW	Brilliant Green Water
BHI	Brain Heart Infusion broth
CCI	Chromogenic *Cronobacter* Isolation *agar*
DFI	Druggan-Forsythe-Iversen agar
RSA	RAPID'*Sakazakii* agar

Contents

1 Introduction

In this project a MicroVal validation extension study, based on ISO 16140-2:2016, of an alternative method for the detection of *Cronobacter* spp. in 2 different categories was carried out by Campden BRI as the MicroVal Expert Laboratory.

The Assurance® GDS for *Cronobacter* Tq II (GDS *Cronobacter*) method was previously validated in December 2018 for Infant Formula and Infant Cereals and environmental samples by Q Laboratories (study ref 2017LR77) for 10-375 g samples.

Below is a summary of the different enrichment protocols used for analysis by the alternative method for the initial validation study.

1.1. For all food matrices analyzed, both 10 g and 375 g test portions were analyzed.
1.2. The 10 g test portions were analyzed after 20 hours of enrichment and the 375 g test portions were analyzed after 24 hours of enrichment.
1.3. All environmental samples were analyzed after 18 hours of enrichment.
1.4. For all matrices (except for milk powder without probiotics) and environmental samples, the primary enrichment was BPW (ISO) pre-warmed to 37 ± 1ºC. The milk powder primary enrichment was brilliant green water (BGW) pre-warmed to 37 ± 1ºC.
1.5. Infant cereal with probiotic included the addition of amylase (10 mg/L) to the BPW (ISO). The infant formula with probiotics included the addition of vancomycin (6 mg/L) to the BPW (ISO). For infant cereal with probiotics, both amylase and vancomycin were added to the BPW (ISO).
1.6. Samples were processed from the primary enrichment according to the Assurance GDS *Cronobacter* Tq II method, except for matrices containing milk powder. For these food types, a regrowth step in BHI is performed first, followed by analysis according to the alternative method.

Reference method: ISO 22964:2017

Confirmation during the initial validation study was carried out following 2 options:
ISO 22964:2017/Traditional Confirmation

For the powdered infant formula (with and without probiotics), infant cereal with probiotics and milk powder test portions, a 10 g sample was enriched with 90 mL of Buffered Peptone Water (BPW) ISO formulation. For infant cereal without probiotics, a 10 g sample was enriched with BPW ISO + amylase. For process water, a 25 mL test portion was enriched with 225 mL of BPW ISO. The stainless steel environmental sponges were enriched with 100 mL of BPW ISO. All test portions were allowed to stand at room temperature (20-25 °C) for 60 ± 10 minutes. Subsequently, the infant formula enrichments were incubated at 34 to 38°C for 18 ± 2 hours.

Following incubation, 0.1 mL of primary enrichment was transferred into 10 mL of *Cronobacter* Screening Broth (CSB) with 0.1mL of vancomycin solution (0.1% v/v). CSB tubes were incubated at 41.5 ± 1ºC for 24 ± 2 hours. Following incubation, a loopful of the secondary enrichments were streaked to Chromogenic *Cronobacter* Isolation agar (CCI) and incubated at 41.5 ± 1ºC for 24 ± 2 hours. One suspected colony from each CCI plate was transferred to Tryptone Soya Agar (TSA) and incubated at 37 ± 7°C for 18 to 24 hours. Typical morphology for CCI is a small to medium sized (1mm to 3mm) blue-green to blue colony. Atypical morphology on CCI is often white or white with a green center, grey or black. After incubation, typical isolates were purified on TSA and identified using the Bruker MALDI following the MicroVal certified method 2017LR72.

Direct Streak Confirmation (Alternative Confirmation)

All samples were also confirmed by conducting a direct streak to Chromogenic *Cronobacter* Isolation Agar (CCI) and incubating at 41.5 ± 1ºC for 24 ± 2 hours. After incubation of the CCI, all typical colonies (1 mm to 3 mm and blue to blue-green in color) were struck to Tryptic Soy Agar (TSA). The TSA plates were incubated at 37 ± 1ºC for 18-24 hours. Following incubation of the TSA, an oxidase test was conducted along with final confirmation on the Bruker MALDI following the MicroVal certified method 2017LR72.

Scope of the validation study was: Infant formula and infant cereal and environmental samples

Samples were prepared in accordance with ISO 6887: parts1, 4, and 5 and ISO 22964:2017 for infant formula (with and without probiotics), infant cereal (with and without probiotics), milk powder (without probiotics), other non-probioitc ingredients, environmental surfaces, process water and dust sweepings.

Categories included:

- Infant Formula and Infant Cereals
- Environmental Samples

Criteria evaluated during the study:

- Method Comparison Study (MCS)
  - Sensitivity study
  - Relative level of detection study
  - Inclusivity and exclusivity study

- Interlaboratory Study (ILS)
  - Specificity
  - Sensitivity
  - Relative Trueness
  - False positive ratio

Overall, the conclusions for the initial Method Comparison Study were:

The observed values for ND-PD and ND+PD for the individual categories and for all categories meet the acceptability limits (observed values ≤ AL).

The observed values for ND-PD for the individual categories and for all categories meet the acceptability limits (observed values ≤ AL).

The RLOD values (using the confirmed alternative method results) meet the acceptability limit, which is 1.5 for paired studies / 2.5 for unpaired studies, for all categories tested.

The alternative Assurance GDS® *Cronobacter* Tq II detection method is selective and specific

2 Method protocols

The Initial Method Comparison Study was carried out using 10 and 375 gram portions of sample material for food matrices. The 10 gram portions were evaluated after 20 hours of primary enrichment and the 375 gram portions were evaluated after 24 hours of primary enrichment. The environmental samples were evaluated after 18 hours of primary enrichment. For all matrices, except milk powder without probiotics, primary enrichment for the method is BPW (ISO), but certain matrices require supplementation of the media. The milk powder without probiotics utilizes a primary enrichment of brilliant green water.

For some food types, the 10 gram test portions were evaluted as paired samples as the reference and the alternative method share the initial (pre)-enrichment step. All resulting data were treated as paired data (EN-ISO 16140-2).

For all of the 375 gram portions and select 10 gram portions, there is no shared initial (pre)-enrichment step for the reference and the alternative method. All resulting data were treated as unpaired data (EN-ISO 16140-2).

2.1 Reference method

See the flow diagram in Annex A.

Sample preparations used in the reference method and the alternative method were done according to ISO 6887-series and ISO 22964:2017 for infant formula (with and without protiobitics) - 10 gram portions, infant cereal (with and without probiotics) -10 gram portions, milk powder (without probiotics) -10 gram portions, other non-probiotic ingredients – 10 gram portions, environmental surfaces – sponges/swabs, process water - 25 mL portions, and dust sweepings - 25 gram portions.

2.2 Alternative method

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

See the Assurance GDS *Cronobacter* Tq II kit insert in Annex C.

The alternative method principle is based on PCR.

The Assurance GDS *Cronobacter* Tq II is an automated nucleic acid amplification system for the detection of *Cronobacter* spp. in infant formula, infant cereals, non-probiotic ingredients and environmental samples.

The food samples were prepared for analysis following an IMS concentration procedure and analyzed in accordance with Assurance GDS *Cronobacter* Tq II package insert.

The Assurance GDS *Cronobacter* Tq II method is applicable to select food matrices. Results are available in just 22 hours for 10 gram portions and in 26 hours for 375 gram portions, including enrichment, sample preparation, and analysis. Food types include powdered infant formula, infant cereal, infant formula/cereal ingredients, as well as environmental samples. Table 1 provides an overview of the food types, test portion size, enrichment protocol and time point of analysis.

Table 1. Overview of the Method Analysis

Category	Type	Enrichment Broth[1,2]	Study Design	Analysis Time Point	Sample Size	Protocol[3]
Infant Formula and Infant Cereals	Infant formula without probiotics	BPW	Paired	20 Hours	10 g	**1**
		BPW	Unpaired	24 Hours	375 g	**2**
	Infant cereals without probiotics	BPW + a	Paired	20 Hours	10 g	**3**
		BPW + a	Unpaired	24 Hours	375 g	**4**
	milk powder without probiotics	BGW	Unpaired	20 Hours	10 g	**9**
		BGW	Unpaired	24 Hours	375 g	**10**
	Other non-probiotic ingredients	BPW	Paired	20 Hours	10 g	**1**
		BPW	Unpaired	24 Hours	375 g	**2**
	Infant formula with probiotics	BPW + v	Unpaired	20 Hours	10 g	**5**
		BPW + v	Unpaired	24 Hours	375 g	**6**
	Infant cereals with probiotics	BPW + a + v	Unpaired	20 Hours	10 g	**7**
		BPW + a + v	Unpaired	24 Hours	375 g	**8**
Environmental samples	Surfaces (swab, sponge)	BPW	Paired	18 Hours	sampling device	**11**
	Process water	BPW	Paired	18 Hours	25 ml	**11**
	Dust, sweepings	BPW	Paired	18 Hours	25 g	**11**

[1]BPW: Buffered peptone water (pre-warmed 37 ±1°C) v: Vancomycin (6 mg/L);
BGW: Brilliant Green Water (0.02 g/l); a: amylase (10 mg/l)
[2]The addition of amylase in the enrichment broth is not to be considered as a specific protocol; this means that the two protocols can be combined to have the required number of samples.
[3] See Annex B for Protocol information.

2.3 Study design

The Method Comparison Study for infant formula and infant cereals were carried out using both 10 and 375 gram portions of sample material for the alternative method. The environmental samples were analyzed using a 25 mL or g sample for the alternative method.

For some of the 10 gram portions, the reference and the alternative method share the initial (pre)-enrichment step, therefore the same test portion (Item) was used for the two methods. All resulting data were treated as **paired** data (EN-ISO 16140-2).

For some of the 10 gram and all of the 375 gram portions, there is no shared initial (pre)-enrichment step for the reference and the alternative method. Different test portions coming from the same batch of product (Item) were used for the two methods. All resulting data were treated as **unpaired** data (EN-ISO 16140-2).

3 Method comparison study

3.1 Sensitivity Study

The sensitivity study (SE) is the ability of the method selected to detect the analyte by either the reference or the alternative method.

3.1.1 Categories and sample types

A total of 2 Categories were included in this validation study.

A minimum of 60 Items for each Category were tested by both the reference method and the alternative method, with a minimum of 30 positive samples per Category required. Each Category was made up of at least 3 Types, with at least 20 Items representative for that Type.

The categories, the types and the number of samples analyzed are presented in Table 2.

Table 2. List of Categories, Types, and examples of Items to be tested within the sensitivity study.

Category	Type	Sample Size	Protocol[1]	# of Samples Analyzed
Infant Formula and Infant Cereals	Infant formula without probiotics	10 g	**1**	20
		375 g	**2**	20
	Infant cereals without probiotics	10 g	**3**	23
		375 g	**4**	23
	Non-probiotic ingredients (that contain milk powder)[2]	10 g	**9**	60
		375 g	**10**	60
	non-probiotic ingredients[2]	10 g	**1**	20
		375 g	**2**	20
	Infant formula with probiotics	10 g	**5**	32
		375 g	**6**	32
	Infant cereals with probiotics	10 g	**7**	30
		375 g	**8**	30
Environmental samples	Surfaces (swab, sponge)	sampling device	**11**	22
	Process water	25 ml	**11**	21
	Dust, sweepings	25 g	**11**	21

[1]See Annex B for Protocol information.

[2] Non-probiotic ingredients were evaluted using 4 different protocols, based on samples size and if the product contains milk powder. See Annex B for details on protocols.

A total of 434 samples were analyzed. The distribution of positive and negative samples per tested category and type is given respectively in Table 3.

Table 3 - Distribution per tested category and type

Category	Type	Sample Size	Positive Samples[1]	Negative Samples	# of Samples Analyzed
Infant Formula and Infant Cereals	Infant formula without probiotics	10 g	8	12	20
	Infant cereals without probiotics	10 g	13	10	23
	Non-probiotic ingredients (that contain milk powder)[2]	10 g	34	26	60
	non-probiotic ingredients[2]	10 g	12	8	20
	Infant formula with probiotics	10 g	17	15	32

Category	Type	Sample Size	Positive Samples[1]	Negative Samples	# of Samples Analyzed
	Infant cereals with probiotics	10 g	11	19	30
	Total		**95**	**90**	**185**
	Infant formula without probiotics	375 g	9	11	20
	Infant cereals without probiotics	375 g	13	10	23
	Non-probiotic ingredients (that contain milk powder)[2]	375 g	34	26	60
	non-probiotic ingredients[2]	375 g	14	6	20
	Infant formula with probiotics	375 g	17	15	32
	Infant cereals with probiotics	375 g	13	17	30
	Total		**100**	**85**	**185**
Environmental samples	Surfaces (swab, sponge)	sampling device	12	10	22
	Process water	25 ml	11	10	21
	Dust, sweepings	25 g	8	13	21
	Total		**31**	**33**	**64**
All Categories			**226**	**208**	**434**

[1]Postive by either the alternative or the reference method.

3.1.2 Test sample preparation

A total of 13% of the samples evaluated were naturally contaminated with the target analyte.

Artificial contaminations were done by seeding protocols.

For infant cereal, infant formula, other ingredient test products and dust sweepings, a lyophilized culture was used. When inoculating the food types, the strains were stressed using two injury protocols. The level of injury was evaluated by plating on a non-selective agar (TSA) and a selective agar (CCI*)*. As proposed, a ≥ 0.5 log injured cells was obtained. The artificial contaminations are presented in Annex D.

For environmental sponges and process water, inoculation followed protocols as outlined in AOAC Appendix J, as recommended by ISO 16140-2.

For environmental surfaces, inoculation was perforemd by spiking overnight cultures of *Cronobacter* onto a surface and then stored under relevant conditions for 24 hours.

The *Cronobacter* strains used for artificial inoculation originated from comparable sample types as the ones that were inoculated. Strains used to inoculate items were used on a maximum of 5 different items.

In total, 191 samples were artificially contaminated by seeding, using 39 different strains. Each item seeded resulted in a positive result. Most of the spiking inoculations, after injury protocols on the inoculum, were lower or equal to 5 CFU/sample.

Regardless of presumptive result, all test portions analyzed by the alternative method were confirmed following ISO 22964 reference method, beginning with a transfer to CSB secondary enrichment broth. In addition, a direct streak was performed to CCI agar from the primary enrichment. Typical colonies were confirmed according to ISO 22964. See Annex A for confirmation steps of the ISO 22964 reference method.

In order to improve the practicability of the method to user labs, enrichment broths for the alternative method were stored for 72 hours at 5 ± 3 ºC. All presumptive positive and discrepant results were reanalyzed with the Assurance GDS *Cronobacter* Tq II after the 72 hour hold time. All samples were reconfirmed at 72 hours following the ISO 22964 reference method, and the alternative confirmation.

3.1.3 Confirmation protocols

ISO 22964:2017/Traditional Confirmation

For the powdered infant formula (with and without probiotics), infant cereal with probiotics and milk powder test portions, a 10 g sample was enriched with 90 mL of Buffered Peptone Water (BPW) ISO formulation. For infant cereal without probiotics, a 10 g sample was enriched with BPW ISO + amylase. For process water, a 25 mL test portion was enriched with 225 mL of BPW ISO. The stainless steel environmental sponges were enriched with 100 mL of BPW ISO. All test portions were allowed to stand at room temperature (20-25 °C) for 60 ± 10 minutes. Subsequently, the infant formula enrichments were incubated at 34 to 38°C for 18 ± 2 hours.

Following incubation, 0.1 mL of primary enrichment was transferred into 10 mL of *Cronobacter* Screening Broth (CSB) with 0.1mL of vancomycin solution (0.1% v/v). CSB tubes were incubated at 41.5 ± 1ºC for 24 ± 2 hours. Following incubation, a loopful of the secondary enrichments were streaked to Chromogenic *Cronobacter* Isolation agar (CCI) and incubated at 41.5 ± 1ºC for 24 ± 2 hours. One suspected colony from each CCI plate was transferred to Tryptone Soya Agar (TSA) and incubated at 37 ± 7°C for 18 to 24 hours. Typical morphology for CCI is a small to medium sized (1mm to 3mm) blue-green to blue colony. Atypical morphology on CCI is often white or white with a green center, grey or black. After incubation, typical isolates were purified on TSA and identified using the Bruker MALDI following the MicroVal certified method 2017LR72.

Direct Streak Confirmation (Alternative Confirmation)

All samples were also confirmed by conducting a direct streak to Chromogenic *Cronobacter* Isolation Agar (CCI) and incubating at 41.5 ± 1ºC for 24 ± 2 hours. After incubation of the CCI, all typical colonies (1 mm to 3 mm and blue to blue-green in color) were struck to Tryptic Soy Agar (TSA). The TSA plates were incubated at 37 ± 1ºC for 18-24 hours. Following incubation of the TSA, an oxidase test was conducted along with final confirmation on the Bruker MALDI following the MicroVal certified method 2017LR72.

3.1.4 Sensitivity study results

All raw data on the sensitivity study are presented in Annex E. Sample numbers in **bold** indicate artificial inoculation of the sample (see Annex D for details on artificial inoculation).

Table 4 shows the summary of results of the reference method and the alternative methods for all Categories.

Table 5 shows the Interpretation of sample results between the reference and alternative method (based on the confirmed alternative method).

Table 4 - Summary of sensitivity study results – *all categories*

Result	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement (R+/A+) **PA = 219**	Positive deviation (R-/A+) **PD = 7**
Alternative method negative (A-)	Negative deviation (R+/A-). **ND = 0**	Negative agreement (R-/A-) **NA = 208**

Table 5 – Interpretation of sample results between the reference and alternative method (based on the confirmed alternative method)

Category	Type	Sample Size	PA	NA[1]	PD	ND[2]	PPNA[3]	PPND[3]	Total
Infant Formula and Infant Cereals	Infant formula without probiotics	10 g	8	12	0	0	0	0	20
	Infant cereals without probiotics	10 g	13	10	0	0	0	0	23
	Non-probiotic ingredients (that contain milk powder)	10 g	33	26	1	0	0	0	60
	non-probiotic ingredients	10 g	12	8	0	0	0	0	20
	Infant formula with probiotics	10 g	17	15	0	0	0	0	32
	Infant cereals with probiotics	10 g	11	19	0	0	0	0	30
	Total		**94**	**90**	**1**	**0**	**0**	**0**	**185**
	Infant formula without probiotics	375 g	8	11	1	0	0	0	20
	Infant cereals without probiotics	375 g	13	10	0	0	0	0	23
	Non-probiotic ingredients (that contain milk powder)	375 g	33	26	1	0	0	0	60
	non-probiotic ingredients	375 g	12	6	2	0	0	0	20

Category	Type	Sample Size	PA	NA[1]	PD	ND[2]	PPNA[3]	PPND[3]	Total
	Infant formula with probiotics	375 g	17	15	0	0	0	0	32
	Infant cereals with probiotics	375 g	11	17	2	0	0	0	30
	Total		**94**	**85**	**6**	**0**	**0**	**0**	**185**
Environmental samples	Surfaces (swab, sponge)	sampling device	12	10	0	0	0	0	22
	Process water	25 ml	11	10	0	0	0	0	21
	Dust, sweepings	25 g	8	13	0	0	0	0	21
	Total		**31**	**33**	**0**	**0**	**0**	**0**	**63**
All Categories			**219**	**208**	**7**	**0**	**0**	**0**	**434**

*The direct streak and the ISO reference method confirmation procedures produced the same result for all samples.

[1]NA: Including PPNA, [2]ND: Including PPND, [3]FP = PPNA + PPND

3.1.5 Sensitivity study calculations

The sensitivity study parameters as specified in Table 6 were calculated for all Categories and Types, and the overview is given in Table 7.

Table 6 – Formula to calculate the sensitivity parameters

Sensitivity for the alternative method	$SE_{alt} = \frac{(PA+PD)}{(PA+ND+PD)} \times 100\%$
Sensitivity for the reference method	$SE_{ref} = \frac{(PA+ND)}{(PA+ND+PD)} \times 100\%$
Relative trueness	$RT = \frac{(PA+NA)}{N} \times 100\%$
False positive ratio for the alternative method	$FPR = \frac{(FP)}{NA} \times 100\%$

Table 7 - Overview calculated sensitivity parameters per Category and Type

Category	Type	Sample Size	PA	NA[1]	PD	ND[2]	FP[3]	SE alt %	SE ref %	RT %	FPR %
Infant Formula and Infant Cereals	Infant formula without probiotics	10 g	8	12	0	0	0	100.0	100.0	100.0	0.0
	Infant cereals without probiotics	10 g	13	10	0	0	0	100.0	100.0	100.0	0.0
	Non-probiotic ingredients (that contain milk powder)	10 g	33	26	1	0	0	100.0	97.1	98.3	0.0
	non-probiotic ingredients	10 g	12	8	0	0	0	100.0	100.0	100.0	0.0
	Infant formula with probiotics	10 g	17	15	0	0	0	100.0	100.0	100.0	0.0

	Infant cereals with probiotics	10 g	11	19	0	0	0	100.0	100.0	100.0	0.0
	Total		**94**	**90**	**1**	**0**	**0**	**100.0**	**98.9**	**99.5**	**0.0**
	Infant formula without probiotics	375 g	8	11	1	0	0	100.0	88.9	95.0	0.0
	Infant cereals without probiotics	375 g	13	10	0	0	0	100.0	100.0	100.0	0.0
	Non-probiotic ingredients (that contain milk powder)	375 g	33	26	1	0	0	100.0	97.1	98.3	0.0
	non-probiotic ingredients	375 g	12	6	2	0	0	100.0	85.7	90.0	0.0
	Infant formula with probiotics	375 g	17	15	0	0	0	100.0	100.0	100.0	0.0
	Infant cereals with probiotics	375 g	11	17	2	0	0	100.0	84.6	93.3	0.0
	Total		**94**	**85**	**6**	**0**	**0**	**100.0**	**94.0**	**96.8**	**0.0**
Environmental samples	Surfaces (swab, sponge)	sampling device	12	10	0	0	0	100.0	100.0	100.0	0.0
	Process water	25 ml	11	10	0	0	0	100.0	100.0	100.0	0.0
	Dust, sweepings	25 g	8	13	0	0	0	100.0	100.0	100.0	0.0
	Total		**31**	**33**	**0**	**0**	**0**	**100.0**	**100.0**	**100.0**	**0.0**
All Categories			**219**	**208**	**7**	**0**	**0**	**100.0**	**96.9**	**98.4**	**0.0**

*The direct streak and the ISO reference method confirmation procedures produced the same result for all samples.

