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Validation of standard method EN ISO 22964:2017 — Microbiology of the food chain — Horizontal method for the detection of *Cronobacter* spp.

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ABSTRACT

The new version of the ISO standard method for detection of *Cronobacter* spp. (EN ISO 22964:2017) was validated in the frame of the European Commission Mandate M381 to CEN. Seventeen laboratories from nine countries participated in the interlaboratory studies to determine the performance characteristics of the method. The performance of the method was evaluated using matrices for which the presence of *Cronobacter* spp. is considered to be of serious concern, such as infant formula and its ingredients and representatives of categories cited in the EC Regulation 2073/2005 on microbiological criteria for foodstuffs for *Cronobacter* spp. The five matrices included in the validation were: two types of powdered infant food formulas (with and without probiotics); lactose; starch and environmental samples (swabs). The samples were each tested at two different levels of contamination, plus a negative control. Inoculation levels ranged from 4 to 95 CFU/sample. Each participant examined eight replicates of each level of inoculation, a total of 24 samples per matrix type.

Specificity was calculated for each matrix used in the validation, with results ranging between 99 and 100%. Sensitivity of the method was calculated for samples in which no fractional recovery was expected and the values that were obtained ranged between 65 and 100%, depending on the matrix, the inoculation level and the interfering microbiota present in the samples. LOD_{50} value was calculated for three food items (the two powdered infant formulas and the starch) with values between 0.8 and 1.1 CFU/sample.

1. Introduction

Cronobacter spp. are foodborne pathogens of special concern in infants, causing sepsis, meningitis, necrotising enterocolitis as a result of the consumption of contaminated infant formula powder (Farmer et al., 1980). *Cronobacter* spp. have the ability to persist in dry environments including powdered foods (Kalyantanda et al., 2015; Osaili and Forsythe, 2009) and can also affect immunocompromised adults.

European Union regulation (EC) No. 2073/2005 as amended by regulation (EU) No. 365/2010, requires the absence of *Cronobacter* spp. in 30 units of 10 g test portions of dried infant formulae and dried dietary foods for special medical purposes intended for infants below six months of age. No further identification of *Cronobacter* to the level of species is necessary (Anonymous, 2007, 2010). European Regulation prescribes the revised Standard horizontal method ISO 22964:2017 (Anonymous, 2017a) for the detection of *Cronobacter* spp. to address the potential risk to infants and neonates who may consume dried infant formulae and dried dietary foods.

The revision and validation of the standard has been prepared by the European Committee for Standardization (CEN), Technical Committee TC 275, Food Analysis, Working Group WG 6, Microbiology of the Food Chain, in collaboration with ISO/TC 34, Food products, Subcommittee SC 9. When compared with the previous version of this standard (ISO/TS 22964:2006) (IDF/RM 210:2006) "Milk and milk products - Detection of *Enterobacter sakazakii*" (Anonymous, 2006), the scope has been extended to the detection of *Cronobacter* spp. in food products for humans, feeding animals and environmental samples.

Since the reclassification of *E. sakazakii* as the genus, "*Cronobacter*," by Iversen et al., 2008a, there has been a need to change the scope of the former ISO/TS 22964:2006 to include the new genus that at present includes 7 species: *Cronobacter sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, *C. dublinensis*, *C. universalis* and *C. condimenti* (Forsythe et al.,

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2014; Iversen et al., 2008a; Joseph et al., 2012). Since then, there has been a proposal to reclassifying three *Enterobacter* species (*Enterobacter pulveris*, *Enterobacter helveticus* and *Enterobacter turicensis*) into the *Cronobacter* genus (Brady et al., 2013). However, Stephan et al., 2014 re-examined these three species and classified them as *Franconibacter pulveris*, *Franconibacter helveticus* and *Siccibacter turicensis* respectively.

As it was shown that several isolates of *Cronobacter* spp. do not grow well in the media and under conditions described in ISO TS 22964:2006, several modifications were introduced in the new standard (EN ISO 22964:2017) (Iversen et al., 2008b). In EN ISO 22964:2017, both culture media and temperature of incubation have been changed. The selective broth mLST (modified Lauryl Sulphate Broth) has been replaced by CSB (*Cronobacter* Selective Broth) and the isolation agar ESIA (*Enterobacter sakazakii* Isolation Agar) has been replaced by CCI (Chromogenic *Cronobacter* Isolation) agar. The temperature for the incubation of these two new media is 41.5 ± 1 °C. Also, several confirmation tests have been replaced by other tests as is shown in another section of this article.

CSB does not contain sodium lauryl sulphate or high levels of NaCl which could affect the recovery of sensitive strains (Iversen et al., 2008b). The antibiotic vancomycin remains in the new media that also contains sucrose, which is fermented by *Cronobacter* spp., producing in most of the cases a change in colour of the media. Unlike ESIA medium, CCI agar does not contain crystal violet and the amount of sodium deoxycholate has been reduced. On the other hand, it contains sodium thiosulphate and ammonium iron (III) citrate to differentiate *Cronobacter* spp. from hydrogen sulphide-producing *Enterobacteriaceae*.

Regarding the confirmation tests, the new revision of the method has eliminated the test for the production of yellow pigment after culturing on TSA (Tryptone Soy Agar) at 25 °C due to the fact that several isolates are not able to produce this characteristic (Besse et al., 2006) and included other tests such as the hydrolysis of 4-Nitrophenyl α-D glucopyranoside, D-arabitol for better differentiation with other *Enterobacteriaceae* (Iversen et al., 2007).

This article reports the methodology and results obtained in the validation of the EN ISO 22964:2017, in which five strains of *Cronobacter* spp., one for each of the five matrices, have been used.

2. Materials and methods

2.1. Design of the trials

Two rounds of interlaboratory studies (ILS) were organized in 2013, with a total of 17 laboratories from nine countries participating. The first round was dispatched in October 2013 and contained samples of two types of infant formula. The second round was dispatched in November 2013 and contained samples of lactose, starch and swabs. All laboratories were accredited and performed the analysis of *Cronobacter* spp. regularly.

The ILS were performed according to a common design elaborated by WG 3 Method Validation and WG 2 Statistics of ISO/TC 34/SC 9. Most of the participant laboratories were part of the working group TAG 14 organized for development and validation of the method and participated in meetings where the methodology and logistics were agreed and discussed previously to the beginning of the ILS. The five types of matrices used to validate the method were artificially inoculated to achieve two levels of contamination: a low level intended to obtain a fractional recovery (approximately 50% positive samples) and a high level containing ten times higher inoculum. Each type of matrix was inoculated with a different strain of *Cronobacter* spp. and some of the samples contained interfering microorganisms that were intentionally added or were naturally present as background microbiota.

Each participant received 24 samples of each type of matrix, eight replicates at three inoculum levels (high, low, and no inoculation).

Table 1
Confirmation tests for *Cronobacter* spp. according to the new version ISO 22964:2017.

Confirmation test	Expected result
Oxidase	−
Hydrolysis of 4-nitrophenyl α-D-glucopyranoside substrate	+
L-Lysine decarboxylase	−
L-Ornithine decarboxylase	−[a]
Methyl Red