[1]NA: Including PPNA, [2]ND: Including PPND, [3]FP = PPNA + PPND

3.1.6 Discordant results

The positive deviations that were observed during the sensitivity evaluation are listed below in Table 8.

Table 8 – Discordant Results

Category	Type	Sample Size	Sample nº	Alternative method results	(additional) Confirmatory test results	Inoculating Organism (CFU/Sample)
Infant Formula and Infant Cereals	Non-probiotic ingredients (that contain milk powder)	10g	46	+	+	Natural Contamination
	Infant formula without probiotics	375g	9	+	+	*Cronobacter sakazakii* CCUG 28860 (3.4)
	Non-probiotic ingredients	375g	46	+	+	*Cronobacter sakazakii* CCUG 10788 (2.1)

	(that contain milk powder)					
	Non-probiotic ingredients	375g	11	+	+	Natural Contamination
	Non-probiotic ingredients	375g	13	+	+	*Cronobacter sakazakii* FSL F6-027 (4.2)
	Infant cereals with probiotics	375g	3	+	+	Natural Contamination
	Infant cereals with probiotics	375g	26	+	+	*Cronobacter sakazakii* CCUG 14558 (2.2)

Seven positive deviations were observed. Three of the deviations were associated with naturally contaminated samples, and four of the deviations were observed with artificial contaminated samples. Each deviation associated with an artificially contaminated sample was observed with a different strain.

Six (6) of the 7 positive deviations were associated with 375 g test portions, indicating that the larger volume of sample analysed and the extended incubation temperature may have contributed to the detection of low level positives in the test product.

The analysis of discordant results according to ISO 16140-2:2016 for a paired and unpaired study is given in Tables 9 and 10.

Table 9 - Interpretation of the sensitivity study results (paired study)

Category	Food type	Negative Deviations (ND^1)	Positive deviations (PD)	ND^1-PD	Acceptability Limit (AL)	ND^1+PD	Acceptability Limit (AL)
Infant Formula and Infant Cereals	Infant formula without probiotics (10 g)	0	0				
Total		0	0	0	3	0	6
Infant Formula and Infant Cereals	Infant cereals without probiotics (10g)	0	0				
Total		0	0	0	3	0	6
Environmental Samples	Surfaces (swab, sponge)	0	0				

	Process water	0	0				
	Dust, sweepings	0	0				
Total		0	0	0	3	0	6
All Categories		0	0	0	4	0	8

[1] ND: including PPND

Table 10– Interpretation of the sensitivity study results (unpaired study)

Category	Food Type	Negative Deviations (ND[1])	Positive Deviations (PD)	ND[1]-PD	Acceptability Limit (AL)
Infant Formula and Infant Cereals Infant Formula and Infant Cereals	Non-probiotic ingredients (that contain milk powder) (10g)	0	1		
Total:		0	1	-1	3
Infant Formula and Infant Cereals Infant Formula and Infant Cereals	non-probiotic ingredients (10g)	0	0		
Total		0	0	0	3
Infant Formula and Infant Cereals Infant Formula and Infant Cereals	Infant formula with probiotics (10g)	0	0		
Total		0	0	0	3
Infant Formula and Infant Cereals Infant Formula and Infant Cereals	Infant cereals with probiotics (10g)	0	0		
Total		0	0	0	3
Infant Formula and Infant Cereals Infant Formula and Infant Cereals	Infant formula without probiotics (375g)	0	1		3
	non-probiotic ingredients (375g)	0	2		3
Total		0	3	-3	3
Infant Formula and Infant Cereals Infant Formula and Infant Cereals	Infant cereals without probiotics (375g)	0	0		
Total		0	0	0	3

Infant Formula and Infant Cereals Infant Formula and Infant Cereals	Non-probiotic ingredients (that contain milk powder) (375g)	0	1		
Total		0	1	-1	3
Infant Formula and Infant Cereals Infant Formula and Infant Cereals	Infant formula with probiotics (375g)	0	0		
Total		0	0	0	3
Infant Formula and Infant Cereals Infant Formula and Infant Cereals	Infant cereals with probiotics (375g)	0	2		
Total		0	2	-2	3
All categories		0	7	-7	3

[1] ND: including PPND

3.1.7 Conclusion sensitivity study

Paired Testing: The observed values for ND-PD and ND+PD for the individual categories and for all categories meet the acceptability limits (observed values ≤ AL).

Unpaired Testing: The observed values for ND-PD for the individual categories and for all categories meet the acceptability limits (observed values ≤ AL).

3.1.8 Enrichment broth storage

Enrichment broth BPW (ISO) storage was done at 2-8 °C for 72 hrs.

All positive (219) samples were tested again after enrichment broth storage. Zero (0) changes were observed.

Table 11 - Interpretation of the sensitivity study results after storage of the enrichment broth (paired study)

Category	**Food type**	**Negative Deviations (ND[1])**	**Positive deviations (PD)**	**ND[1]-PD**	**Acceptability Limit (AL)**	**ND[1]+PD**	**Acceptability Limit (AL)**
Infant Formula and Infant Cereals	Infant formula without probiotics (10 g)	0	0				
Total		0	0	0	3	0	6
Infant Formula and	Infant cereals	0	0				

Infant Cereals	without probiotics (10g)						
Total		0	0	0	3	0	6
Environmental Samples	Surfaces (swab, sponge)	0	0				
	Process water	0	0				
	Dust, sweepings	0	0				
Total		0	0	0	3	0	6
All Categories		0	0	0	4	0	8

[1] ND: including PPND

Table 12 – Interpretation of the sensitivity study results after storage of the enrichment broth (unpaired study)

Category	Food Type	Negative Deviations (ND[1])	Positive Deviations (PD)	ND[1]-PD	Acceptability Limit (AL)
Infant Formula and Infant Cereals Infant Formula and Infant Cereals	Non-probiotic ingredients (that contain milk powder) (10g)	0	1		
Total:		0	1	-1	3
Infant Formula and Infant Cereals Infant Formula and Infant Cereals	non-probiotic ingredients (10g)	0	0		
Total		0	0	0	3
Infant Formula and Infant Cereals Infant Formula and Infant Cereals	Infant formula with probiotics (10g)	0	0		
Total		0	0	0	3

Infant Formula and Infant Cereals Infant Formula and Infant Cereals	Infant cereals with probiotics (10g)	0	0		
Total		0	0	0	3
Infant Formula and Infant Cereals Infant Formula and Infant Cereals	Infant formula without probiotics (375g)	0	1		3
	non-probiotic ingredients (375g)	0	2		3
Total		0	3	-3	3
Infant Formula and Infant Cereals Infant Formula and Infant Cereals	Infant cereals without probiotics (375g)	0	0		
Total		0	0	0	3
Infant Formula and Infant Cereals Infant Formula and Infant Cereals	Non-probiotic ingredients (that contain milk powder) (375g)	0	1		
Total		0	1	-1	3
Infant Formula and Infant Cereals Infant Formula and Infant Cereals	Infant formula with probiotics (375g)	0	0		
Total		0	0	0	3
Infant Formula and Infant Cereals Infant Formula and Infant Cereals	Infant cereals with probiotics (375g)	0	2		
Total		0	2	-2	3
All categories		0	7	-7	3

[1] ND: including PPND

Conclusion for the enrichment broth storage (Paired Testing):

The observed values for ND-PD and ND+PD for the individual categories and for all categories meet the acceptability limits (observed values ≤ AL).

Conclusion for the enrichment broth storage (Unpaired Testing):

The observed values for ND-PD for the individual categories and for all categories meet the acceptability limits (observed values ≤ AL).

3.2 Relative level of detection study

The relative level of detection is the level of detection at P = 0.50 (LOD_{50}) of the alternative method divided by the level of detection at P = 0.50 (LOD_{50}) of the reference method.

3.2.1 Categories, sample types and strains

One sample type and one relevant target micro-organism for this sample type was chosen for each of the Categories in this validation study, as shown in Table 13.

Table 13 - List of selected types and strains per category, as tested within the relative level of detection study.

Category	Type	Sample size	Study design	Analysis Time Points	Inoculated strain	Strain origin	Storage conditions before analysis
Infant Formula and Infant Cereals	Infant formula without probiotics	10 g	Paired	20 Hours	*Cronobacter sakazakii* QL[1] 11007-9	Rice Flour	Lyophilized strain 2 weeks at room temperature
		375 g	Unpaired	24 Hours			
	Milk powder without probiotics	10 g	Unpaired	20 Hours	*Cronobacter dublinensis* QL 17031-2	Infant Formula	Lyophilized strain 2 weeks at room temperature
		375 g	Unpaired	24 Hours			
	Infant cereals with probiotics	10 g	Unpaired	20 Hours	*Cronobacter sakazakii* QL 123015-2	Rice Flour	Lyophilized strain 2 weeks at room temperature
		375 g	Unpaired	24 Hours			
Environmental samples	Process water	25 mL	Paired	18 Hours	*Cronobacter muytjensii* QL 17031-6	Environmental Isolate	Seeding 48 h at 5 ± 3°C

[1]QL-Q Laboratories, Inc Culture Collection

3.2.2 Test sample preparations

Three levels of artificial contamination were prepared for each type:

- Negative control level: One unnoculated in order to get 5 test portions,
- Low level: One inoculated between 0.2 and 2.0 CFU/sample in order to get 20 test portions providing fractional recovery (5-15 positive results out of 20),
- High level: One inoculated between 2.0 and 5.0 CFU/sample in order to get 5 test portions contaminated at a higher level.

A bulk lot of the matrix was inoculated at each level, homogenized and stored as described in Table 14.

3.2.3 RLOD study results

The tabulated raw data on the RLOD study are given in Annex G.

The RLOD calculations were performed using the Excel spread sheet (version 06-07-2015) of the international standard as described in ISO 16140-2: 2016.

The RLOD per Category is given in Table 14.

Table 14 – Presentation of RLOD before and after confirmation of the alternative method results

Type (Category)	RLOD using the alternative method results	RLOD using the confirmed direct streak results	RLOD using the confirmed alternative method results
Infant formula without probiotics: 10g (Infant Formula and Infant Cereals) **Paired Analysis**	1.000	1.000	1.000
Infant formula without probiotics: 375g (Infant Formula and Infant Cereals) **Unpaired Analysis**	0.698	0.698	0.698
Milk Powder without probiotics: 10g (Infant Formula and Infant Cereals) **Unpaired Analysis**	0.843	0.843	0.843
Milk Powder without probiotics: 375g (Infant Formula and Infant Cereals) **Unpaired Analysis**	0.721	0.721	0.721
Infant cereal with probiotics:10g (Infant Formula and Infant Cereals) **Unpaired Analysis**	0.807	0.807	0.807
Infant cereal with probiotics:375g (Infant Formula and Infant Cereals) **Unpaired Analysis**	1.000	1.000	1.000
Process Water (Environmental Surfaces) **Paired Analysis**	1.000	1.000	1.000

Combined	0.858	0.858	0.858

3.2.4 Conclusion RLOD study

The RLOD values (using the confirmed alternative method results) meet the acceptability limit, 1.5 for paired test portions and 2.5 for unpaired test portions for all categories tested.

3.3 Inclusivity/exclusivity study

Inclusivity is the ability of the alternative method to detect the target analyte from a wide range of strains.

Exclusivity is the lack of interference from a relevant range of non-target strains of the alternative method.

3.3.1 Protocols

Inclusivity: Fifty (50) *Cronobacter* strains were freshly cultured in BPW (ISO) medium at 37 ± 1°C. Dilutions were made in order to inoculate 10 -100 CFU/ 225ml prewarmed BGW + milk powder. Each sample was incubated for 20 h at 37 ± 1°C. The alternative method protocol was then performed. Isolates were plated onto CCI to confirm the presence of *Cronobacter* species.

Exclusivity: Thirty (30) strains were freshly cultured in BHI medium at 37 ± 1°C. Dilutions were made in order to inoculate about 10^5 CFU/ 225ml BPW (ISO) broth. The BPW (ISO) broth was incubated for 20 h at 37 ± 1°C. The alternative method was then performed (no sample material was added). Isolates were plated onto CCI to confirm the absence of *Cronobacter* species.

3.3.2 Results inclusivity and exclusivity study

All raw data on inclusivity and exclusivity are given in Annex F.

A total of 50 strains were tested for **inclusivity**. 50 of these strains showed the expected positive result. 0 strains showed a negative result.

A total of 30 strains were tested for **exclusivity**. 30 of these strains showed the expected negative result. 0 strains showed a positive result.

3.3.3 Conclusion inclusivity and exclusivity study

The alternative Assurance GDS® *Cronobacter* Tq II detection method is both selective and specific for the detection of *Cronobacter* species.

4 Conclusions Method Comparison Study

Overall, the conclusions for the Method Comparison Study are:

The observed values for ND-PD and ND+PD for the individual categories and for all categories meet the acceptability limits (observed values ≤ AL).

The observed values for ND-PD for the individual categories and for all categories meet the acceptability limits (observed values ≤ AL).

The RLOD values (using the confirmed alternative method results) meet the acceptability limit, which is 1.5 for paired studies / 2.5 for unpaired studies, for all categories tested.

The alternative Assurance GDS® *Cronobacter* Tq II detection method is selective and specific.

5 Interlaboratory Study

5.1 General Overview

In this collaborative study, one food type, powdered infant formula (milk based with iron and DHA) containing probiotics (*Lactobacillus reuteri*) was evaluated. The matrix was obtained from a local retailer and screened negative for the presence of *Cronobacter* by the ISO 22964:2017 reference method. The matrix was artificially contaminated with a lyophilized culture of *Cronobacter sakazakii* Q Laboratories (QL) isolate 17031.4 (origin – powdered infant formula) at two inoculation levels: a high inoculation level of approximately 2-5 colony-forming units (CFU)/test portion and a low inoculation level of approximately 0.2-2 CFU/test portion. A set of uninoculated control test portions (0 CFU/test portion) were also included.

The Assurance GDS *Cronobacter* Tq and ISO 22964:2017 share a different pre-enrichment for this food type, therefore the study was designed using unpaired samples. A total of 72 samples were evaluated per collaborator, 36 for the reference method and 36 for the alternative method. Within each sample set were 12 replicate test portions from each of the three inoculation levels. A total of 11 collaborators from 8 locations particpated. Collaborators were also sent a test portion for determining the total background microflora of the probiotic by running a Lactic Acid Bacteria (LAB) test as outlined in the Compendium for the Microbiological Examination of Food Products reference method on the day samples were received. Table 15 provides the results of the LAB analysis for each of the collaborators.

Table 15: Results of the Background Microflora Evaluation

Collaborator	LAB Results (CFU/g)	Collaborator	LAB Results (CFU/g)
1	9.5×10^5	7	4.6×10^6
2	1.2×10^6	8	5.1×10^6
3	2.9×10^6	9	1.8×10^6
4	2.4×10^6	10	8.2×10^6
5	6.5×10^5	11	4.9×10^6
6	9.8×10^6		

5.1.1 Preparation of Inocula and Test Portions

The *Cronobacter sakazakii* isolate used in this evaluation was lyophilized prior to inoculation. The culture was propagated onto Tryptic Soy Agar with 5% Sheep Blood (SBA) from a Q Laboratories frozen stock culture stored at -70°C. To prepare the culture for lyophilization, a single, well isolated colony from SBA was transferred into brain heart infusion (BHI) broth and incubated at 37 ± 2 °C for 18-24 hours. The culture was diluted in a sterile cryoprotectant, reconstituted 10% non-fat dry milk (NFDM), and freeze dried for 48-72 hours. A bulk lot of the test matrix was inoculated with the culture at a high level expected to yield all positive results. An aliquot of the high-level inoculum was further mixed with uninoculated powdered infant formula to produce the low-level inoculum. After inoculation,

the matrix was held for a minimum of 2 weeks at ambient temperature (20 - 25ºC). The inoculated test product was packaged into separate 10 g samples in sterile Whirl-Pak® bags and shipped to the collaborators.

5.1.2 Test Portion Distribution

All samples were labeled with a randomized, blind-coded 3-digit number affixed to the sample container. Test portions were shipped on a Wednesday via overnight delivery according to the Category B Dangerous Goods shipment regulations set forth by the International Air Transport Association (IATA). Upon receipt, samples were held by the collaborating laboratory at ambient temperature (20-25°C) until the following Monday when analysis was initiated.

In addition to each of the test portions and LAB sample, collaborators received a data logger. Data loggers were programmed to monitor the temperature of the shipment to ensure it did not fall outside the range of 18-25°C. Participants were instructed to send the data file from the logger to the Expert Lab upon receipt of the samples. Results from the temperature probes from each collaborator are presented in Table 16.

Table 16: Temperature Records for Collaborator Shipments

Collaborator	Temperature Record (ºC)	Collaborator	Temperature Record (ºC)
1	24.3	7	20.2
2	20.8	8	23.3
3	22.5	9	21.9
4	23.6	10	21.4
5	24.8	11	20.8
6	22.7		

5.1.3 Stability of Test Portions

Prior to shipping test portions, the stability of the inoculum in the matrix had been verified by the Expert Lab by confirming the viability of ten replicates of a bulk lot of the inoculated food type at 14, 21 and 28 days.

5.1.4 Inoculum Levels

To determine the level of *Cronobacter* in the food type, a 5-tube most probable number (MPN) was conducted by the coordinating laboratory on the day of the initiation of analysis using the ISO 22964 reference method. The MPN for each contamination level was determined by analyzing 5 x 20 g test portions, the reference method test portions from the collaborating laboratories (132 x 10 g) and 5 x 5 g test portions. The MPN and 95% confidence intervals were calculated using the LCF MPN Calculator, Version 1.6, (http://www.lcfltd.com/customer/LCFMPNCalculator.exe), provided by AOAC Research Institute (RI). Table 17 presents the inoculum results for the ILS evaluation.

Table 17: MPN Summary Table for ILS Study

Infant Formula with Probiotics (10 g) *Cronobacter sakazakii* QL 17031.4					
Low Level Inoculum (0.2-2 MPN/Test Portion)					
	A	**B**	**C**	**D**	**E**
5 x 20 g	**+**	**-**	**+**	**+**	**-**
132 x 10 g (Reference Samples)	**46/132**				
5 x 5 g	**-**	**-**	**+**	**+**	**-**
MPN/Test portion	**0.43**				
Low Conf. Limit MPN/Test Portion	**0.32**				
High Conf. Limit MPN/Test Portion	**0.56**				
High Level Inoculum (2-10 MPN/Test Portion)					
	A	**B**	**C**	**D**	**E**
5 x 20 g	**+**	**+**	**+**	**+**	**+**
132 x 10 g (Reference Samples)	**132/132**				
5 x 5 g	**+**	**+**	**-**	**+**	**-**
MPN/Test portion	**5.03**				
Low Conf. Limit MPN/Test Portion	**3.37**				
High Conf. Limit MPN/Test Portion	**7.51**				

5.1.5 Results

Table 18 summarizes the collaborative study results for participant.each matrix. For each level (L0, L1, L2) 12 unpaired test portions were evaluted by each collaborator. Results for the raw data can be found in Appendix J. The average LAB result obtained by the collaborators was 4.4 x 10^6 CFU/g with low and high data points of 9.5 x 10^5 CFU/g and 9.8 x 10^6 CFU/g.

Table 18: Summary of ILS Results

Collaborator	Alternative Method Presumptive Result			Alternative Method Confirmed Result			Reference Method		
	L0	L1	L2	L0	L1	L2	L0	L1	L2
1	0	4	12	0	4	12	0	3	12
2	0	5	12	0	5	12	0	3	12
3	0	4	12	0	4	12	0	3	12
4	0	5	12	0	5	12	0	6	12
5	0	5	12	0	5	12	0	5	12
6	0	6	12	0	6	12	0	4	12
7	1	6	12	0	4	12	0	5	12
8	0	7	12	0	5	12	0	6	12
9	0	3	12	0	3	12	0	4	12
10	0	4	12	0	4	12	0	3	12
11	0	6	12	0	6	12	0	4	12
Total	1	55	132	0	51	132	0	46	132

The Expert Lab analyzed a set of test portions on the day of the ILS shipment and a second set on the day of testing initiation. Table 19 provides the results from the quality control testing performed by the Expert Lab.

Table 19: Summary of Expert Laboratory Results

Collaborator	Alternative Method Presumptive Result			Alternative Method Confirmed Result			Reference Method		
	L0	L1	L2	L0	L1	L2	L0	L1	L2
Day of Shipment	0	6	12	0	6	12	0	5	12
Day of ILS Analysis	0	5	12	0	5	12	0	5	12

5.1.6 *Calculation of Specificity Percentage*

The specificity of the reference and alternative method are calculated using level L0. These values are obtained from the following equations, where N_ is the total number of tests at L0, and P_0 and CP_0 are the number of false positive results with the uninoculated controls.

$$SP_{ref} = \left(1 - \left(\frac{P_0}{N_-}\right)\right) x\ 100\ \% = \qquad SP_{alt} = \left(1 - \left(\frac{CP_0}{N_-}\right)\right) x\ 100\ \% =$$

A 100.0% specificity was obtained for the reference method and 99.2% specificity for the alternative method.

5.1.7 *Calculation of Sensitivity (SEalt, SEref), relative trueness (RT) and false positive ration for the alternative method (FPR)*

From the data obtained in the ILS, the following parameters outlined in Table 20 were determined:

Table 20: Criteria for evaluating all collaborators for an unpaired study

Results of the (reference or alternative) method per sample			
Reference Method	Alaternative Method Result	Confirmed Alternative Method	Interpretation
+	+	+	Positive Agreement (PA)
+	+	-	Negative Deviation due to false positive of the alternative method result (ND)
-	-	-	Negative Agreement (NA)
-	-	+	Negative Agreemetn due to false negative alternative method result (NA)
+	-	-	Negative Deviation (ND)
+	-	+	Negative Deviation due to false negative alternative method result (ND)
-	+	+	Positive Deviation

-	+	-	Negative Agreemetn due to false postive alternative method result

Table 21: Summary of agreements and deviations for the ILS for L1

	Reference Method Positive (R+)	**Reference Method Negative (R-)**
Alternative Method Positive (A+)	Positive Agreement (+/+) 51	Positive Deviation (-/+) 4
Alternative Method Negative (A-)	Negative Deviation (+/-) 0	Negative Agreement (-/-) 77

Using the values in Table 21, the sensitivity of the reference and alternative methods were determined, along with relative trueness and false positive rate. Calculations for

each statistic is provided below. Additionally, the requirement of fractional positive results was obtained for both the alternative and reference method. A summary of results in presented in Table 22.

$$SE_{alt} = \frac{(PA+PD)}{(PA+PD+ND)} \; x \; 100\% =$$

Figure 1: Calculation for the Sensitivity of the Alternative Method

$$SE_{ref} = \frac{(PA+ND)}{(PA+PD+ND)} \; x \; 100\% =$$

Figure 2: Calculation for the Sensitivity of the Reference Method

$$RT = \frac{(PA+NA)}{N} \; x \; 100\% =$$

Figure 3: Calculation for the Relative Trueness

$$FPR = \frac{FP}{NA} \; x \; 100\% =$$

Figure 4: Calculation for the False Positive Rate of the Alternative Method

Table 22: Summary of Statistical Analysis of the ILS Study

Sensitivity for the Alternative Method	**100 %**
Sensitivity for the Reference Method	**92.7%**

Relative Trueness	**97.0%**
False Positive Ratio for the Alternative Method	**3.0%**

5.1.8 Interpretation of data

For an unpaired study, the difference between (ND – PD) at L1 shall not be higher than the AL. The AL is defined as [(ND-PD)max] and is calculated using the following equations and the sum of (ND+PD) are calculated for the level where fractional recovery was obtained (L_1). The values found for (ND – PD) and (ND + PD) shall not be higher than the acceptability limits (AL).

$$(p+)_{\text{ref}} = \frac{P_x}{N_x}$$

Where

P_X is the number of samples with a positive result obtained with the reference method at the fractional level for all collaborators

N_X is the number of samples tested at the fractional level with the reference method by all collaborators

$$(p+)_{\text{alt}} = \frac{CP_x}{N_x}$$

Where

CP_X is the number of samples with a confirmed positive result obtained with the alternative method at the fractional level for all collaborators

N_X is the number of samples tested at the fractional level with the alternative method by all collaborators

$$(\text{ND-PD})_{\text{max}} = \sqrt{3N_x \times \left((p+)_{\text{ref}} + (p+)_{\text{alt}} - 2\left((p+)_{\text{ref}} \times (p+)_{\text{alt}}\right)\right)}$$

Where

N_X is the number of samples tested at the fractional level with the reference method by all collaborators

The results of the alternative method when compared to the AL are presented in Table 23.

Table 23: Evaluation of the AL for the alternative method

(ND – PD)	-4
$(ND\text{-}PD)_{max}$	13.6

The value obtained during the ILS is lower than the AL as determined by $(ND\text{-}PD)_{max}$ indicated the alternative method.

5.1.9 Evaluation of the RLOD between Laboratories

The RLOD for the ILS was calculated using the available Excel spread sheet (http://standards.iso.org/iso/16140) as listed in ISO 1614-2:2016. These results are provided for informational purposes only. See Table 24 for results of the RLOD.

Table 24: RLOD of the ILS

RLOD	RLODL	RLODU	b-ln(RLOD)	sd(b)	z-Test statistic	p-value
0.877	0.582	1.322	-0.1322	0.205	0.638	1.476

5.2 Conclusion of the Interlaboratory study

The data obtained during the ILS meets the criteria set forth in ISO 16140-2:2016 and indicates that the alternative method, the Assurance GDS for *Cronobacter* tq is considered equivalent to the ISO 22964:2017 reference method.

6 Current extension study

The extension study carried out by Campden BRI involved the evaluation of a new confirmation method in combination with 2 different isolation methods.

This study followed the MicroVal interpretation guidelines for ISO 16140-Part 6 (2019) issued in 2020 for an extension study for a new confirmation method from a previously validated qualitative method. This extension configuration was previously validated in the Assurance® GDS for *E. coli* O157:H7 Tq (2020), where an inclusivity and exclusivity study and a limited number of sensitivity samples were used to assess the performance of the new confirmation method. In the extension study, samples were analysed by the alternative method only to verify the performance of the new colony isolation and colony confirmation procedures.

6.1 Introduction

6.1.1 Alternative method

GDS *Cronobacter* is an automated nucleic acid amplification system for the detection of *Cronobacter* in infant nutritional formula, ingredients, and environmental samples. This PCR-based method targets DNA specific to *Cronobacter* spp. in a procedure spilt into 3 stages:

1) Sample enrichment of a 1:10 dilution in Buffered Peptone Water (BPW), or appropriate diluent, prewarmed to 36°C. Details of the media and incubation times used for the different sample types included in the study are listed below.

Food Type	Media	Sample size	Enrichment Time
Infant Formula without Probiotics	BPW	10 – 25 g	20 – 28 h
		26 – 375 g	24 – 32 h
Infant Cereals without Probiotics	BPW+a	10 – 25 g	20 – 28 h
		26 – 375 g	24 – 32 h
Non-probiotic Ingredients (except dry milk)	BPW	10 – 25 g	20 – 28 h
		26 – 375 g	24 – 32 h
Infant Formula with Probiotics	BPW+v	10 – 25 g	20 – 28 h
		26 – 375 g	24 – 32 h
Infant Cereals with Probiotics	BPW+v+a	10 – 25 g	20 – 28 h
		26 – 375 g	24 – 32 h
Dry milk (including Nonfat Dry Milk, NFDM)	BGW	10 – 25 g	20 – 28 h
		26 – 375 g	24 – 32 h

Key
v=vancomycin
a= amylase

2) Immunomagnetic concentration of the target organism in the enriched sample using the proprietary PickPen® device. For dry milk samples, an extended enrichment protocol involving a secondary incubation in BHI broth for 2-4 h at 36°C before IMS, outlined in the kit insert, was carried out.

3) Amplification and detection of the target DNA by real time PCR in the Assurance® GDS Rotor-Gene® thermocycler using set PCR parameters predefined in the instrument settings.

Confirmation of presumptive positive colonies for the extension study was performed using two different isolation methods available to the end user:

- Direct streak of the 1:10 enriched sample onto one chromogenic agar plate from a choice of 3 different isolation media: Chromogenic *Cronobacter* Isolation agar (CCI), Druggan-Forsythe-Iversen agar (DFI), and RAPID'*Sakazakii* agar (RSA).

  Typical isolated colonies are analysed by 2 available options:
  i) Biochemical galleries
  ii) Appropriate ISO 16140-6 (2019) validated confirmation method, such as MALDI ToF (MALDI Biotyper® complete solution, Bruker)

For the direct streak method used for dry milk powder samples, isolate only onto CCI plates after same-day analysis by GDS.

- Aliquot the remaining GDS concentration reagent retained in the resuspension plate onto one chromogenic agar plate from a choice of 2 different isolation media: CCI and DFI. Typical isolated colonies are analysed by 2 available options:
  i) Biochemical galleries
  ii) Appropriate ISO 16140-6 (2019) validated confirmation method such as MALDI ToF (MALDI Biotyper® complete solution, Bruker)

A selection of infant formula, infant cereals and dried milk products were tested in the sensitivity study as a representative number of the samples listed in the validation.

All three isolation media listed above were included in the extension study to assess their performance in the confirmation step. Details of the confirmation plates used for the product types included in the study are listed in the table, below:

Sample type	Direct streak enrichment at time of GDS analysis			Direct streak enrichment following storage at 2-8°C for 72h			Plate resuspension IMS at time of GDS analysis			Plate resuspension IMS following storage at 2-8°C for 48h		
	CCI	DFI *	RSA *	CCI *	DFI *	RSA *	CCI	DFI	RSA*	CCI	DFI	RSA*
Infant Formula without Probiotics	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓	
Infant Cereals without Probiotics	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓	
Non-probiotic Ingredients (except dry milk)	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓	
Infant Formula with Probiotics	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓	
Infant Cereals with Probiotics	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓	
Dry milk (including NFDM)	✓						✓	✓		✓	✓	

* Specific chromogenic plates were excluded for individual food type/isolation method, per kit insert. These exclusions are indicated as grey-coloured cells in table.

6.1.2 Reference Method

A reference method was not required for this extension study as the analysis for the confirmation method is proposed to be performed with the alternative protocol only.

6.1.3 Scope

Scope of the extension validation study was: Infant Formula and Infant Cereals and Environmental Samples

Categories included:

- Infant Formula and Infant Cereals
- Environmental Samples

Criteria evaluated during the study have been:

- Method Comparison Study (MCS)
  - Sensitivity study (limited scope, refer to section 3.2 for details)
  - Relative level of detection study (already completed, refer to initial study, 2017LR77, for details)
  - Inclusivity and exclusivity study

  Interlaboratory Study (ILS)[1] (already completed, refer to initial study, 2017LR77, for details)

Summarised, results and conclusions on the Extension Study are:

Data from the direct streak protocol for isolation and confirmation of *Cronobacter* gave good agreement with the expected results for the 46 samples tested in the sensitivity study. Data from the resuspension plate protocol also gave good agreement with the expected results for the 46 samples tested in the sensitivity study.

Analysis of the data revealed that the Acceptance Limits (AL) for the inclusivity study were met for the 100 isolates analysed. The calculated ND-PD and ND+PD were lower than the AL required for 100 inclusivity isolates.

In the exclusivity study the AL were met. The calculated ND-PD and ND+PD were lower than the AL required for 100 exclusivity isolates.

This report corresponds to the method comparison study and gathers the observed data and interpretations according to the MicroVal interpretation guidelines for ISO 16140-Part 6 (2019) issued in 2020 to validate an extension study for a new confirmation method from a previously validated qualitative method. The alternative Assurance® GDS for *Cronobacter* Tq II detection method with the new confirmation method is selective and specific.

6.2 Method protocols

The Method Comparison Study was carried out using 375 gram portions of test samples to ensure that 10 g tests portion sizes are also covered in the scope of the current validation.

6.2.1 Reference method

The reference method was not required for this extension study, as the analysis for the confirmation method was proposed to be performed with the alternative protocol only.

6.2.2 Alternative method

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

See the Assurance® GDS for *Cronobacter* Tq II kit insert in Annex B.

The Assurance® GDS for *Cronobacter* Tq II assay is an automated nucleic acid amplification system for the detection of *Cronobacter* in infant nutritional formula, ingredients, and environmental samples. This PCR-based method targets DNA specific to *Cronobacter* spp. in a procedure spilt into 3 stages:

1) Sample enrichment of a 1:10 dilution in Buffered Peptone Water (BPW), or appropriate diluent, prewarmed to 36°C. Details of the media and incubation times to be used for the different sample types included in the study are listed below.
2) Immunomagnetic concentration of the target organism in the enriched sample using the proprietary PickPen® device. For dry milk samples, an extended enrichment protocol involving a secondary incubation in BHI broth for 2-4 h at 36°C before IMS, outlined in the kit insert, was performed.

3) Amplification and detection of the target DNA by real time PCR in the Assurance® GDS Rotor-Gene® thermocycler using set PCR parameters predefined in the instrument settings.

Food Type	Media	Sample size	Enrichment Time
Infant Formula without Probiotics	BPW	10 –25 g	20 – 28 h
		26 – 375 g	24 – 32 h
Infant Cereals without Probiotics	BPW+a	10 – 25 g	20 – 28 h
		26 – 375 g	24 – 32 h
Non-probiotic Ingredients (except dry milk)	BPW	10 – 25 g	20 – 28 h
		26 – 375 g	24 – 32 h
Infant Formula with Probiotics	BPW+v	10 – 25 g	20 – 28 h
		26 – 375 g	24 – 32 h
Infant Cereals with Probiotics	BPW+v+a	10 – 25 g	20 – 28 h
		26 – 375 g	24 – 32 h
Dry milk	BGW	10 – 25 g	20 – 28 h
		26 – 375 g	24 – 32 h

Key
v=vancomycin
a= amylase

6.3 Study design

As the reference and alternative methods in the protocol are performed with different test portions and do not have a common enrichment procedure, this is an unpaired data study. However, reference method enrichment was not required for the proposed extension study, as the analysis for the confirmation method was performed with the alternative protocol only. Also, since only modifications to the isolation and confirmation methods were validated, no GDS analysis of samples was performed.

During the validation, only confirmation was performed on the enriched samples to validate the performance of the new proposed colony isolation methods in combination with biochemical galleries, as well as MALDI ToF (using the Bruker MALDI Biotyper®)

The Method Comparison Study was carried out using 375 gram test portions of the sample.

The samples were prepared for analysis and diluted in accordance with ISO 6887 (all parts) unless specified differently in the alternative method.

See **Table 1** for specific enrichments used in the validation study.

Table 1: Details specific pre-enrichments used in the validation study

Category	Sample type	Appropriate part of ISO 6887- to be used	Preparation needed
Infant Formula and Infant Cereals	Infant formula without probiotics and non-probiotic ingredients	1 and 4	1:10 dilution pre-warmed BPW
	Infant cereals without probiotics and non-probiotic ingredients	1 and 4	1:10 dilution pre-warmed BPW supplemented with amylase
	Infant formula with probiotics	1 and 4	1:10 dilution pre-warmed BPW supplemented with vancomycin
	Infant cereals with probiotics	1 and 4	1:10 dilution pre-warmed BPW supplemented with vancomycin and amylase
	Dry milk	1 and 5	1:10 dilution pre-warmed BGW

6.4 Method comparison study

6.4.1 Sensitivity Study

The sensitivity study (SE) is the ability of the method selected to detect the analyte by either the reference or the alternative method. In this study, confirmation only after enrichment was carried out to verify the ability of a new confirmation procedure for the alternative method.

Categories and sample types

For this extension study, a defined number of representative samples were analysed only for the infant formula and cereals category. Included were 25% of the total number of samples analysed in the full sensitivity study. Details of the samples tested are listed in the **Table 2** below.

Table 2. List of Categories, Types, and examples of Items to be tested within the reduced sensitivity study.

Categories	Types	No samples to be tested (per type)	Special preparations
Infant formula and cereals	Infant formula without probiotics	5	1:10 dilution pre-warmed BPW
	Infant cereals without probiotics	5	1:10 dilution pre-warmed BPW supplemented with amylase
	Non-probiotic ingredients (excluding dry milk)	5	1:10 dilution pre-warmed BPW
	Infant formula with probiotics	8	1:10 dilution pre-warmed BPW supplemented with vancomycin
	Infant cereals with probiotics	8	1:10 dilution pre-warmed BPW supplemented with vancomycin and amylase
	Dry milk	15	1:10 dilution pre-warmed BGW

Total number of samples tested = 46

Forty-six (46) samples were analysed in the sensitivity study. The distribution of positive and negative samples per tested category and type are given, respectively, in **Tables 3a and b**.

Table 3a - Distribution per tested category and type for the direct streak

Category	**Type**		**Positive samples per agar type**		
			CCI	**DFI**	**RSA**
Infant formula and cereals without probiotics	a	Infant formula without probiotics	5	5	5
	b	Infant cereals without probiotics	5	5	5
	c	Non-probiotic ingredients (excluding dry milk)	5	5	5
		Total	***15***	***15***	***15***
Infant formula and cereals with probiotics	a	Infant formula with probiotics	8	8	8
	b	Infant cereals with probiotics	7	8	8
		Total	***15***	***16***	***16***
Dry milk	a	Dry milk	15	NT	NT
		Total	***15***	***0***	***0***
Total			**45**	**31**	**31**

Key NT – not tested

Table 3 - Distribution per tested category and type for the resuspension plate confirmation

Category	**Type**		**Positive samples**		
			CCI	**DFI**	**RSA**
Infant formula and cereals without probiotics	a	Infant formula without probiotics	5	5	NT
	b	Infant cereals without probiotics	5	5	NT
	c	Non-probiotic ingredients (excluding dry milk)	4	4	NT
		Total	***14***	***14***	***0***
Infant formula and cereals with probiotics	a	Infant formula with probiotics	8	8	NT
	b	Infant cereals with probiotics	7	8	NT

		Total	***15***	***16***	***0***
Dry milk	a	Dry milk	13	15	NT
		Total	***13***	***15***	***0***
Total			**42**	**45**	**0**

Key NT – not tested

Test sample preparation

All samples included in the sensitivity study were artificially contaminated using a seeding protocol. The inoculum was prepared by drying the culture onto the sample which was then stored for 2 weeks and diluted to the appropriate level prior to testing.

The isolates used for artificial inoculations preferably originated from comparable sample types as the ones to be inoculated. Each particular strain was used to contaminate up to 5 different items. The 46 samples were artificially contaminated using 10 different strains with 1 seeding protocol.

Most of the seeding inoculations were lower or equal to 5 CFU/sample, with samples contaminated at no higher than 8 cfu per 375g test portion.

Confirmation protocols

The confirmation of presumptive positive colonies was carried out using the 2 different isolation methods available to the end user:

- Direct streak of the 1:10 enriched sample onto one chromogenic agar plate from a choice of 3 different isolation media: Chromogenic *Cronobacter* Isolation *agar* (CCI), Druggan-Forsythe-Iversen agar (DFI), and RAPID'*Sakazakii* agar (RSA). Typical isolated colonies are analysed by 2 available options:
  - i) Biochemical galleries
  - ii) Appropriate ISO 16140-6 (2019) validated confirmation method, such as MALDI ToF (MALDI Biotyper® complete solution, Bruker)

For the direct streak method used for dry milk powder samples, isolate only onto CCI plates after same day analysis by GDS.

- Aliquot the remaining GDS concentration reagent retained in theresuspension plate onto one chromogenic agar plate from a choice of 2 different isolation media (CCI and DFI). Typical isolated colonies are analysed by 2 available options:
  - i) Biochemical galleries
  - ii) Appropriate ISO 16140-6 (2019) validated confirmation method such as MALDI ToF (MALDI Biotyper® complete solution, Bruker)

In addition to the MALDI ToF, biochemical galleries were used for confirmation on a representative selection of the samples tested during the study. All three replicate chromogenic plates were analysed by MALDI ToF. One of three replicate chromogenic plates was analysed by biochemical galleries, as the inoculating organisms were already biochemically confirmed as *Cronobacter*. A summary of the colonies to be confirmed by each of the isolation and confirmation method combinations are given in **Tables 4a and 4b**, below.

In order to verify the stability of initial enrichments during chilled storage (72h at 2-8°C, as stated in the kit insert), the direct streak confirmation protocol was repeated on the sample enrichments of positive and discordant samples following storage for 72h at 2-8°C. At the request of the client, the confirmation protocol was also performed on positive and discordant samples using remaining concentration reagent, following storage of the IMS resuspension plate for 48h at 2-8°C.

Table 4a: Number of colonies confirmed for each agar and confirmation method combination during this study - direct streaks from the initial sample enrichment

Category	Food type	No samples	No. of colonies confirmed for each agar and confirmation method combination						Number of biochemical confirmations per food type
			CCI		DFI		RSA		
			Maldi	API	Maldi	API	Maldi	API	
Infant formula and cereals	Infant formula without probiotics	5	5	5	5	-	5	-	15
	Infant cereals without probiotics	5	5	-	5	5	5	-	
	Non-probiotic ingredients (exc. dry milk)	5	5	-	5	-	5	5	
	Total	15	15	5	15	5	15	5	
	Infant formula with probiotics	8	8	4	8	4	8	-	16
	Infant cereals with probiotics	8	8	4	8	-	8	4	
	Total	16	16	8	16	4	16	4	
	Dry milk	15	15	5	np	np	np	np	5
	Total	15	15	5	15	5	15	5	
	Overall total	46	46	18	31	9	31	9	36

Key np = not plated

Key NT = not tested

Table 4b: Number of colonies confirmed for each agar and confirmation method combination during this study - IMS streaks from the IMS concentration reagent

Category	Food type	No samples	No. of colonies confirmed for each agar and confirmation method combination						Number of biochemical confirmations per food type
			CCI		DFI		RSA		
			Maldi	API	Maldi	API	Maldi	API	
Infant formula and cereals	Infant formula without probiotics	5	5	5	5	-*	np	np	10
	Infant cereals without probiotics	5	5	-	5	5	np	np	
	Non-probiotic ingredients (exc. dry milk)	5	5	-	5	-	np	np	
	Total	15	15	5	15	5	0	0	
	Infant formula with probiotics	8	8	4	8	4	np	np	12
	Infant cereals with probiotics	8	8	4	8	-	np	np	
	Total	16	16	8	16	4	0	0	
	Dry milk	15	15	5	15	5	np	np	10
	Total	15	15	5	15	5	0	0	
	Overall total	46	46	18	46	14	0	0	32

Key np = not plated

Sensitivity study results

All raw data on the sensitivity study are given in Excel spreadsheet “2017LR77 extension raw data”, using 4 spreadsheet tabs: sensitivity 0h direct, sensitivity 72h direct, sensitivity 0h IMS and sensitivity 48h IMS.

	Excel Tab			
	sensitivity 0h direct	sensitivity 72h direct	sensitivity 0h IMS	sensitivity 48h IMS
Data	Direct streak enrichme at time of GDS analysi	Direct streak enrichme following storage at 2-8°C for 72h	Plate resuspension IMS at time of GDS analysis	Plate resuspension IM following storage at 2-8°C for 48h

A summary of the results obtained for each of the isolation media and confirmation technique combination used are given in **Tables 5a and 5b**, below.

Table 5a: Summary of the confirmed results obtained from the direct streak confirmation protocol for the defined isolation media and confirmation method combinations

Category	Food type	No of positive confirmed samples for each agar and confirmation method combination					
		CCI		DFI		RSA	
		Maldi	Biochemical	Maldi	Biochemical	Maldi	Biochemical
Infant formula and cereals	Infant formula without probiotics	5/5	5/5	5/5	nt	5/5	nt
	Infant cereals without probiotics	5/5	nt	5/5	5/5	5/5	nt
	Non-probiotic ingredients (exc. dry milk)	5/5	nt	5/5	nt	5/5	5/5
	Total	15	5	15	5	15	5
	Infant formula with probiotics	8/8	4/4	8/8	4/4	8/8	nt
	Infant cereals with probiotics	7/8	3/4	8/8	nt	8/8	4/4
	Total	15	7	16	4	16	4
	Dry milk	15/15	5/5	*np*	*np*	*np*	*np*
	Total	15	5	0	0	0	0
	Overall total	45/46	17/18	31/31	9/9	31/31	9/9

Key nt = not tested, np = not plated

For CCI confirmation plates, using the direct streak protocol, *Cronobacter* was successfully isolated and confirmed in all 38 samples in the following food types (infant formula without probiotics, infant formula with probiotics, infant cereals without probiotics, non-probiotic ingredients (exc. dry milk), and dry milk); confirmation was performed using MALDI ToF. For infant cereals with probiotics, 7 out of the 8 samples analysed gave the anticipated result. In the sample that gave discrepant results, no presumptive positive colonies were isolated on this agar type. The 17 representative samples with typical colonies, also confirmed using biochemical confirmations, gave the anticipated results.

The results from the direct streak protocol for DFI and RSA plates showed that all 31 samples isolated and confirmed *Cronobacter* across all food types analysed (infant formula without probiotics, infant formula with probiotics, infant cereals without probiotics, infant cereals with probiotics, non-probiotic ingredients (exc.

dry milk)); confirmation was performed using MALDI ToF. The 9 representative samples confirmed using biochemical confirmation also gave the anticipated results.

Data from the direct streak protocol gave good agreement with the expected results for the 46 samples tested in the sensitivity study for all three plate types (CCI, DFI, RSA).

Table 5b: Summary of the confirmed results obtained from the resuspension plate confirmation protocol for the defined isolation media and confirmation method combinations

		No of positive confirmed samples for each agar and confirmation method combination					
		CCI		**DFI**		**RSA**	
Category	**Food type**	**Maldi**	**Biochemical**	**Maldi**	**Biochemical**	**Maldi**	**Biochemical**
Infant formula and cereals	Infant formula without probiotics	5/5	5/5	5/5	nt	*np*	*np*
	Infant cereals without probiotics	5/5	nt	5/5	5/5	*np*	*np*
	Non-probiotic ingredients (exc. dry milk)	4/5	nt	4/5	nt	*np*	*np*
	Total	14	5	14	5	*np*	*np*
	Infant formula with probiotics	8/8	4/4	8/8	4/4	*np*	*np*
	Infant cereals with probiotics	7/8	4/4	8/8	-	*np*	*np*
	Total	15	8	16	4	*np*	*np*
	Dry milk	13/15	5/5	15	5/5	*np*	*np*
	Total	13	5	15	5	*np*	*np*
	Overall total	42/46	18/18	45/46	14/14	*np*	*np*

Key nt = not tested, np = not plated

On CCI confirmation plates, using the resuspension IMS protocol, *Cronobacter* was successfully isolated and confirmed from 42 out of 46 samples; confirmation was performed using MALDI ToF. Four samples did not isolate presumptive positive colonies. One sample with discrepant results was from non-probiotic ingredients (excluding dry milk); this sample also lacked confirmation with DFI. One sample did not confirm and was in infant cereals with probiotics. Lastly, two additional samples were not confirmed and were in the dry milk food type. The 18 representative samples confirmed using biochemical confirmation from CCI plates also gave the anticipated results.

Data from the resuspension plate confirmation workflow showed that *Cronobacter* was successfully confirmed in 45 out of the 46 samples streaked onto DFI plates; confirmation was performed using MALDI ToF. The sample with the discrepant result on DFI was from non-probiotic ingredients (excluding dry milk). All 14 selected samples taken from the DFI plates also confirmed correctly using biochemical confirmation.

Analysis of the results from the resuspension plate protocol gave good agreement with the expected results for the 46 samples tested for all two plate types (CCI, DFI).

All raw data on the sensitivity study are given in Annex E. Sample numbers in **bold** indicate artificial inoculation of the sample (see Annex D for details on artificial inoculation).

Discordant results

Negative deviations are listed in **Table 6**.

Table 6 - Negative deviations

Type	**Sample nº**	**Isolation method**	**Agar type**	**Alternative method results**	**Confirmatory test results**	**Inoculation (CFU/Sample)**
Infant cereals with probiotics	S26	DS	CCI	-	MALDI ToF and Biochemical tests	*Cronobacter sakazakii* CRA 17636 (5)
Non probiotic ingredients (excluding dry mi	S11	RP	CCI	-	MALDI ToF and Biochemical tests	*Cronobacter turicensis* CRA 17663 (4)
Infant cereals with probiotics	S26	RP	CCI	-	MALDI ToF and Biochemical tests	*Cronobacter sakazakii* CRA 17636 (5)
Dry milk	S41	RP	CCI	-	MALDI ToF	*Cronobacter dublinens* subsp. Dublinensis DSM 18705 (5)
	S42					
Non probiotic ingredients (excluding dry mi	S11	RP	DFI	-	MALDI ToF and Biochemical tests	*Cronobacter turicensis* CRA 17663 (4)

Key DS= direct streak, RP = resuspension plate

More discordant results occurred from CCI agar compared to DFI agar, though both isolation media were overall effective. The data also revealed that the direct streak method was more successful for to the

isolation of *Cronobacter* from the test portions analysed than the resuspension plate method, though, again, both methods overall were effective.

One point to note was that two strains used for the artificial inoculation *Cronobacter sakazakii* CRA 17636 and *Cronobacter turicensis* CRA 17663 were not isolated from the same sample using either CCI or DFI agars. A possible explanation for this could be that the selective media was inhibitory to the growth of the injured cells present in the samples.

Conclusion sensitivity study

Data from the direct streak protocol gave good agreement with the expected results for the 46 samples tested in the sensitivity study.

Data from the resuspension plate protocol gave good agreement with the expected results for the 46 samples tested in the sensitivity study.

Enrichment broth and resuspension plate storage

Enrichment broth storage

Enrichment broth storage was performed for all samples at 2-8 °C for 72h.

All positive and discordant samples were tested again after enrichment broth storage. No changes were observed for RSA following the 27h storage period and 1 change was observed for the CCI and DFI agars, respectively (see **Table 7a** for details).

Table 7a Observed changes in results before and after storage of the enrichment broth with MALDI ToF confirmation

Sample nr.	*Result before storage*			*Result after storage*		
	Alt. method	*Confirmation*	*Interpretation*	*Alt. method*	*Confirmation*	*Interpretation*
CCI						
S26	*Neg*	*Neg*	*SD*	*Pos*	*Pos*	*SA*
DFI						
S26	*Pos*	*Pos*	*SA*	*Neg*	*Neg*	*SD*

Key: SA= sensitivity agreement, SD= sensitivity discordant

Resuspension plate storage was performed for all samples at 2-8 °C for 48h.

All positive and discordant samples were tested again after storage of the resuspension plate. 3 changes were observed for CCI agar and 3 changes for DFI agar (see **Table 7b** for details).

Table 7b Observed changes in results before and after storage of the resuspension plate with MALDI ToF confirmation

Sample nr.	*Result before storage*			*Result after storage*		
	Alt. method	*Confirmation*	*Interpretation*	*Alt. method*	*Confirmation*	*Interpretation*
CCI						
S41	*Neg*	*Neg*	*SD*	*Pos*	*Pos*	*SA*
S42	*Neg*	*Neg*	*SD*	*Pos*	*Pos*	*SA*
S47	*Pos*	*Pos*	*SA*	*Neg*	*Neg*	*SD*
DFI						
S11	*Neg*	*Neg*	*SD*	*Pos*	*Pos*	*SA*
S26	*Pos*	*Pos*	*SA*	*Neg*	*Neg*	*SD*
S42	*Pos*	*Pos*	*SA*	*Neg*	*Neg*	*SD*

Key: SA= sensitivity agreement, SD= sensitivity discordant

6.4.2 *Relative level of detection study*

This has already been completed in the initial study ref 2017LR77; therefore, this is not required for the extension study.

6.4.3 *Inclusivity/exclusivity study*

Inclusivity is the ability of the alternative method to detect the target analyte from a wide range of strains.

Exclusivity is the lack of interference from a relevant range of non-target strains of the alternative method.

Protocols

As the Assurance® GDS for *Cronobacter* Tq II kit has already been validated in the original study ref 2017LR77, the new confirmation protocol was validated following the MicroVal interpretation guidelines issued in 2020 for extension study for a new confirmation method with isolated colonies.

Inclusivity Study:

Due to the limited availability of *Cronobacter* isolates, 100 blind coded strains were analysed by the alternative confirmation method only. All 100 isolates were enriched in BGW supplemented with milk powder for 20 hours and with a direct streak onto all 3 chromogenic plate types (CCI, DFI, RSA) followed by MALDI ToF and biochemical analysis of colony picks.

Since this study used pure culture analysis of 100 known *Cronobacter* isolates, representative plates from each of the agar types were selected for analysis by MALDI ToF as described in the confirmation scheme, below.

Media type	Number of colonies taken forward for confirmation with MALDI		
	CCI	DFI	RSA
CCI only	30		
DFI only		30	
RSA only			30
CCI, DFI, and RSA	10	10	10

Biochemical confirmation was carried out on any *Cronobacter* colonies that did not give a MALDI ToF positive result to resolve the discrepancies.

Exclusivity Study:

One hundred (100) blind coded non-target organisms, composed of non-*Cronobacter* spp., coliforms, and other Gram-negative bacteria, were analysed by the alternative confirmation method only. Exclusivity isolates were incubated in BPW-ISO for 20 hours and streaked onto all 3 chromogenic plate types (CCI, DFI, RSA) followed by MALDI ToF on all typical colonies.

All results for the inclusivity and exclusivity studies were tabulated and interpreted according to ISO 16140-2.

6.4.4 Results inclusivity and exclusivity study
All raw data on inclusivity and exclusivity are given in an excel spreadsheet, “2017LR77 extension raw data” using spreadsheet tab "inclusivity and exclusivity”.

Results Inclusivity:

The analysis of the confirmation workflow was broken down into 2 stages: the initial screen to check for typical colonies followed by MALDI ToF analysis of typical colonies.

A summary of the isolates giving non-typical isolates from the direct streaks of the BGW enrichment for the three *Cronobacter* isolation media is shown below:

Media	**Number of strains giving non-typical colonies on the selective isolation plates**

CCI	**3/100**
DFI	**1/100**
RSA	**0/100**

CCI Agar

97 out of the 100 strains tested showed the expected positive result. There were 3 *Cronobacter sakazakii* strains that did not give typical colonies (CRA 5181, 5196 and 5197). These 3 isolates were analysed by MALDI ToF and were confirmed to be *Cronobacter* spp.

DFI Agar

99 out of the 100 strains analysed showed the expected positive result. There was 1 *Cronobacter sakazakii* strain (CRA 17639, CCUG 28863) which did not give typical colonies. This isolate was analysed by MALDI ToF and confirmed to be *Cronobacter* spp.

RSA Agar

All 100 strains showed the expected positive result. The 40 representative isolates analysed by MALDI ToF were confirmed to be *Cronobacter* spp.

In addition, a subset of the inclusivity panel, taken from the three selective media (as described earlier), were confirmed using MALDI ToF. A summary of the method comparison for the new confirmation workflow is given in **Table 8**.

Table 8: Summary of the method comparison study results for MALDI ToF for the new confirmation protocol

Plate type	N	PA	ND	PD	ND-PD	ND+PD
CCI only	30	30	0	0	0	0
DFI only	30	30	0	0	0	0
RSA only	30	30	0	0	0	0
CCI, DFI, and RSA	10	10	0	0	0	0
Total	**100**	**100**	**0**	**0**	**0**	**0**

Inclusivity Results evaluation

Details of the study evaluation are shown in **Table 9** below:

Table 9: Evaluation of method comparison study results for the inclusivity study

Protocol	ND-PD	AL	Evaluation	ND-PD	AL	Evaluation
Direct streak from the ini enrichment	0	3	Accepted	0	3	Accepted

The AL for the inclusivity study were met for the 100 isolates analysed. The calculated ND-PD and ND+PD were lower than the AL required for 100 inclusivity isolates.

Results Exclusivity:

A total of 100 strains were tested for exclusivity.

The analysis of exclusivity isolates involved an initial streak of the BPW enriched cultures onto three *Cronobacter* isolation media to check for the presence of typical colonies. Any isolates that gave unexpected results were taken forward for analysis by MALDI ToF to verify the identity of the isolate. A summary of the isolates giving typical colonies on the *Cronobacter* isolation media is given below:

Media	Number of strains giving typical colonies on the selective isolation plates
CCI	**1/100**
DFI	**2/100**
RSA	**1/100**

CCI Agar

99 isolates showed the expected negative result. The one isolate that gave typical colonies on CCI was *Franconibacter helveticus* CRA 17678 (CCUG 54944/ DSM 18396). This isolate was analyzed by MALDI as *Franconibacter helveticus*, and therefore confirmed as negative for *Cronobacter* spp.

DFI Agar

98 isolates showed the expected negative result. The two isolates that gave typical colonies on DFI were *Franconibacter helveticus* CRA 17678 (CCUG 54944/ DSM 18396) and *Klebsiella oxytoca* CRA 15926 (ATCC 13182). These isolates were analysed by MALDI as *Franconibacter helveticus* and *Klebsiella oxytoca*, respectively. Hence these isolates were confirmed as negative for *Cronobacter* spp.

RSA Agar

99 isolates showed the expected negative result. The one isolate that gave typical colonies on RSA was *Franconibacter helveticus* CRA 17678 (CCUG 54944/ DSM 18396). This isolate was analysed by MALDI as *Franconibacter helveticus*, and therefore confirmed as negative for *Cronobacter* spp.

The results from the exclusivity study revealed that all 100 isolates tested on the three isolation agar types gave the expected result after MALDI ToF confirmation had been carried out. A summary of the method comparison for the new confirmation workflow is given in **Table 10**.

Table 10: Summary of the method comparison study results for MALDI ToF for the new confirmation protocol

Media type	N	PA	ND	PD	ND-PD	ND+PD
CCI	100	0	0	0	0	0
DFI	100	0	0	0	0	0
RSA	100	0	0	0	0	0
Total	**100**	**0**	**0**	**0**	**0**	**0**

Exclusivity Results evaluation

Details of the study evaluation are shown in **Table 11** below:

Table 11: Evaluation of method comparison study results for the exclusivity study

Protocol	**ND-PD**	**AL**	**Evaluation**	**ND-PD**	**AL**	**Evaluation**
Direct streak from the in enrichment	0	2	Accepted	0	2	Accepted

The AL for the exclusivity study were met. The calculated ND-PD and ND+PD were lower than the AL required for 100 exclusivity isolates.

Conclusion of inclusivity and exclusivity study

Analysis of the data revealed that the AL for the inclusivity study were met for the 100 isolates analysed. The calculated ND-PD and ND+PD were lower than the AL required for 100 inclusivity isolates. In the exclusivity study the AL were met. The calculated ND-PD and ND+PD were lower than the AL required for 100 exclusivity isolates.

6.5 Conclusions of Method Comparison Study

Overall, the conclusions for the Method Comparison Study are:

Data from the direct streak protocol gave good agreement with the expected results for the 46 samples tested in the sensitivity study.

Data from the resuspension plate protocol gave good agreement with the expected results for the 46 samples tested in the sensitivity study.

Analysis of the data revealed that the AL for the inclusivity study were met for the 100 isolates analysed. The calculated ND-PD and ND+PD were lower than the AL required for 100 inclusivity isolates.

In the exclusivity study the AL were also met. The calculated ND-PD and ND+PD were lower than the AL required for 100 exclusivity isolates.

The alternative Assurance® GDS for *Cronobacter* Tq II detection method with the new confirmation method is selective and specific.

Date 18/05/23

Signature Suzanne Jordan

Dr Suzanne Jordan

ANNEX A1: Flow diagram of the reference method for initial study

(Samples are prepared according to ISO 6887-1, 4 and 5)

↓

Infant formula and ingredients:

10 g + 90 mL BPW ISO;

(Certain food types, infant cereal, will require the addition of amylase to the media)

Environmental samples:

1 swab + 10 mL BPW ISO

or 1 sponge + 100 mL BPW ISO

or 25 g or 25 mL + 225 mL BPW ISO

↓

Incubation 18 h ± 2 h

at 34 – 38°C

↓

0.1 mL + 10 mL CSB

↓

Incubation 24 h ± 2 h

at 41.5°C ± 1°C

↓

Streaking onto CCI Agar plate

↓

Incubation 24 h ± 2 h

at 41.5°C ± 1°C

↓

Confirmatory test on one typical colony,

and four other colonies

(typical colony: blue to blue-green)

↓

Streaking onto TSA-YE

↓

Incubation 18 h – 24 h

at 37°C ± 1°C

↓

Biochemical confirmation

(oxidase, mini-galleries)

ANNEX A2: Flow diagram of the alternative method for the initial study

Flow Charts of the Assurance GDS *Cronobacter* Tq Enrichment Protocols

Infant formula without probiotics and non-probiotics ingredients (except milk powders)

Protocol 1
10 g + 90 mL pre-warmed[1] BPW
(d 1/10)
↓
20 h - 24 h at 34 - 38°C

Protocol 2
375g + 3375 mL pre-warmed BPW
(d 1/10)
↓
24 h - 28 h at 34 - 38°C

Infant formula with probiotics

Protocol 5
10 g + 90 mL pre-warmed double strength BPW
+ vancomycin (6 mg/L) (d 1/10)
↓
20 h - 24 h at 34 - 38°C

Protocol 6
375 g + 3375 mL pre-warmed BPW
+ vancomycin (6 mg/L) (d 1/10)
↓
24 h - 28 h at 34 - 38°C

Infant cereals without probiotics

Protocol 3
10 g + 90 mL pre-warmed BPW
+ amylase (10 mg/L) (d 1/10)
↓
20 h - 24 h at 34 - 38°C

Protocol 4
375 g + 3375 mL pre-warmed BPW
+ amylase (10 mg/L) (d 1/10)
↓
24 h - 28 h at 34 - 38°C

Infant cereals with probiotics

Protocol 7
10 g + 90 mL pre-warmed BPW
+ vancomycin (6 mg/l) + amylase (10 mg/L)
(d 1/10)
↓
20 h - 24 h at 34 - 38°C

Protocol 8
375 g + 3375 mL pre-warmed BPW
+ vancomycin (6 mg/l) + amylase (10 mg/L)
(d 1/10)
↓
24 h - 28 h at 34 - 38°C

Ingredients: milk powder

Protocol 9
10 g + 90 mL pre-warmed Brilliant Green Water
(d 1/10)
↓
20 h - 24 h at 34 - 38°C

Protocol 10
375 g + 3375 mL pre-warmed Brilliant Green Water
(d 1/10)
↓
24 h - 28 h at 34 - 38°C

Environmental samples

[1] Pre-warming of all media will be conducted at 34-38°C

Protocol 11
Swab + 10 ml BPW
Sponge + 100 ml BPW
25 g or 25 ml + 225 ml BPW
↓
18 h - 20 h at 34 - 38°C

Qualitative methods – Assurance GDS for Cronobacter TqII extension study Ref 2017 LR77 MCS report v1 02/03/23

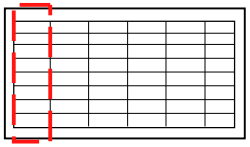
MICROVAL® NEN

Infant Formula & Cereals / Environmental Samples

Reagent Prep:

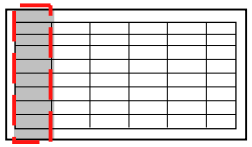
Add the appropriate volume of the specified reagent using the indicated repeater pipette tip and cover each row with an adhesive strip.

Sample Block



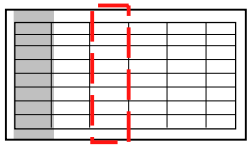
Concentration Reagent – 20 µL
(0.5 mL tip)

Sample Block



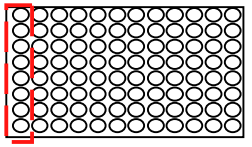
Wash Solution – 700 µL
(10 mL tip)
Add to wells with Concentration Reagent

Sample Block



Wash Solution – 1 mL
(10 mL tip)

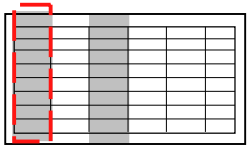
Resuspension Plate



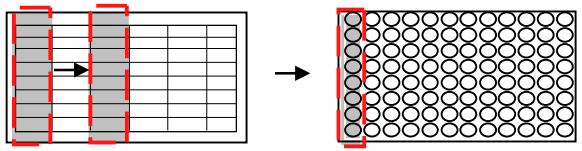
Resuspension Buffer Tq– 45 µL
(0.5 mL tip)

Sample Prep:

Add 0.3 mL of enriched sample to wells containing concentration reagent/wash. Cover and vortex for 10 – 20 min.

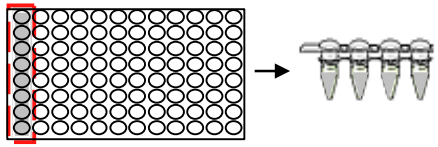


Use the PickPen to transfer samples through wash solution to the resuspension plate.



Amplification & Detection:

Transfer 30 µL of each sample from resuspension plate to Amplification Tubes Tq.



Place amp tubes in Assurance GDS Rotor-Gene and start.

Qualitative methods – Assurance GDS for Cronobacter TqII extension study Ref 2017 LR77 MCS report v1 02/03/23

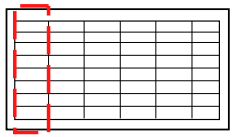
MICROVAL® NEN

Non-Fat Dry Milk Samples

Reagent Prep:

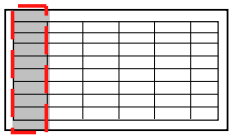
Add the appropriate volume of the specified reagent using the indicated repeater pipette tip and cover each row with an adhesive strip.

Sample Block



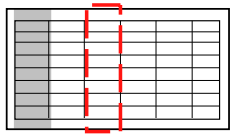
Concentration Reagent – 20 µL
(0.5 mL tip)

Sample Block



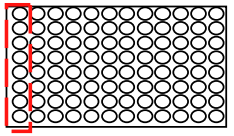
Wash Solution – 700 µL
(10 mL tip)
Add to wells with Concentration Reagent

Sample Block



BHI – 0.5 mL
(10 mL tip)

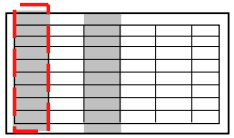
Resuspension Plate



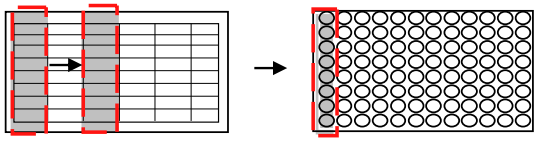
Resuspension Buffer Tq– 45 µL
(0.5 mL tip)

Sample Prep:

Add 0.3 mL of enriched sample to wells containing concentration reagent/wash.
Cover and vortex for 10 – 20 min.

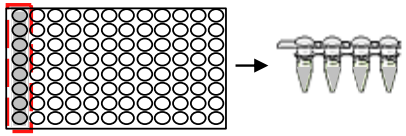


Use the PickPen to transfer samples into BHI, release particles.
Cover and incubate for 2 – 4 h @ 36 ± 2 °C.
Use PickPen to transfer samples to the resuspension plate.



Amplification & Detection:

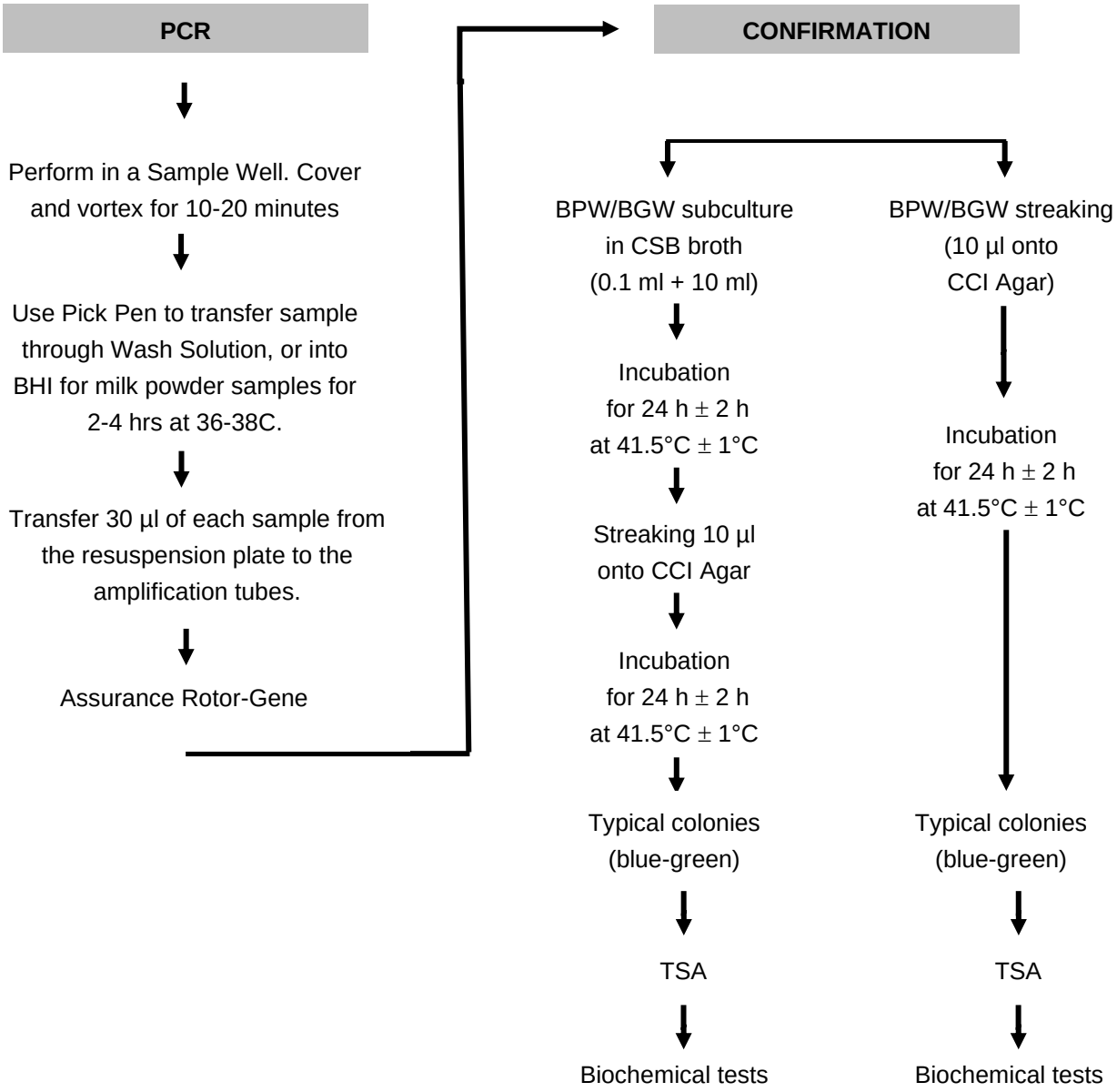
Transfer 30 µL of each sample from resuspension plate to Amplification Tubes Tq.



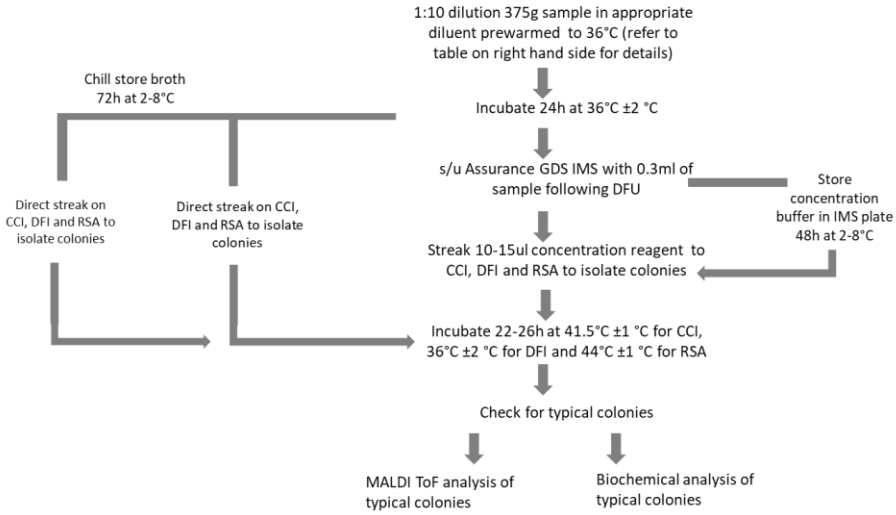
Place amp tubes in Assurance GDS Rotor-Gene and start.

Flow Charts of the Assurance GDS *Cronobacter* Tq PCR Methods

PickPen IMS Procedure



ANNEX B1: Flow diagram of the alternative method – Infant formula and cereals, except dry milk (incl. NFDM) for the extension study

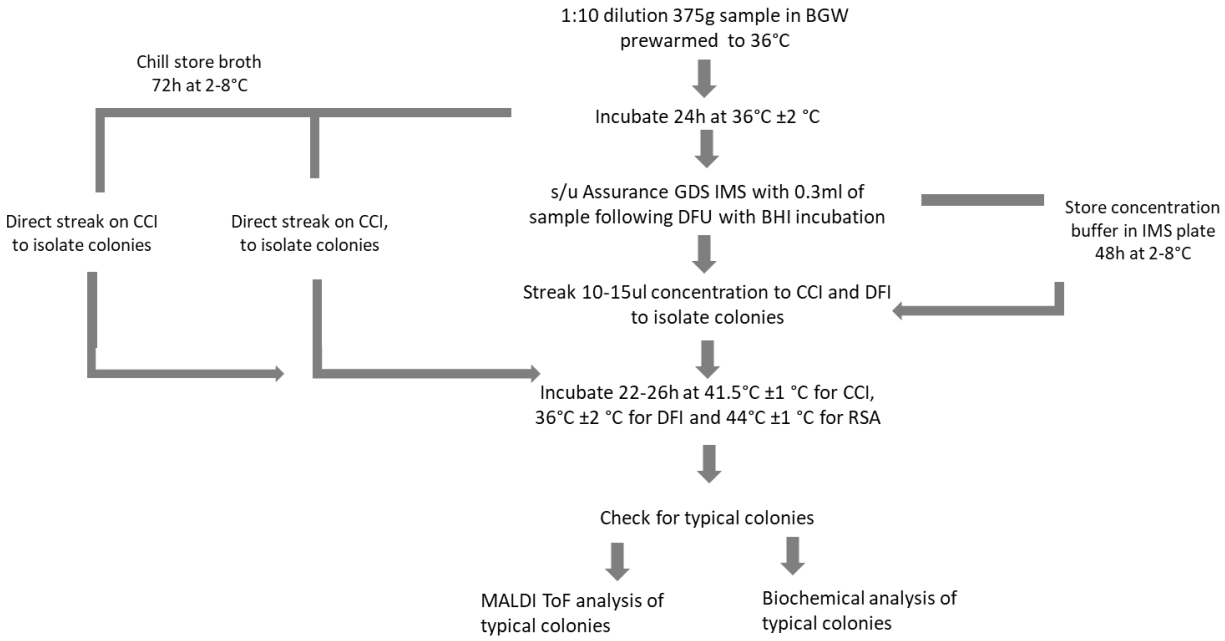


*the full incubation range is 24-32h, however for the validation study, the minimum time of 24h was used

Sample diluents to used for analysis

Types	Special preparations
Infant formula without probiotics	1:10 dilution pre-warmed BPW
Infant cereals without probiotics	1:10 dilution pre-warmed BPW supplemented with amylase
Infant formula with probiotics	1:10 dilution pre-warmed BPW supplemented with vancomycin
Infant cereals with probiotics	1:10 dilution pre-warmed BPW supplemented with vancomycin and amylase

ANNEX B2: Flow diagram of the alternative method – – dry milk (incl. NFDM) for the extension study



*the full incubation range is 24-32h, however for the validation study, the minimum time of 24h was used

ANNEX C: Kit insert(s)

Please refer to separate pdf for details.

ANNEX D: Artificial contaminations for the initial study

Category	Item Type	Sample nº	Strain	Origin	Injury Protocol	Injury Evaluation (Log10)	Inoculation (CFU/Sample)	Alternative Method Result	Reference Method Result
Infant Formula and Infant Cereal	Infant formula without probiotic Soy based	1	*Cronobacter sakazakii* QL 17031.4	Infant Formula	Lyophilized culture: 2 week hold at 20-25ºC	0.62	5.1	Positive	Positive
	Infant formula without probiotic Pure Bliss	3	*Cronobacter sakazakii* QL 17031.4	Infant Formula	Lyophilized culture: 2 week hold at 20-25ºC	0.62	5.1	Positive	Positive
	Infant formula without probiotic Neosure	5	*Cronobacter sakazakii* QL 17031.4	Infant Formula	Lyophilized culture: 2 week hold at 20-25ºC	0.62	5.1	Positive	Positive
	Infant formula without probiotic Infant	6	*Cronobacter sakazakii* QL 17031.4	Infant Formula	Lyophilized culture: 2 week hold at 20-25ºC	0.62	5.1	Positive	Positive
	Infant formula without probiotic Toddler	7	*Cronobacter sakazakii* QL 17031.4	Infant Formula	Lyophilized culture: 2 week hold at 20-25ºC	0.62	5.1	Positive	Positive
	Infant formula without probiotic 12-24 Months	9	*Cronobacter sakazakii* CCUG 28860	Formula	Lyophilized culture: 2 week hold at 20-25ºC	0.53	3.4	Positive	Positive
	Infant formula without probiotic Stage 1	11	*Cronobacter sakazakii* CCUG 28860	Formula	Lyophilized culture: 2 week hold at 20-25ºC	0.53	3.4	Positive	Positive
	Infant formula without probiotic Stage 2	12	*Cronobacter sakazakii* CCUG 28860	Formula	Lyophilized culture: 2 week hold at 20-25ºC	0.53	3.4	Positive	Positive
	Infant formula without probiotic Stage 3	17	*Cronobacter sakazakii* CCUG 28860	Formula	Lyophilized culture: 2 week hold at 20-25ºC	0.53	3.4	Positive	Positive

Category	Item Type	Sample nº	Strain	Origin	Injury Protocol	Injury Evaluation (Log10)	Inoculation (CFU/Sample)	Alternative Method Result	Reference Method Result
Infant Formula and Infant Cereal	Infant cereal without probiotic Organic rice	2	*Cronobacter sakazakii* QL 123015.1A	Rice Flour	Lyophilized culture: 2 week hold at 20-25ºC	0.61	2.6	Positive	Positive
	Infant cereal without probiotic Single grain	6	*Cronobacter sakazakii* QL 123015.1A	Rice Flour	Lyophilized culture: 2 week hold at 20-25ºC	0.61	2.6	Positive	Positive
	Infant cereal without probiotic Oatmeal	7	*Cronobacter sakazakii* QL 123015.1A	Rice Flour	Lyophilized culture: 2 week hold at 20-25ºC	0.61	2.6	Positive	Positive
	Infant cereal without probiotic Multigrain	12	*Cronobacter sakazakii* QL 123015.1A	Rice Flour	Lyophilized culture: 2 week hold at 20-25ºC	0.61	2.6	Positive	Positive
	Infant cereal without probiotic Multigrain Banana	13	*Cronobacter sakazakii* QL 11007.9	Rice Flour	Lyophilized culture: 2 week hold at 20-25ºC	0.68	4.1	Positive	Positive
	Infant cereal without probiotic Multigrain Apple	14	*Cronobacter sakazakii* QL 11007.9	Rice Flour	Lyophilized culture: 2 week hold at 20-25ºC	0.68	4.1	Positive	Positive
	Infant cereal without probiotic Wholegrain	17	*Cronobacter sakazakii* QL 11007.9	Rice Flour	Lyophilized culture: 2 week hold at 20-25ºC	0.68	4.1	Positive	Positive
	Infant cereal without probiotic Wholegrain oatmeal	18	*Cronobacter sakazakii* QL 11007.9	Rice Flour	Lyophilized culture: 2 week hold at 20-25ºC	0.68	4.1	Positive	Positive
	Infant cereal without probiotic Wholegrain apple	19	*Cronobacter sakazakii* QL 11007.9	Rice Flour	Lyophilized culture: 2 week hold at 20-25ºC	0.68	4.1	Positive	Positive
	Infant cereal without probiotic Single grain rice	20	*Cronobacter turicensis* QL 17031.5	Infant Formula	Lyophilized culture: 2 week hold at 20-25ºC	0.72	5.7	Positive	Positive
	Infant cereal without probiotic Single grain raisin	21	*Cronobacter turicensis* QL 17031.5	Infant Formula	Lyophilized culture: 2 week hold at 20-25ºC	0.72	5.7	Positive	Positive

Category	Item Type	Sample nº	Strain	Origin	Injury Protocol	Injury Evaluation (Log10)	Inoculation (CFU/Sample)	Alternative Method Result	Reference Method Result
	Infant cereal without probiotic Single grain banana	22	*Cronobacter turicensis* QL 17031.5	Infant Formula	Lyophilized culture: 2 week hold at 20-25ºC	0.72	5.7	Positive	Positive
	Infant cereal without probiotic Single grain apple	23	*Cronobacter turicensis* QL 17031.5	Infant Formula	Lyophilized culture: 2 week hold at 20-25ºC	0.72	5.7	Positive	Positive
Infant Formula and Infant Cereal	Instant milk powder	6	*Cronobacter sakazakii* FSL F6-024	Finished RTE Product (dairy)	Lyophilized culture: 2 week hold at 20-25ºC	0.53	4.1	Positive	Positive
	Dry milk mix	7	*Cronobacter sakazakii* FSL F6-024	Finished RTE Product (dairy)	Lyophilized culture: 2 week hold at 20-25ºC	0.53	4.1	Positive	Positive
	Whole milk powder	11	*Cronobacter sakazakii* FSL F6-024	Finished RTE Product (dairy)	Lyophilized culture: 2 week hold at 20-25ºC	0.53	4.1	Positive	Positive
	Nonfat dry milk powder	12	*Cronobacter sakazakii* FSL F6-024	Finished RTE Product (dairy)	Lyophilized culture: 2 week hold at 20-25ºC	0.53	4.1	Positive	Positive
	Malted milk powder	13	*Cronobacter sakazakii* FSL F6-024	Finished RTE Product (dairy)	Lyophilized culture: 2 week hold at 20-25ºC	0.53	4.1	Positive	Positive
	Skim milk powder	14	*Cronobacter malonaticus* FSL F6-030	Finished RTE Product (dairy)	Lyophilized culture: 2 week hold at 20-25ºC	0.59	5.7	Positive	Positive
	Full cream milk powder	16	*Cronobacter malonaticus* FSL F6-030	Finished RTE Product (dairy)	Lyophilized culture: 2 week hold at 20-25ºC	0.59	5.7	Positive	Positive
	Organic nonfat dry milk powder	17	*Cronobacter malonaticus* FSL F6-030	Finished RTE Product (dairy)	Lyophilized culture: 2 week hold at 20-25ºC	0.59	5.7	Positive	Positive
	Powdered goat milk	21	*Cronobacter malonaticus* FSL F6-030	Finished RTE Product (dairy)	Lyophilized culture: 2 week hold at 20-25ºC	0.59	5.7	Positive	Positive

Category	Item Type	Sample nº	Strain	Origin	Injury Protocol	Injury Evaluation (Log10)	Inoculation (CFU/Sample)	Alternative Method Result	Reference Method Result
	Low heat skim milk powder	22	*Cronobacter malonaticus* FSL F6-030	Finished RTE Product (dairy)	Lyophilized culture: 2 week hold at 20-25ºC	0.59	5.7	Positive	Positive
	Deep milk powder	29	*Cronobacter dublinensis* CCUG 55851	Milk powder, production facility	Lyophilized culture: 2 week hold at 20-25ºC	0.75	3.3	Positive	Positive
	Instant nonfat dry milk powder	30	*Cronobacter dublinensis* CCUG 55851	Milk powder, production facility	Lyophilized culture: 2 week hold at 20-25ºC	0.75	3.3	Positive	Positive
	Organic goat milk powder	33	*Cronobacter dublinensis* CCUG 55851	Milk powder, production facility	Lyophilized culture: 2 week hold at 20-25ºC	0.75	3.3	Positive	Positive
	Camel milk powder	37	*Cronobacter dublinensis* CCUG 55851	Milk powder, production facility	Lyophilized culture: 2 week hold at 20-25ºC	0.75	3.3	Positive	Positive
	Organic camel milk powder	39	*Cronobacter dublinensis* CCUG 55851	Milk powder, production facility	Lyophilized culture: 2 week hold at 20-25ºC	0.75	3.3	Positive	Positive
	Fat-free milk powder	40	*Cronobacter sakazakii* CCUG 10788	Tin of milk	Lyophilized culture: 2 week hold at 20-25ºC	0.56	5.0	Positive	Positive
	GMO-free milk powder	41	*Cronobacter sakazakii* CCUG 10788	Tin of milk	Lyophilized culture: 2 week hold at 20-25ºC	0.56	5.0	Positive	Positive

Category	Item Type	Sample nº	Strain	Origin	Injury Protocol	Injury Evaluation (Log10)	Inoculation (CFU/Sample)	Alternative Method Result	Reference Method Result
Infant Formula and Infant Cereal	100% instant nonfat dry milk	43	*Cronobacter sakazakii* CCUG 10788	Tin of milk	Lyophilized culture: 2 week hold at 20-25ºC	0.56	5.0	Positive	Positive
	Pure skim milk powder	44	*Cronobacter sakazakii* CCUG 10788	Tin of milk	Lyophilized culture: 2 week hold at 20-25ºC	0.56	5.0	Positive	Positive
	Low fat milk powder	46	*Cronobacter sakazakii* CCUG 10788	Tin of milk	Lyophilized culture: 2 week hold at 20-25ºC	0.56	5.0	Positive	Positive
	Real skim milk, fat free	48	*Cronobacter muytjensii* FSL F6-031	Finished RTE Product (dairy)	Lyophilized culture: 2 week hold at 20-25ºC	0.63	4.7	Positive	Positive
	Dry milk, instant nonfat dry milk powder	50	*Cronobacter muytjensii* FSL F6-031	Finished RTE Product (dairy)	Lyophilized culture: 2 week hold at 20-25ºC	0.63	4.7	Positive	Positive
	Organic fat free milk powder	51	*Cronobacter muytjensii* FSL F6-031	Finished RTE Product (dairy)	Lyophilized culture: 2 week hold at 20-25ºC	0.63	4.7	Positive	Positive
	Nonfat powdered goat milk	52	*Cronobacter muytjensii* FSL F6-031	Finished RTE Product (dairy)	Lyophilized culture: 2 week hold at 20-25ºC	0.63	4.7	Positive	Positive
	Organic coconut milk powder	53	*Cronobacter muytjensii* FSL F6-031	Finished RTE Product (dairy)	Lyophilized culture: 2 week hold at 20-25ºC	0.63	4.7	Positive	Positive
	Premium organic milk powder	56	*Cronobacter sakazakii* FSL F6-032	Finished RTE Product (dairy)	Lyophilized culture: 2 week hold at 20-25ºC	0.67	3.0	Positive	Positive
	High heat nonfat dry milk	58	*Cronobacter sakazakii* FSL F6-032	Finished RTE Product (dairy)	Lyophilized culture: 2 week hold at 20-25ºC	0.67	3.0	Positive	Positive
	Hormone free non-fat dry milk powder	59	*Cronobacter sakazakii* FSL F6-032	Finished RTE Product (dairy)	Lyophilized culture: 2 week hold at 20-25ºC	0.67	3.0	Positive	Positive

Category	Item Type	Sample nº	Strain	Origin	Injury Protocol	Injury Evaluation (Log10)	Inoculation (CFU/Sample)	Alternative Method Result	Reference Method Result
	Whole goat milk powder	60	*Cronobacter sakazakii* FSL F6-032	Finished RTE Product (dairy)	Lyophilized culture: 2 week hold at 20-25ºC	0.67	3.0	Positive	Positive

Category	Item Type	Sample nº	Strain	Origin	Injury Protocol	Injury Evaluation (Log10)	Inoculation (CFU/Sample)	Alternative Method Result	Reference Method Result
Infant Formula and Infant Cereal	Organic lactose	2	*Cronobacter sakazakii* CCUG 64760	Environment, Industry	Lyophilized culture: 2 week hold at 20-25ºC	0.57	5.8	Positive	Positive
	Biotin	3	*Cronobacter sakazakii* CCUG 64760	Environment, Industry	Lyophilized culture: 2 week hold at 20-25ºC	0.57	5.8	Positive	Positive
	Folic acid	4	*Cronobacter sakazakii* CCUG 64760	Environment, Industry	Lyophilized culture: 2 week hold at 20-25ºC	0.57	5.8	Positive	Positive
	Whey protein	6	*Cronobacter sakazakii* CCUG 64760	Environment, Industry	Lyophilized culture: 2 week hold at 20-25ºC	0.57	5.8	Positive	Positive
	Mixed tocopherol concentrate	7	*Cronobacter sakazakii* CCUG 64760	Environment, Industry	Lyophilized culture: 2 week hold at 20-25ºC	0.57	5.8	Positive	Positive
	Calcium phosphate	10	*Cronobacter sakazakii* FSL F6-027	Environment, Food	Lyophilized culture: 2 week hold at 20-25ºC	0.64	4.2	Positive	Positive
	Manganese sulfate	13	*Cronobacter sakazakii* FSL F6-027	Environment, Food	Lyophilized culture: 2 week hold at 20-25ºC	0.64	4.2	Positive	Positive
	Potassium chloride	14	*Cronobacter sakazakii* FSL F6-027	Environment, Food	Lyophilized culture: 2 week hold at 20-25ºC	0.64	4.2	Positive	Positive

	Sodium citrate	15	*Cronobacter sakazakii* FSL F6-027	Environment, Food	Lyophilized culture: 2 week hold at 20-25ºC	0.64	4.2	Positive	Positive
	Zinc sulfate	18	*Cronobacter sakazakii* FSL F6-027	Environment, Food	Lyophilized culture: 2 week hold at 20-25ºC	0.64	4.2	Positive	Positive

Category	Item Type	Sample nº	Strain	Origin	Injury Protocol	Injury Evaluation (Log10)	Inoculation (CFU/Sample)	Alternative Method Result	Reference Method Result
Infant Formula and Infant Cereal	Infant formula with probiotics: Hypoallergenic	1	*Cronobacter malonaticus* CCUG 28859	Formula	Lyophilized culture: 2 week hold at 20-25ºC	0.79	4.6	Positive	Positive
	Infant formula with probiotics: Organic 0 months+	3	*Cronobacter malonaticus* CCUG 28859	Formula	Lyophilized culture: 2 week hold at 20-25ºC	0.79	4.6	Positive	Positive
	Infant formula with probiotics: Soothe	4	*Cronobacter malonaticus* CCUG 28859	Formula	Lyophilized culture: 2 week hold at 20-25ºC	0.79	4.6	Positive	Positive
	Infant formula with probiotics: Total comfort	10	*Cronobacter malonaticus* CCUG 28859	Formula	Lyophilized culture: 2 week hold at 20-25ºC	0.79	4.6	Positive	Positive
	Infant formula with probiotics: Good start	14	*Cronobacter malonaticus* CCUG 28859	Formula	Lyophilized culture: 2 week hold at 20-25ºC	0.79	4.6	Positive	Positive
	Infant formula with probiotics: Milk based	16	*Cronobacter condimenti* QL 17031.1	Infant formula	Lyophilized culture: 2 week hold at 20-25ºC	0.55	2.1	Positive	Positive
	Infant formula with probiotics: Fat malabsorption problems	17	*Cronobacter condimenti* QL 17031.1	Infant formula	Lyophilized culture: 2 week hold at 20-25ºC	0.55	2.1	Positive	Positive
	Infant formula with probiotics: For fussiness	19	*Cronobacter condimenti* QL 17031.1	Infant formula	Lyophilized culture: 2 week hold at 20-25ºC	0.55	2.1	Positive	Positive

Category	Item Type	Sample nº	Strain	Origin	Injury Protocol	Injury Evaluation (Log10)	Inoculation (CFU/Sample)	Alternative Method Result	Reference Method Result
	Infant formula with probiotics: For spit up	21	*Cronobacter condimenti* QL 17031.1	Infant formula	Lyophilized culture: 2 week hold at 20-25ºC	0.55	2.1	Positive	Positive
	Infant formula with probiotics: Stage 1	23	*Cronobacter condimenti* QL 17031.1	Infant formula	Lyophilized culture: 2 week hold at 20-25ºC	0.55	2.1	Positive	Positive
	Infant formula with probiotics: Stage 2	24	*Cronobacter dublinensis* QL 17031.2	Infant formula	Lyophilized culture: 2 week hold at 20-25ºC	0.66	3.9	Positive	Positive
	Infant formula with probiotics: Stage 3	25	*Cronobacter dublinensis* QL 17031.2	Infant formula	Lyophilized culture: 2 week hold at 20-25ºC	0.66	3.9	Positive	Positive
	Infant formula with probiotics: 0-12 Months	26	*Cronobacter dublinensis* QL 17031.2	Infant formula	Lyophilized culture: 2 week hold at 20-25ºC	0.66	3.9	Positive	Positive
	Infant formula with probiotics: Organic Lactose	28	*Cronobacter dublinensis* QL 17031.2	Infant formula	Lyophilized culture: 2 week hold at 20-25ºC	0.66	3.9	Positive	Positive
	Infant formula with probiotics: With iron	30	*Cronobacter dublinensis* QL 17031.2	Infant formula	Lyophilized culture: 2 week hold at 20-25ºC	0.66	3.9	Positive	Positive
	Infant formula with probiotics: Sensitive	31	*Cronobacter sakazakii* CCUG 28867	Formula	Lyophilized culture: 2 week hold at 20-25ºC	0.60	6.1	Positive	Positive
	Infant formula with probiotics: Stage 2 organic	32	*Cronobacter sakazakii* CCUG 28867	Formula	Lyophilized culture: 2 week hold at 20-25ºC	0.60	6.1	Positive	Positive

Category	Item Type	Sample nº	Strain	Origin	Injury Protocol	Injury Evaluation (Log10)	Inoculation (CFU/Sample)	Alternative Method Result	Reference Method Result
Infant Formula and Infant Cereal	Infant Cereal with probiotics: Oatmeal	2	*Cronobacter sakazakii* ATCC 29544	Child's throat	Lyophilized culture: 2 week hold at 20-25ºC	0.72	5.1	Positive	Positive
	Infant formula with probiotics: Rice and banana apple	5	*Cronobacter sakazakii* ATCC 29544	Child's throat	Lyophilized culture: 2 week hold at 20-25ºC	0.72	5.1	Positive	Positive
	Infant formula with probiotics: Organic with DHA	7	*Cronobacter sakazakii* ATCC 29544	Child's throat	Lyophilized culture: 2 week hold at 20-25ºC	0.72	5.1	Positive	Positive
	Infant formula with probiotics: Organic	9	*Cronobacter sakazakii* ATCC 29544	Child's throat	Lyophilized culture: 2 week hold at 20-25ºC	0.72	5.1	Positive	Positive
	Infant formula with probiotics: Oatmeal peach and apple	13	*Cronobacter sakazakii* ATCC 29544	Child's throat	Lyophilized culture: 2 week hold at 20-25ºC	0.72	5.1	Positive	Positive
	Infant formula with probiotics: Banana	14	*Cronobacter malonaticus* QL 17031.3	Infant formula	Lyophilized culture: 2 week hold at 20-25ºC	0.67	3.4	Positive	Positive
	Infant formula with probiotics: Multi-Grain	16	*Cronobacter malonaticus* QL 17031.3	Infant formula	Lyophilized culture: 2 week hold at 20-25ºC	0.67	3.4	Positive	Positive
	Infant formula with probiotics: Supported sitting	20	*Cronobacter malonaticus* QL 17031.3	Infant formula	Lyophilized culture: 2 week hold at 20-25ºC	0.67	3.4	Positive	Positive
	Infant formula with probiotics: Organic oatmeal	22	*Cronobacter malonaticus* QL 17031.3	Infant formula	Lyophilized culture: 2 week hold at 20-25ºC	0.67	3.4	Positive	Positive
	Infant formula with probiotics: Oatmeal banana strawberry	24	*Cronobacter malonaticus* QL 17031.3	Infant formula	Lyophilized culture: 2 week hold at 20-25ºC	0.67	3.4	Positive	Positive
	Infant formula with probiotics: Single grain rice	25	*Cronobacter sakazakii* CCUG 14558	Human throat, Child	Lyophilized culture: 2 week hold at 20-25ºC	0.69	2.2	Positive	Positive

	Infant formula with probiotics: Oats and quinoa	26	*Cronobacter sakazakii* CCUG 14558	Human throat, Child	Lyophilized culture: 2 week hold at 20-25ºC	0.69	2.2	Positive	Positive

Category	Item Type	Sample nº	Strain	Origin	Injury Protocol	Injury Evaluation (Log10)	Inoculation (CFU/Sample)	Alternative Method Result	Reference Method Result
Environmental Surfaces	Sponge (food industry)	2	*Cronobacter sakazakii* FSL F6-025	Environment, Food	Seeding 48 h at 5 ± 3°C	/	6.3	Positive	Positive
	Sponge (food industry)	6	*Cronobacter sakazakii* FSL F6-025	Environment, Food	Seeding 48 h at 5 ± 3°C	/	6.3	Positive	Positive
	Swab (food industry)	7	*Cronobacter sakazakii* FSL F6-025	Environment, Food	Seeding 48 h at 5 ± 3°C	/	6.3	Positive	Positive
	Swab (food industry)	11	*Cronobacter sakazakii* FSL F6-025	Environment, Food	Seeding 48 h at 5 ± 3°C	/	6.3	Positive	Positive
	Sponge (food industry)	14	*Cronobacter muytjensii* QL 17031.6	Environmental Isolate	Seeding 48 h at 5 ± 3°C	/	5.9	Positive	Positive
	Swab (food industry)	18	*Cronobacter muytjensii* QL 17031.6	Environmental Isolate	Seeding 48 h at 5 ± 3°C	/	5.9	Positive	Positive

Category	Item Type	Sample nº	Strain	Origin	Injury Protocol	Injury Evaluation (Log10)	Inoculation (CFU/Sample)	Alternative Method Result	Reference Method Result
Environmental Surfaces	Process water (food industry)	1	*Cronobacter dublinensis* CCUG 58095	Water Fountain Basin	Seeding 48 h at 5 ± 3°C	/	3.9	Positive	Positive

Category	Item Type	Sample nº	Strain	Origin	Injury Protocol	Injury Evaluation (Log10)	Inoculation (CFU/Sample)	Alternative Method Result	Reference Method Result
	Process water (food industry)	2	*Cronobacter dublinensis* CCUG 58095	Water Fountain Basin	Seeding 48 h at 5 ± 3°C	/	3.9	Positive	Positive
	Process water (food industry)	3	*Cronobacter dublinensis* CCUG 58095	Water Fountain Basin	Seeding 48 h at 5 ± 3°C	/	3.9	Positive	Positive
	Process water (food industry)	7	*Cronobacter dublinensis* CCUG 58095	Water Fountain Basin	Seeding 48 h at 5 ± 3°C	/	3.9	Positive	Positive
	Process water (food industry)	13	*Cronobacter dublinensis* CCUG 58095	Water Fountain Basin	Seeding 48 h at 5 ± 3°C	/	3.9	Positive	Positive
	Process water (food industry)	14	*Cronobacter malonaticus* CCUG 28869	Dish Brush	Seeding 48 h at 5 ± 3°C	/	4.4	Positive	Positive
	Process water (food industry)	15	*Cronobacter malonaticus* CCUG 28869	Dish Brush	Seeding 48 h at 5 ± 3°C	/	4.4	Positive	Positive
	Process water (food industry)	16	*Cronobacter malonaticus* CCUG 28869	Dish Brush	Seeding 48 h at 5 ± 3°C	/	4.4	Positive	Positive
	Process water (food industry)	18	*Cronobacter malonaticus* CCUG 28869	Dish Brush	Seeding 48 h at 5 ± 3°C	/	4.4	Positive	Positive
	Process water (food industry)	19	*Cronobacter malonaticus* CCUG 28869	Dish Brush	Seeding 48 h at 5 ± 3°C	/	4.4	Positive	Positive
	Process water (food industry)	21	*Cronobacter sakazakii* CCUG 28868	Dish Brush	Seeding 48 h at 5 ± 3°C	/	5.3	Positive	Positive

Category	Item Type	Sample nº	Strain	Origin	Injury Protocol	Injury Evaluation (Log10)	Inoculation (CFU/Sample)	Alternative Method Result	Reference Method Result
Environmental Surfaces	Dust (food industry)	7	*Cronobacter sakazakii* FSL F6-036	Environment, Food	Lyophilized culture: 2 week hold at 20-25ºC	0.61	5.4	Positive	Positive
	Dust (food industry)	11	*Cronobacter sakazakii* FSL F6-036	Environment, Food	Lyophilized culture: 2 week hold at 20-25ºC	0.61	5.4	Positive	Positive
	Dust (food industry)	19	*Cronobacter sakazakii* FSL F6-036	Environment, Food	Lyophilized culture: 2 week hold at 20-25ºC	0.61	5.4	Positive	Positive
	Dust (food industry)	21	*Cronobacter sakazakii* FSL F6-036	Environment, Food	Lyophilized culture: 2 week hold at 20-25ºC	0.61	5.4	Positive	Positive

ANNEX E: Raw data sensitivity study for the initial study

Infant Formula without Probiotics															
Item Type	**Sample nº**	**Reference Method: ISO 22964[1]**				**Alternative Method: GDS *Cronobacter* Tq II**									
						PCR		Confirmation[2]							
								10 g		375 g					
		CCI	Oxidase	Bruker MALDI	Final Result	10 g	375 g	CCI	Confirmation Procedures[3]	CCI	Confirmation Procedures[3]	Final Result (10 g)	Final Result (375 g)	Agreement (10 g)	Agreement (375 g)
Soy based	1	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
0-12 Months	2	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Pure Bliss	3	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Non-GMO	4	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Neosure	5	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Infant	6	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Toddler	7	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Stage 1 Non-GMO	8	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
12-24 Months	9	-	/	/	-	-	+	-	/	+	+	-	+	NA	PD
Stage 2 Non-GMO	10	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Stage 1	11	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Stage 2	12	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Stage 3 Non-GMO	13	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
With iron	14	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
With iron, 0-12 Months	15	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Non-GMO with Iron	16	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Stage 3	17	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic with lactose	18	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Organic Stage 1	19	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Organic Stage 2	20	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA

1. A paired analysis for the 10 g samples was conducted between the alternative and the reference method.
2. The alternative method samples were also subjected to a direct streak to CCI from the primary enrichment and confirmed following the ISO 22964:2017 reference method. All results were identical to the ISO 22964:2017 reference method.
3. The confirmation procedures included a spot oxidase test and final confirmation on the Bruker Biotyper MALDI.

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Infant Cereal without Probiotics															
Item Type	**Sample nº**	**Reference Method: ISO 22964[1]**				**Alternative Method: GDS *Cronobacter* Tq II**									
						PCR		Confirmation[2]							
								10 g		375 g					
		CCI	Oxidase	Bruker MALDI	Final Result	10 g	375 g	CCI	Confirmation Procedures[3]	CCI	Confirmation Procedures[3]	Final Result (10 g)	Final Result (375 g)	Agreement (10 g)	Agreement (375 g)
Oatmeal baby cereal	1	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Organic rice	2	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic with DHA	3	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Oatmeal Banana	4	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Step 1	5	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Single grain	6	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Oatmeal	7	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Step 2	8	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Step 3	9	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Stage 1	10	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Stage 2	11	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Multigrain	12	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Multigrain banana	13	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Multigrain apple	14	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Stage 3	15	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Organic with wholegrain	16	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Wholegrain	17	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Wholegrain oatmeal	18	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Wholegrain apple	19	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Single grain rice	20	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Single grain raisin	21	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Single grain banana	22	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Single grain apple	23	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA

1. A paired analysis for the 10 g samples was conducted between the alternative and the reference method.
2. The alternative method samples were also subjected to a direct streak to CCI from the primary enrichment and confirmed following the ISO 22964:2017 reference method. All results were identical to the ISO 22964:2017 reference method.
3. The confirmation procedures included a spot oxidase test and final confirmation on the Bruker Biotyper MALDI.

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Non-Probiotic Ingredients (that contain milk powder)															
Item Type	**Sample nº**	**Reference Method: ISO 22964**				**Alternative Method: GDS *Cronobacter* Tq II**									
						PCR		Confirmation[1]				Final Result (10 g)	Final Result (375 g)	Agreement (10 g)	Agreement (375 g)
								10 g		375 g					
		CCI	Oxidase	Bruker MALDI	Final Result	10 g	375 g	CCI	Confirmation Procedures[2]	CCI	Confirmation Procedures[2]				
Nonfat dairy milk powder	1	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Cow full cream milk powder, vitamin D	2	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Cow full cream milk powder, vitamin A	3	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Milk powder, vitamin D	4*	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Milk powder, vitamin A	5	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Instant milk powder	6	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Dry milk mix	7	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Sweetened chocolate milk powder	8	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Powdered milk, Grade A	9	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Fortified milk powderr	10	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Whole milk powder	11	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Nonfat dry milk powder	12	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Malted milk powder	13	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Skim milk powder	14	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Hormone free milk powder	15	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA

Full cream milk powder	16	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic nonfat dry milk powder	17	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA

1. The alternative method samples were also subjected to a direct streak to CCI from the primary enrichment and confirmed following the ISO 22964:2017 reference method. All results were identical to the ISO 22964:2017 reference method.
2. The confirmation procedures included a spot oxidase test and final confirmation on the Bruker Biotyper MALDI.

*Indicates a naturally contaminated sample

Non-Probiotic Ingredients (that contain milk powder)															
Item Type	**Sample nº**	**Reference Method: ISO 22964**				**Alternative Method: GDS *Cronobacter* Tq II**									
						PCR		Confirmation[1]							
								10 g		375 g					
		CCI	Oxidase	Bruker MALDI	Final Result	10 g	375 g	CCI	Confirmation Procedures[2]	CCI	Confirmation Procedures[2]	Final Result (10 g)	Final Result (375 g)	Agreement (10 g)	Agreement (375 g)
Gluten free milk powder	18	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Pure skim milk powder	19*	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Lowfat dry milk	20	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Powdered goat milk	21	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Low heat skim milk powder	22	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Buttermilk powder	23*	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Milk powder for adults	24	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Real milk powder	25	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Coconut milk powder, 5.25 oz	26	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Fat free skim milk powder	27	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Instant nonfat dry milk, 3 pouches	28	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Deep milk powder	29	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Instant nonfat dry milk powder	30	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA

Bovine milk powder	31	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Nonfat dry milk powder, 22 oz	32	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Organic goat milk powder	33	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Skim milk powder, 16 oz	34	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Canned milk, 16 oz	35	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA

1. The alternative method samples were also subjected to a direct streak to CCI from the primary enrichment and confirmed following the ISO 22964:2017 reference method. All results were identical to the ISO 22964:2017 reference method.
2. The confirmation procedures included a spot oxidase test and final confirmation on the Bruker Biotyper MALDI.

*Indicates a naturally contaminated sample

Non-Probiotic Ingredients (that contain milk powder)															
Item Type	**Sample nº**	**Reference Method: ISO 22964**				**Alternative Method: GDS *Cronobacter* Tq II**									
						PCR		Confirmation[1]							
								10 g		375 g					
		CCI	Oxidase	Bruker MALDI	Final Result	10 g	375 g	CCI	Confirmation Procedures[2]	CCI	Confirmation Procedures[2]	Final Result (10 g)	Final Result (375 g)	Agreement (10 g)	Agreement (375 g)
Powdered milk case pack	36	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Camel milk powder	37	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Milk powder, 7.2 oz	38	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Organic camel milk powder	39	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Fat-free milk powder	40	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
GMO-free milk powder	41	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Nonfat dry milk powder, 12 oz	42	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
100% instant nonfat dry milk	43	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Pure skim milk powder	44	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Instant nonfat dry milk, 9.6 oz	45	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA

Instant nonfat dry milk, 3.2 oz	46	-	/	/	-	+	+	+	+	+	+	+	+	PD	PD
Nonfat dry milk, 48 oz	47	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Real skim milk, fat free	48	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Powdered goat milk, vitamin D	49*	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Dry milk instant nonfat dry milk powder	50	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic fat free milk powder	51	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Nonfat powdered goat milk	52	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic coconut milk powder	53	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA

1. The alternative method samples were also subjected to a direct streak to CCI from the primary enrichment and confirmed following the ISO 22964:2017 reference method. All results were identical to the ISO 22964:2017 reference method.
2. The confirmation procedures included a spot oxidase test and final confirmation on the Bruker Biotyper MALDI.

*Indicates a naturally contaminated sample

Non-Probiotic Ingredients (that contain milk powder)

Item Type	Sample nº	Reference Method: ISO 22964				Alternative Method: GDS *Cronobacter* Tq II									
						PCR		Confirmation[1]							
								10 g		375 g					
		CCI	Oxidase	Bruker MALDI	Final Result	10 g	375 g	CCI	Confirmation Procedures[2]	CCI	Confirmation Procedures[2]	Final Result (10 g)	Final Result (375 g)	Agreement (10 g)	Agreement (375 g)
Organic goat milk powder	54*	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Nonfat dry milk powder, large can	55	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Premium organic milk powder	56	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Powdered milk sachet	57	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
High heat nonfat dry milk powder	58	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Hormone free nonfat dry milk powder	59	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Whole goat milk powder	60	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA

1. The alternative method samples were also subjected to a direct streak to CCI from the primary enrichment and confirmed following the ISO 22964:2017 reference method. All results were identical to the ISO 22964:2017 reference method.
2. The confirmation procedures included a spot oxidase test and final confirmation on the Bruker Biotyper MALDI.

*Indicates a naturally contaminated sample

Qualitative methods – Assurance GDS for Cronobacter TqII extension study Ref 2017 LR77 MCS report v1 02/03/23

MICROVAL® NEN

Non-Probiotic Ingredients															
Item Type	**Sample nº**	**Reference Method: ISO 22964**				**Alternative Method: GDS *Cronobacter* Tq II**									
						PCR		Confirmation[1]							
								10 g		375 g					
		CCI	Oxidase	Bruker MALDI	Final Result	10 g	375 g	CCI	Confirmation Procedures[2]	CCI	Confirmation Procedures[2]	Final Result (10 g)	Final Result (375 g)	Agreement (10 g)	Agreement (375 g)
Organic palm oil	1	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Organic lactose	2	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Biotin	3	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Folic acid	4	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic coconut oil	5	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Whey protein	6	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Mixed tocopherol concentrate	7	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic soy lecithin	8	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Cupric sulfate	9	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Calcium phosphate	10	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic soy oil	11	-	/	/	-	-	+	-	/	+	+	-	+	NA	PD
Scizochytrium oil (DHA)	12	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Manganese sulfate	13	-	/	/	-	-	+	-	/	+	+	-	+	NA	PD
Potassium chloride	14	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Sodium citrate	15	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Taurine	16*	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Ascorbyl palmitate	17	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Zinc sulfate	18	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Viamin A Palmitate	19*	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Vitamin D	20*	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA

1. The alternative method samples were also subjected to a direct streak to CCI from the primary enrichment and confirmed following the ISO 22964:2017 reference method. All results were identical to the ISO 22964:2017 reference method.
2. The confirmation procedures included a spot oxidase test and final confirmation on the Bruker Biotyper MALDI.

*Indicates a naturally contaminated sample

MICROVAL® NEN

Infant Formula with Probiotics															
Item Type	**Sample nº**	**Reference Method: ISO 22964**				**Alternative Method: GDS *Cronobacter* Tq II**									
						PCR		Confirmation[1]							
								10 g		375 g					
		CCI	Oxidase	Bruker MALDI	Final Result	10 g	375 g	CCI	Confirmation Procedures[2]	CCI	Confirmation Procedures[2]	Final Result (10 g)	Final Result (375 g)	Agreement (10 g)	Agreement (375 g)
Hypoallergenic	1	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic Combiotic	2	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Organic 0 months+	3	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Soothe	4	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Closest to breast milk	5	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Milk based for spit up	6	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Sensitive	7	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Combiotic Stage 1	8	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Combiotic Stage 2	9	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Total comfort	10	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Combiotic Stage 3	11	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Gentle start	12	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Easy to digest	13	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Good start stage 1	14	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Good start stage 2	15	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Milk based	16	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Fat malabsorption problems	17	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Good start stage 3	18	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
For fussiness	19	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Combiotic Infant	20	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA

1. The alternative method samples were also subjected to a direct streak to CCI from the primary enrichment and confirmed following the ISO 22964:2017 reference method. All results were identical to the ISO 22964:2017 reference method.
2. The confirmation procedures included a spot oxidase test and final confirmation on the Bruker Biotyper MALDI.

Infant Formula with Probiotics															
Item Type	**Sample nº**	**Reference Method: ISO 22964**				**Alternative Method: GDS *Cronobacter* Tq II**									
						PCR		Confirmation[1]							
								10 g		375 g					
		CCI	Oxidase	Bruker MALDI	Final Result	10 g	375 g	CCI	Confirmation Procedures[2]	CCI	Confirmation Procedures[2]	Final Result (10 g)	Final Result (375 g)	Agreement (10 g)	Agreement (375 g)
For spit up	21	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Comfort	22	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Stage 1	23	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Stage 2	24	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Stage 3	25	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
0-12 Months	26	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Comfort special	27	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Organic Lactose	28	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Birth – 12 months	29	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
With iron	30	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Sensitive	31	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Stage 2 organic	32	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA

1. The alternative method samples were also subjected to a direct streak to CCI from the primary enrichment and confirmed following the ISO 22964:2017 reference method. All results were identical to the ISO 22964:2017 reference method.
2. The confirmation procedures included a spot oxidase test and final confirmation on the Bruker Biotyper MALDI.

Qualitative methods – Assurance GDS for Cronobacter TqII extension study Ref 2017 LR77 MCS report v1 02/03/23

MICROVAL® NEN

Infant Cereal with Probiotics															
Item Type	**Sample nº**	**Reference Method: ISO 22964**				**Alternative Method: GDS *Cronobacter* Tq II**									
						PCR		Confirmation[1]				Final Result (10 g)	Final Result (375 g)	Agreement (10 g)	Agreement (375 g)
								10 g		375 g					
		CCI	Oxidase	Bruker MALDI	Final Result	10 g	375 g	CCI	Confirmation Procedures[2]	CCI	Confirmation Procedures[2]				
Banana cereal	1	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Oatmeal	2	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic with DHA Oatmeal	3*	-	/	/	-	-	+	-	-	+	+	-	+	NA	PD
Organic Mutli-Grain	4	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Rice and banana apple	5	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Rice banana oatmeal	6	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Organic with DHA	7	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic oatmeal with choline	8	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Organic	9	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Step 2	10	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Crawler	11	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Organic Variety Pack	12	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Oatmeal peach and apple	13	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Banana	14	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Pre and Probiotic	15	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Multi-Grain	16	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Pre and Probiotic Multigrain	17	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Singlegrain variety pack	18	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA

Multigrain variety pack	19	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Support sitting	20	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA

1. The alternative method samples were also subjected to a direct streak to CCI from the primary enrichment and confirmed following the ISO 22964:2017 reference method. All results were identical to the ISO 22964:2017 reference method.
2. The confirmation procedures included a spot oxidase test and final confirmation on the Bruker Biotyper MALDI.

*Indicates a naturally contaminated sample

Infant Cereal with Probiotics															
Item Type	**Sample n°**	**Reference Method: ISO 22964**				**Alternative Method: GDS *Cronobacter* Tq II**									
						PCR		Confirmation[1]							
								10 g		375 g					
		CCI	Oxidase	Bruker MALDI	Final Result	10 g	375 g	CCI	Confirmation Procedures[2]	CCI	Confirmation Procedures[2]	Final Result (10 g)	Final Result (375 g)	Agreement (10 g)	Agreement (375 g)
Pre and Probiotic singlegrain	21	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Organic oatmeal	22	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Sprouted whole grain brown rice	23	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Oatmeal banana strawberry	24	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Single grain rice	25	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Oats and quinoa	26	-	/	/	-	-	+	-	/	+	+	-	+	NA	PD
Quinoa	27	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Rice	28	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Wheat	29	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA
Sprouted whole grain buckwheat	30	-	/	/	-	-	-	-	/	-	/	-	-	NA	NA

1. The alternative method samples were also subjected to a direct streak to CCI from the primary enrichment and confirmed following the ISO 22964:2017 reference method. All results were identical to the ISO 22964:2017 reference method.
2. The confirmation procedures included a spot oxidase test and final confirmation on the Bruker Biotyper MALDI.

Surfaces (Sponges and Swabs)											
Item Type	**Sample nº**	**Reference Method: ISO 22964[1]**				**Alternative Method: GDS *Cronobacter* Tq II**					
		CCI	Oxidase	Bruker MALDI	Final Result	PCR	Confirmation[2] Sampling Device		Final Result (Sampling Device)	Agreement (Sampling Device)	
						Sampling Device	CCI	Confirmation Procedures[3]			
Sponge (food industry)	1	-	-	-	-	-	-	/	-	NA	
Sponge (food industry)	2	+	+	+	+	+	+	+	+	PA	
Swab (food industry)	3	-	-	-	-	-	-	/	-	NA	
Swab (food industry)	4	-	-	-	-	-	-	/	-	NA	
Swab (food industry)	5	-	-	-	-	-	-	/	-	NA	
Sponge (food industry)	6	+	+	+	+	+	+	+	+	PA	
Swab (food industry)	7	+	+	+	+	+	+	+	+	PA	
Sponge (food industry)	8	-	-	-	-	-	-	/	-	NA	
Sponge (food industry)	9	-	-	-	-	-	-	/	-	NA	
Sponge (food industry)	10	-	-	-	-	-	-	/	-	NA	
Swab (food industry)	11	+	+	+	+	+	+	+	+	PA	
Sponge (food industry)	12	-	-	-	-	-	-	/	-	NA	
Sponge (food industry)	13	-	-	-	-	-	-	/	-	NA	
Sponge (food industry)	14	+	+	+	+	+	+	+	+	PA	
Swab (food industry)	15*	+	+	+	+	+	+	+	+	PA	
Swab (food industry)	16	-	-	-	-	-	-	/	-	NA	
Swab (food industry)	17*	+	+	+	+	+	+	+	+	PA	
Swab (food industry)	18	+	+	+	+	+	+	+	+	PA	
Swab (food industry)	19*	+	+	+	+	+	+	+	+	PA	
Swab (food industry)	20*	+	+	+	+	+	+	+	+	PA	
Sponge (food industry)	21*	+	+	+	+	+	+	+	+	PA	
Sponge (food industry)	22*	+	+	+	+	+	+	+	+	PA	

1. A paired analysis was conducted between the alternative and the reference method.
2. The alternative method samples were also subjected to a direct streak to CCI from the primary enrichment and confirmed following the ISO 22964:2017 reference method. All results were identical to the ISO 22964:2017 reference method.
3. The confirmation procedures included a spot oxidase test and final confirmation on the Bruker Biotyper MALDI.

*Indicates a naturally contaminated sample

Process Water										
Item Type	**Sample nº**	**Reference Method: ISO 22964[1]**				**Alternative Method: GDS *Cronobacter* Tq II**				
		CCI	Oxidase	Bruker MALDI	Final Result	PCR	Confirmation[2] / Sampling Device		Final Result (25 mL)	Agreement (25 mL)
						25 mL	CCI	Confirmation Procedures[3]		
Process water (food industry)	1	+	+	+	+	+	+	**+**	+	PA
Process water (food industry)	2	+	+	+	+	+	+	**+**	+	PA
Process water (food industry)	3	+	+	+	+	+	+	**+**	+	PA
Process water (food industry)	4	-	-	-	-	-	-	**/**	-	NA
Process water (food industry)	5	-	-	-	-	-	-	**/**	-	NA
Process water (food industry)	6	-	-	-	-	-	-	**/**	-	NA
Process water (food industry)	7	+	+	+	+	+	+	**+**	+	PA
Process water (food industry)	8	-	-	-	-	-	-	**/**	-	NA
Process water (food industry)	9	-	-	-	-	-	-	**/**	-	NA
Process water (food industry)	10	-	-	-	-	-	-	**/**	-	NA
Process water (food industry)	11	-	-	-	-	-	-	**/**	-	NA
Process water (food industry)	12	-	-	-	-	-	-	**/**	-	NA
Process water (food industry)	13	+	+	+	+	+	+	**+**	+	PA
Process water (food industry)	14	+	+	+	+	+	+	**+**	+	PA
Process water (food industry)	15	+	+	+	+	+	+	**+**	+	PA
Process water (food industry)	16	+	+	+	+	+	+	**+**	+	PA
Process water (food industry)	17	-	-	-	-	-	-	**/**	-	NA
Process water (food industry)	18	+	+	+	+	+	+	**+**	+	PA
Process water (food industry)	19	+	+	+	+	+	+	**+**	+	PA
Process water (food industry)	20	-	-	-	-	-	-	**/**	-	NA
Process water (food industry)	21	+	+	+	+	+	+	**+**	+	PA

1. A paired analysis was conducted between the alternative and the reference method.
2. The alternative method samples were also subjected to a direct streak to CCI from the primary enrichment and confirmed following the ISO 22964:2017 reference method. All results were identical to the ISO 22964:2017 reference method.
   The confirmation procedures included a spot oxidase test and final confirmation on the Bruker Biotyper MALDI.

Dust Sweepings										
Item Type	**Sample nº**	**Reference Method: ISO 22964[1]**				**Alternative Method: GDS *Cronobacter* Tq II**				
		CCI	Oxidase	Bruker MALDI	Final Result	PCR	Confirmation[2] / Sampling Device		Final Result (25 g)	Agreement (25 g)
						25 g	CCI	Confirmation Procedures[3]		
Dust (food industry)	1	-	-	-	-	-	-	/	-	NA
Dust (food industry)	2	-	-	-	-	-	-	/	-	NA
Dust (food industry)	3*	+	+	+	+	+	+	+	+	PA
Dust (food industry)	4	-	-	-	-	-	-	/	-	NA
Dust (food industry)	5	-	-	-	-	-	-	/	-	NA
Dust (food industry)	6	-	-	-	-	-	-	/	-	NA
Dust (food industry)	7	+	+	+	+	+	+	+	+	PA
Dust (food industry)	8	-	-	-	-	-	-	/	-	NA
Dust (food industry)	9	-	-	-	-	-	-	/	-	NA
Dust (food industry)	10	-	-	-	-	-	-	/	-	NA
Dust (food industry)	11	+	+	+	+	+	+	+	+	PA
Dust (food industry)	12*	+	+	+	+	+	+	+	+	PA
Dust (food industry)	13	-	-	-	-	-	-	/	-	NA
Dust (food industry)	14	-	-	-	-	-	-	/	-	NA
Dust (food industry)	15	-	-	-	-	-	-	/	-	NA
Dust (food industry)	16	-	-	-	-	-	-	/	-	NA
Dust (food industry)	17*	+	+	+	+	+	+	+	+	PA
Dust (food industry)	18	-	-	-	-	-	-	/	-	NA
Dust (food industry)	19	+	+	+	+	+	+	+	+	PA
Dust (food industry)	20*	+	+	+	+	+	+	+	+	PA
Dust (food industry)	21	+	+	+	+	+	+	+	+	PA

1. A paired analysis was conducted between the alternative and the reference method.
2. The alternative method samples were also subjected to a direct streak to CCI from the primary enrichment and confirmed following the ISO 22964:2017 reference method. All results were identical to the ISO 22964:2017 reference method.
   The confirmation procedures included a spot oxidase test and final confirmation on the Bruker Biotyper MALDI.
   *Indicates a naturally contaminated sample

Qualitative methods – Assurance GDS for Cronobacter TqII extension study Ref 2017 LR77 MCS report v1 02/03/23

MICROVAL® NEN

Infant Formula without Probiotics															
Item Type	**Sample nº**	**Reference Method: ISO 22964[1]**				**Alternative Method: GDS *Cronobacter* Tq II: 72 hour hold at 2-8ºC**									
						PCR		Confirmation[2]							
								10 g		375 g					
		CCI	Oxidase	Bruker MALDI	Final Result	10 g	375 g	CCI	Confirmation Procedures[3]	CCI	Confirmation Procedures[3]	Final Result (10 g)	Final Result (375 g)	Agreement (10 g)	Agreement (375 g)
Soy based	1	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Pure Bliss	3	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Neosure	5	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Infant	6	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Toddler	7	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
12-24 Months	9	-	/	/	-	-	+	-	/	+	+	-	+	NA	PD
Stage 1	11	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Stage 2	12	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Stage 3	17	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA

1. A paired analysis for the 10 g samples was conducted between the alternative and the reference method.
2. The alternative method samples were also subjected to a direct streak to CCI from the primary enrichment and confirmed following the ISO 22964:2017 reference method. All results were identical to the ISO 22964:2017 reference method.
3. The confirmation procedures included a spot oxidase test and final confirmation on the Bruker Biotyper MALDI.

Infant Cereal without Probiotics															
Item Type	**Sample nº**	**Reference Method: ISO 22964[1]**				**Alternative Method: GDS *Cronobacter* Tq II: 72 hour hold at 2-8ºC**									
						PCR		Confirmation[2]							
								10 g		375 g					
		CCI	Oxidase	Bruker MALDI	Final Result	10 g	375 g	CCI	Confirmation Procedures[3]	CCI	Confirmation Procedures[3]	Final Result (10 g)	Final Result (375 g)	Agreement (10 g)	Agreement (375 g)
Organic rice	2	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Single grain	6	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Oatmeal	7	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Multigrain	12	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Multigrain banana	13	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Multigrain apple	14	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Wholegrain	17	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Wholegrain oatmeal	18	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Wholegrain apple	19	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Single grain rice	20	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Single grain raisin	21	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Single grain banana	22	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Single grain apple	23	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA

1. A paired analysis for the 10 g samples was conducted between the alternative and the reference method.
2. The alternative method samples were also subjected to a direct streak to CCI from the primary enrichment and confirmed following the ISO 22964:2017 reference method. All results were identical to the ISO 22964:2017 reference method.
3. The confirmation procedures included a spot oxidase test and final confirmation on the Bruker Biotyper MALDI.

Non-Probiotic Ingredients (that contain milk powder)															
Item Type	**Sample nº**	**Reference Method: ISO 22964**				**Alternative Method: GDS *Cronobacter* Tq II: 72 hour hold at 2-8ºC**									
						PCR		Confirmation[1]							
								10 g		375 g					
		CCI	Oxidase	Bruker MALDI	Final Result	10 g	375 g	CCI	Confirmation Procedures[2]	CCI	Confirmation Procedures[2]	Final Result (10 g)	Final Result (375 g)	Agreement (10 g)	Agreement (375 g)
Organic milk powder	4*	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Instant milk powder	6	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Dry milk mix	7	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Whole milk powder	11	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Nonfat dry milk powder	12	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Malted milk powder	13	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Skim milk powder	14	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Full cream milk powder	16	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic nonfat dry milk powder	17	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic skim milk powder	19*	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Powdered goat milk	21	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Low heat skim milk powder	22	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic nonfat dry milk powder	23*	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Deep milk powder	29	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Instant nonfat dry milk powder	30	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic goat milk powder	33	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Camel milk powder	37	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic camel milk powder	39	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Fat-free milk powder	40	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
GMO-free milk powder	41	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA

1. The alternative method samples were also subjected to a direct streak to CCI from the primary enrichment and confirmed following the ISO 22964:2017 reference method. All results were identical to the ISO 22964:2017 reference method.
2. The confirmation procedures included a spot oxidase test and final confirmation on the Bruker Biotyper MALDI.

*Indicates a naturally contaminated sample

Non-Probiotic Ingredients (that contain milk powder)															
Item Type	**Sample nº**	**Reference Method: ISO 22964**				**Alternative Method: GDS *Cronobacter* Tq II: 72 hour hold at 2-8ºC**									
						PCR		Confirmation[1]							
								10 g		375 g					
		CCI	Oxidase	Bruker MALDI	Final Result	10 g	375 g	CCI	Confirmation Procedures[2]	CCI	Confirmation Procedures[2]	Final Result (10 g)	Final Result (375 g)	Agreement (10 g)	Agreement (375 g)
100% instant nonfat dry milk	43	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Pure skim milk powder	44	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Nonfat dry milk, hormone free	46	-	/	/	-	+	+	+	+	+	+	+	+	PD	PD
Real skim milk, fat free	48	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic whole milk powder	49*	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Dry milk instant nonfat dry milk powder	50	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic fat free milk powder	51	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Nonfat powdered goat milk	52	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic coconut milk powder	53	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic goat milk powder	54*	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Premium organic milk powder	56	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
High heat nonfat dry milk powder	58	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Hormone free nonfat dry milk powder	59	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Whole goat milk powder	60	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA

1. The alternative method samples were also subjected to a direct streak to CCI from the primary enrichment and confirmed following the ISO 22964:2017 reference method. All results were identical to the ISO 22964:2017 reference method.
2. The confirmation procedures included a spot oxidase test and final confirmation on the Bruker Biotyper MALDI.

*Indicates a naturally contaminated sample

Non-Probiotic Ingredients															
		Reference Method: ISO 22964				Alternative Method: GDS *Cronobacter* Tq II: 72 hour hold at 2-8ºC									
						PCR		Confirmation[1]							
								10 g		375 g					
Item Type	**Sample nº**	CCI	Oxidase	Bruker MALDI	Final Result	10 g	375 g	CCI	Confirmation Procedures[2]	CCI	Confirmation Procedures[2]	Final Result (10 g)	Final Result (375 g)	Agreement (10 g)	Agreement (375 g)
Organic lactose	2	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Biotin	3	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Folic acid	4	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Whey protein	6	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Mixed tocopherol concentrate	7	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Calcium phosphate	10	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic sunflower oil	11*	-	/	/	-	-	+	-	/	+	+	-	+	NA	PD
Manganese sulfate	13	-	/	/	-	-	+	-	/	+	+	-	+	NA	PD
Potassium chloride	14	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Sodium citrate	15	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic coconut oil	16*	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Zinc sulfate	18	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic palm oil	19	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic soy oil	20*	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA

1. The alternative method samples were also subjected to a direct streak to CCI from the primary enrichment and confirmed following the ISO 22964:2017 reference method. All results were identical to the ISO 22964:2017 reference method.
2. The confirmation procedures included a spot oxidase test and final confirmation on the Bruker Biotyper MALDI.

*Indicates a naturally contaminated sample

MICROVAL® NEN

Infant Formula with Probiotics															
Item Type	**Sample nº**	**Reference Method: ISO 22964**				**Alternative Method: GDS *Cronobacter* Tq II: 72 hour hold at 2-8ºC**									
						PCR		Confirmation[1]							
								10 g		375 g					
		CCI	Oxidase	Bruker MALDI	Final Result	10 g	375 g	CCI	Confirmation Procedures[2]	CCI	Confirmation Procedures[2]	Final Result (10 g)	Final Result (375 g)	Agreement (10 g)	Agreement (375 g)
Hypoallergenic	1	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic 0 months+	3	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Soothe	4	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Total comfort	10	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Good start	14	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Milk based	16	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Fat malabsorption problems	17	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
For fussiness	19	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
For spit up	21	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Stage 1	23	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Stage 2	24	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Stage 3	25	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
0-12 Months	26	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic Lactose	28	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
With iron	30	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Sensitive	31	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Stage 2 organic	32	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA

1. The alternative method samples were also subjected to a direct streak to CCI from the primary enrichment and confirmed following the ISO 22964:2017 reference method. All results were identical to the ISO 22964:2017 reference method.
2. The confirmation procedures included a spot oxidase test and final confirmation on the Bruker Biotyper MALDI.

Qualitative methods – Assurance GDS for Cronobacter TqII extension study Ref 2017 LR77 MCS report v1 02/03/23

MICROVAL® NEN

Infant Cereal with Probiotics															
Item Type	**Sample nº**	**Reference Method: ISO 22964**				**Alternative Method: GDS *Cronobacter* Tq II: 72 hour hold at 2-8ºC**									
						PCR		Confirmation[1]							
								10 g		375 g					
		CCI	Oxidase	Bruker MALDI	Final Result	10 g	375 g	CCI	Confirmation Procedures[2]	CCI	Confirmation Procedures[2]	Final Result (10 g)	Final Result (375 g)	Agreement (10 g)	Agreement (375 g)
Oatmeal	2	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic oatmeal	3*	-	/	/	-	-	+	-	-	+	+	-	+	PA	PD
Rice and banana apple	5	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic with DHA	7	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic	9	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Oatmeal peach and apple	13	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Banana	14	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Mutli-Grain	16	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Support sitting	20	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Organic oatmeal	22	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Oatmeal banana strawberry	24	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Single grain rice	25	+	+	+	+	+	+	+	+	+	+	+	+	PA	PA
Oats and quinoa	26	-	/	/	-	-	+	-	/	+	+	-	+	NA	PD

1. The alternative method samples were also subjected to a direct streak to CCI from the primary enrichment and confirmed following the ISO 22964:2017 reference method. All results were identical to the ISO 22964:2017 reference method.
2. The confirmation procedures included a spot oxidase test and final confirmation on the Bruker Biotyper MALDI.

*Indicates a naturally contaminated sample

Surfaces (Sponges and Swabs)											
Item Type	**Sample nº**	**Reference Method: ISO 22964[1]**				**Alternative Method: GDS *Cronobacter* Tq II: 72 hour hold at 2-8ºC**					
		CCI	Oxidase	Bruker MALDI	Final Result	PCR	Confirmation[2] Sampling Device		Final Result (Sampling Device)	Agreement (Sampling Device)	
						Sampling Device	CCI	Confirmation Procedures[3]			
Sponge (food industry)	2	+	+	+	+	+	+	+	+	PA	
Sponge (food industry)	6	+	+	+	+	+	+	+	+	PA	
Swab (food industry)	7	+	+	+	+	+	+	+	+	PA	
Swab (food industry)	11	+	+	+	+	+	+	+	+	PA	
Sponge (food industry)	14	+	+	+	+	+	+	+	+	PA	
Swab (food industry)	15*	+	+	+	+	+	+	+	+	PA	
Swab (food industry)	17*	+	+	+	+	+	+	+	+	PA	
Swab (food industry)	18	+	+	+	+	+	+	+	+	PA	
Swab (food industry)	19*	+	+	+	+	+	+	+	+	PA	
Swab (food industry)	20*	+	+	+	+	+	+	+	+	PA	
Sponge (food industry)	21*	+	+	+	+	+	+	+	+	PA	
Sponge (food industry)	22*	+	+	+	+	+	+	+	+	PA	

1. A paired analysis was conducted between the alternative and the reference method.
2. The alternative method samples were also subjected to a direct streak to CCI from the primary enrichment and confirmed following the ISO 22964:2017 reference method. All results were identical to the ISO 22964:2017 reference method.
3. The confirmation procedures included a spot oxidase test and final confirmation on the Bruker Biotyper MALDI.

*Indicates a naturally contaminated sample

Process Water										
		Reference Method: ISO 22964[1]				**Alternative Method: GDS *Cronobacter* Tq II: 72 hour hold at 2-8ºC**				
						PCR	Confirmation[2] Sampling Device			
Item Type	**Sample nº**	CCI	Oxidase	Bruker MALDI	Final Result	25 mL	CCI	Confirmation Procedures[3]	Final Result (25 mL)	Agreement (25 mL)
Process water (food industry)	1	+	+	+	+	+	+	+	+	PA
Process water (food industry)	2	+	+	+	+	+	+	+	+	PA
Process water (food industry)	3	+	+	+	+	+	+	+	+	PA
Process water (food industry)	7	+	+	+	+	+	+	+	+	PA
Process water (food industry)	13	+	+	+	+	+	+	+	+	PA
Process water (food industry)	14	+	+	+	+	+	+	+	+	PA
Process water (food industry)	15	+	+	+	+	+	+	+	+	PA
Process water (food industry)	16	+	+	+	+	+	+	+	+	PA
Process water (food industry)	18	+	+	+	+	+	+	+	+	PA
Process water (food industry)	19	+	+	+	+	+	+	+	+	PA
Process water (food industry)	21	+	+	+	+	+	+	+	+	PA

1. A paired analysis was conducted between the alternative and the reference method.
2. The alternative method samples were also subjected to a direct streak to CCI from the primary enrichment and confirmed following the ISO 22964:2017 reference method. All results were identical to the ISO 22964:2017 reference method.
3. The confirmation procedures included a spot oxidase test and final confirmation on the Bruker Biotyper MALDI.

Dust Sweepings											
Item Type	**Sample nº**	**Reference Method: ISO 22964[1]**				**Alternative Method: GDS *Cronobacter* Tq II: 72 hour hold at 2-8ºC**					
		CCI	Oxidase	Bruker MALDI	Final Result	PCR	Confirmation[2] Sampling Device		Final Result (25 g)	Agreement (25 g)	
						25 g	CCI	Confirmation Procedures[3]			
Dust (food industry)	3*	+	+	+	+	+	+	+	+	PA	
Dust (food industry)	7	+	+	+	+	+	+	+	+	PA	
Dust (food industry)	11	+	+	+	+	+	+	+	+	PA	
Dust (food industry)	12*	+	+	+	+	+	+	+	+	PA	
Dust (food industry)	17*	+	+	+	+	+	+	+	+	PA	
Dust (food industry)	19	+	+	+	+	+	+	+	+	PA	
Dust (food industry)	20*	+	+	+	+	+	+	+	+	PA	
Dust (food industry)	21	+	+	+	+	+	+	+	+	PA	

1. A paired analysis was conducted between the alternative and the reference method.
2. The alternative method samples were also subjected to a direct streak to CCI from the primary enrichment and confirmed following the ISO 22964:2017 reference method. All results were identical to the ISO 22964:2017 reference method.
3. The confirmation procedures included a spot oxidase test and final confirmation on the Bruker Biotyper MALDI.

*Indicates a naturally contaminated sample

ANNEX FRaw data on inclusivity and exclusivity for the initial study

Inclusivity									
Sample nº	Genus	Specie	Source	Origin	Inoculation Level on CCI (CFU/ml)	Alternative Method: GDS *Cronobacter* Tq II			
						PCR	CCI	Oxidase	Bruker MALDI
1	*Cronobacter*	*sakazakii*	ATCC[1] 29544	Child's Throat	49	+	+	+	+
2	*Cronobacter*	*sakazakii*	QL[2] 17031.4	Infant Formula	53	+	+	+	+
3	*Cronobacter*	*sakazakii*	CCUG[3] 10788	Tin of Milk	37	+	+	+	+
4	*Cronobacter*	*sakazakii*	CCUG 14558	Human Throat, Child	44	+	+	+	+
5	*Cronobacter*	*sakazakii*	CCUG 21205	Not Available	61	+	+	+	+
6	*Cronobacter*	*sakazakii*	CCUG 28857	Human Cerebrospinal Fluid	55	+	+	+	+
7	*Cronobacter*	*sakazakii*	CCUG 28858	Human Cerebrospinal Fluid	29	+	+	+	+
8	*Cronobacter*	*sakazakii*	CCUG 28860	Formula	32	+	+	+	+
9	*Cronobacter*	*sakazakii*	CCUG 28861	Human Cerebrospinal Fluid	46	+	+	+	+
10	*Cronobacter*	*sakazakii*	CCUG 28863	Human Cerebrospinal Fluid	41	+	+	+	+
11	*Cronobacter*	*sakazakii*	CCUG 28865	Human Cerebrospinal Fluid	58	+	+	+	+
12	*Cronobacter*	*sakazakii*	CCUG 28866	Human Vagina, newborn	22	+	+	+	+
13	*Cronobacter*	*sakazakii*	CCUG 28867	Formula	34	+	+	+	+
14	*Cronobacter*	*sakazakii*	CCUG 28868	Dish Brush	39	+	+	+	+
15	*Cronobacter*	*sakazakii*	CCUG 28870	Dish Brush	42	+	+	+	+
16	*Cronobacter*	*sakazakii*	CCUG 28821	Human Sputum, Colonization	47	+	+	+	+
17	*Cronobacter*	*sakazakii*	CCUG 29212	Human Cerebrospinal Fluid	33	+	+	+	+
18	*Cronobacter*	*sakazakii*	CCUG 64760	Environment, Industry	24	+	+	+	+
19	*Cronobacter*	*sakazakii*	QL 123015.1A	Rice Flour	43	+	+	+	+
20	*Cronobacter*	*sakazakii*	QL 11007.9	Rice Flour	48	+	+	+	+

1. ATCC – American Type Culture Collection
2. QL – Q Laboratories Culture Collection
3. CCUG – University of Goteborg Culture Collection

Inclusivity									
Sample nº	Genus	Specie	Source	Origin	Inoculation Level on CCI (CFU/ml)	Alternative Method: GDS *Cronobacter* Tq II			
						PCR	CCI	Oxidase	Bruker MALDI
21	*Cronobacter*	*sakazakii*	FSL[1] F6-023	Human Clinical	20	+	+	+	+
22	*Cronobacter*	*sakazakii*	FSL F6-024	Finished RTE Product (dairy)	18	+	+	+	+
23	*Cronobacter*	*sakazakii*	FSL F6-025	Environment, Food	32	+	+	+	+
24	*Cronobacter*	*sakazakii*	FSL F6-027	Environment, Food	55	+	+	+	+
25	*Cronobacter*	*sakazakii*	FSL F6-028	Human Clinical	67	+	+	+	+
26	*Cronobacter*	*sakazakii*	FSL F6-029	Human Clinical	60	+	+	+	+
27	*Cronobacter*	*sakazakii*	FSL F6-032	Finished RTE Product (dairy)	44	+	+	+	+
28	*Cronobacter*	*turicensis*	QL[2] 17031.5	Infant Formula	19	+	+	+	+
29	*Cronobacter*	*turicensis*	CCUG[3] 55852	Neonatal Meningitis	29	+	+	+	+
30	*Cronobacter*	*sakazakii*	FSL F6-036	Environment, Food	37	+	+	+	+
31	*Cronobacter*	*malonaticus*	QL123015.1	Not Available	41	+	+	+	+
32	*Cronobacter*	*malonaticus*	QL 17031.3	Infant Formula	34	+	+	+	+
33	*Cronobacter*	*condimenti*	QL 17031.1	Infant Formula	58	+	+	+	+
34	*Cronobacter*	*dublinensis*	QL 17031.2	Infant Formula	40	+	+	+	+
35	*Cronobacter*	*muytjensii*	QL 17031.6	Environmental Isolate	46	+	+	+	+
36	*Cronobacter*	*dublinensis*	CCUG 58095	Water Fountain Basin	27	+	+	+	+
37	*Cronobacter*	*malonaticus*	FSL F6-030	Finished RTE Product (Dairy)	16	+	+	+	+
38	*Cronobacter*	*muytjensii*	FSL F6-031	Finished RTE Product (Dairy)	36	+	+	+	+
39	*Cronobacter*	*malonaticus*	FSL F6-049	Human Clinical	40	+	+	+	+
40	*Cronobacter*	*malonaticus*	FSL F6-052	Human Clinical	21	+	+	+	+

1. FSL – Cornell Culture Collection
2. QL – Q Laboratories Culture Collection
3. CCUG – University of Goteborg Culture Collection

Inclusivity									
Sample nº	Genus	Specie	Source	Origin	Inoculation Level on CCI (CFU/ml)	Alternative Method: GDS *Cronobacter* Tq II			
						PCR	CCI	Oxidase	Bruker MALDI
41	*Cronobacter*	*universalis*	DSM[1] 27963	Not Available	29	+	+	+	+
42	*Cronobacter*	*dublinensis*	DSM 18706	Not Available	27	+	+	+	+
43	*Cronobacter*	*dublinensis*	CCUG[2] 55851	Milk Powder, Production Facility	33	+	+	+	+
44	*Cronobacter*	*dublinensis*	DSM 18705	Not Available	46	+	+	+	+
45	*Cronobacter*	*condimenti*	DSM 27966	Not Available	51	+	+	+	+
46	*Cronobacter*	*dublinensis*	DSM 18707	Not Available	39	+	+	+	+
47	*Cronobacter*	*malonaticus*	CCUG 28859	Formula	25	+	+	+	+
48	*Cronobacter*	*malonaticus*	CCUG 22864	Human Cerebrospinal Fluid	23	+	+	+	+
49	*Cronobacter*	*malonaticus*	CCUG 28869	Dish Brush	17	+	+	+	+
50	*Cronobacter*	*universalis*	NCTC[3] 9529	Not Available	20	+	+	+	+

1. DSM – Deutsche Sammlung vo Mikroorganismen und Zellkulturen GmbH
2. CCUG – University of Goteborg Culture Collection
3. NCTC – National Collection Type Culture

Exclusivity							
Sample nº	Genus	Specie	Source	Origin	Inoculation Level on TSA (CFU/ml)	Inoculation Level on CCI[3] (CFU/ml)	Alternative Method: GDS *Cronobacter* Tq II PCR
1	*Aeromonas*	*hydrophila*	ATCC[1] 49140	Clinical Isolate	3.7×10^5	0	-
2	*Aeromonas*	*virdans*	QL[2] 17041.8	Raw Milk	2.1×10^5	0	-
3	*Citrobacter*	*farmeri*	ATCC 51633	Human Feces	7.4×10^4	0	-
4	*Citrobacter*	*freundii*	QL 11007.10	Clinical Isolate	2.9×10^5	0	-
5	*Edwardsiella*	*tarda*	QL 11007.11	Clinical Isolate	1.1×10^5	0	-
6	*Enterobacter*	*amnigenus*	ATCC 51816	Milk	4.3×10^5	0	-
7	*Enterobacter*	*cancerogenus*	QL11010.1	Bottled Water	2.0×10^5	0	-
8	*Enterobacter*	*cloacae*	QL 100813-3A	Isolated Soy Protein	6.9×10^4	0	-
9	*Escherichia*	*coli* (O103)	QL 15071-2	Meat Powder	3.2×10^5	0	-
10	*Enterobacter*	*gregoviae*	QL 123009-1	Clinical Isolate	4.9×10^5	0	-
11	*Enterobacter*	*aerogenes*	ATCC 13048	Sputum	1.8×10^5	0	-
12	*Havnia*	*alvei*	ATCC 51815	Milk	$1.7x\ 10^5$	0	-
13	*Klebsiella*	*pneumoniae*	ATCC 10031	Clinical Isolate	2.4×10^5	0	-
14	*Klebsiella*	*oxytoca*	ATCC 43165	Clinical Isolate	3.9×10^5	0	-
15	*Franconibacter*	*helveticus*	QL 17031.9	Environmental Isolate	2.1×10^5	0	-
16	*Franconibacter*	*pulveris*	QL 17031.11	Milk Powder	3.0×10^5	0	-
17	*Siccibacter*	*turicensis*	QL 17031.7	Infant Formula	6.3×10^4	0	-
18	*Proteus*	*mirabilis*	QL 11007.6	Veterinary	5.4×10^4	0	-
19	*Morganella*	*morganii*	ATCC 25829	Human	4.1×10^5	0	-
20	*Pantoea*	*agglomerans*	ATCC 19552	Sewage	3.2×10^5	0	-

1. ATCC – American Type Culture Collection
2. QL – Q Laboratories Culture Collection
3. Only typical colonies were counted

Exclusivity							
Sample nº	Genus	Specie	Source	Origin	Inoculation Level on TSA (CFU/ml)	Inoculation Level on CCI[3] (CFU/ml)	Alternative Method: GDS *Cronobacter* Tq II PCR
21	*Serratia*	*marcescens*	QL[1] 11007.1	Bottled Water	2.2×10^5	0	-
22	*Proteus*	*vulgaris*	ATCC[2] 6380	Clinical Isolate	1.7×10^5	0	-
23	*Escherichia*	*coli*	QL 11010.2	Bottled Water	3.5×10^5	0	-
24	*Serratia*	*marcescens*	QL 11007.1	Bottled Water	2.9×10^5	0	-
25	*Pseudomonas*	*extremorientialis*	QL 17041.1	Raw Milk	3.6×10^5	0	-
26	*Providencia*	*stuartii*	QL 11007.5	Clinical Isolate	1.8×10^5	0	-
27	*Salmonella*	Infantis	ATCC 51741	Pasta	2.8×10^5	0	-
28	*Vibrio*	*vulnificus*	QL 021111A	Seafood Product	3.3×10^5	0	-
29	*Kocuria*	*rhizophila*	ATCC 3941	Soil	6.8×10^4	0	-
30	*Proteus*	*mirabilis*	ATCC 9240	Veterinary	4.0×10^5	0	-

1. QL – Q Laboratories Culture Collection
2. ATCC – American Type Culture Collection
3. Only typical colonies were counted

ANNEX G Raw data Relative Level of Detection

Matrix	**APC[1] (CFU/g)**		***Cronobacter* Pathogen Screen[2] (10 g test portions)**
Powdered Infant Formula without Probiotics	5.4 x 10^2		0/5
Matrix	**APC[1] (CFU/g)**	**LAB[3] (CFU/g)**	**Cronobacter Pathogen Screen[2] (10 g test portions)**
Infant Cereal with Probiotics	2.8 x 10^3	7.2 x 10^8	0/5
Matrix	**APC[1] (CFU/g)**		**Cronobacter Pathogen Screen[2] (10 g test portions)**
Milk Powder without Probiotics	4.9 x 10^3		0/5
Matrix	**APC[1] (CFU/mL)**		**Cronobacter Pathogen Screen[2] (25 mL test portions)**
Process Water	1.8 x 10^4		0/5

[1] APC conducted in accordance with ISO 4833-1.

[2] *Cronobacter* screen conducted following the ISO 22964 reference method

[3]LAB conducted in accordance with CMMEF Chapter 20

Infant Formula without Probiotics							
***Cronobacter sakazakii* QL 11007-9**							
Low Level: 0.37 (0.16, 0.67) MPN/Test Portion							
Sample #	**Assurance GDS® *Cronobacter* Tq II**						**ISO 22964:2017 Result[1]**
	PCR Presumptive Result		**Confirmed**				
			Direct Streak Result		**ISO 22964:2017 Result**		
	10 g	**375 g**	**10 g**	**375 g**	**10 g**	**375 g**	
1	+	-	+	-	+	-	+
2	-	+	-	+	-	+	-
3	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-
5	-	+	-	+	-	+	-
6	-	-	-	-	-	-	-
7	+	-	+	-	+	-	+
8	-	-	-	-	-	-	-
9	+	-	+	-	+	-	+
10	-	-	-	-	-	-	-
11	-	+	-	+	-	+	-
12	-	+	-	+	-	+	-
13	+	-	+	-	+	-	+
14	-	+	-	+	-	+	-
15	-	-	-	-	-	-	-
16	-	+	-	+	-	+	-
17	+	-	+	-	+	-	+
18	-	+	-	+	-	+	-
19	-	-	-	-	-	-	-
20	+	+	+	+	+	+	+
Total	**6/20**	**8/20**	**6/20**	**8/20**	**6/20**	**8/20**	**6/20**
High Level: 3.10 (1.31, 7.35) MPN/Test Portion							
1	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+
Total	**5/5**	**5/5**	**5/5**	**5/5**	**5/5**	**5/5**	**5/5**
Uninoculated							
1	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-
Total	**0/5**	**0/5**	**0/5**	**0/5**	**0/5**	**0/5**	**0/5**

1. A paired analysis was conducted for the 10g test portion size.

Infant Cereal with Probiotics							
***Cronobacter sakazakii* QL 123015-2**							
Low Level: 0.33 (0.16, 0.59) MPN/Test Portion							
Sample #	**Assurance GDS® *Cronobacter* Tq II**						**ISO 22964:2017 Result**
	PCR Presumptive Result		**Confirmed**				
			Direct Streak Result		**ISO 22964:2017 Result**		
	10 g	**375 g**	**10 g**	**375 g**	**10 g**	**375 g**	
1	-	+	-	+	-	+	-
2	-	-	-	-	-	-	-
3	+	-	+	-	+	-	-
4	-	-	-	-	-	-	+
5	-	+	-	+	-	+	-
6	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-
8	-	-	-	-	-	-	+
9	+	-	+	-	+	-	-
10	+	-	+	-	+	-	-
11	-	+	-	+	-	+	-
12	-	+	-	+	-	+	-
13	+	-	+	-	+	-	-
14	-	-	-	-	-	-	-
15	-	+	-	+	-	+	+
16	-	-	-	-	-	-	-
17	-	-	-	-	-	-	+
18	+	-	+	-	+	-	-
19	-	-	-	-	-	-	-
20	+	-	+	-	+	-	+
Total	**6/20**	**5/20**	**6/20**	**5/20**	**6/20**	**5/20**	**5/20**
High Level: 2.32 (1.02, 5.29) MPN/Test Portion							
1	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+
Total	**5/5**	**5/5**	**5/5**	**5/5**	**5/5**	**5/5**	**5/5**
Uninoculated							
1	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-
Total	**0/5**	**0/5**	**0/5**	**0/5**	**0/5**	**0/5**	**0/5**

Milk Powder without Probiotics							
***Cronobacter dublinensis* QL 17031.2**							
Low Level: 0.54 (0.28, 0.92) MPN/Test Portion							
Sample #	**Assurance GDS® *Cronobacter* Tq II**						**ISO 22964:2017 Result**
	PCR Presumptive Result		**Confirmed**				
			Direct Streak Result		**ISO 22964:2017 Result**		
	10 g	**375 g**	**10 g**	**375 g**	**10 g**	**375 g**	
1	+	-	+	-	+	-	-
2	-	+	-	+	-	+	+
3	-	-	-	-	-	-	+
4	+	-	+	-	+	-	-
5	-	+	-	+	-	+	-
6	-	-	-	-	-	-	-
7	-	-	-	-	-	-	+
8	-	-	-	-	-	-	-
9	+	+	+	+	+	+	+
10	+	+	+	+	+	+	-
11	-	-	-	-	-	-	-
12	+	+	+	+	+	+	-
13	-	-	-	-	-	-	+
14	+	+	+	+	+	+	-
15	-	-	-	-	-	-	-
16	+	-	+	-	+	-	-
17	-	+	-	+	-	+	+
18	-	-	-	-	-	-	+
19	+	+	+	+	+	+	-
20	-	+	-	+	-	+	-
Total	**8/20**	**9/20**	**8/20**	**9/20**	**8/20**	**9/20**	**7/20**
High Level: 4.60 (1.78, 11.94) MPN/Test Portion							
1	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+
Total	**5/5**	**5/5**	**5/5**	**5/5**	**5/5**	**5/5**	**5/5**
Uninoculated							
1	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-
Total	**0/5**	**0/5**	**0/5**	**0/5**	**0/5**	**0/5**	**0/5**

Process Water				
***Cronobacter muytjensii* QL 17031.6**				
Low Level: 0.76 (0.44, 1.26) MPN/Test Portion				
Sample #	**Assurance GDS® *Cronobacter* Tq II**			**ISO 22964:2017 Result[1]**
	PCR Presumptive Result	**Confirmed**		
		Direct Streak Result	**ISO 22964:2017 Result**	
1	+	+	+	+
2	-	-	-	-
3	+	+	+	+
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	+	+	+	+
8	-	-	-	-
9	-	-	-	-
10	-	-	-	-
11	-	-	-	-
12	+	+	+	+
13	-	-	-	-
14	+	+	+	+
15	+	+	+	+
16	+	+	+	+
17	-	-	-	-
18	+	+	+	+
19	+	+	+	+
20	+	+	+	+
Total	**10/20**	**10/20**	**10/20**	**10/20**
High Level: 2.98 (1.27, 6.95) MPN/Test Portion				
1	+	+	+	+
2	+	+	+	+
3	+	+	+	+
4	+	+	+	+
5	+	+	+	+
Total	**5/5**	**5/5**	**5/5**	**5/5**
Uninoculated				
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-

5	-	-	-	-
Total	**0/5**	**0/5**	**0/5**	**0/5**

1. A paired analysis was conducted.

Qualitative methods – Assurance GDS for Cronobacter TqII extension study Ref 2017 LR77 MCS report v1 02/03/23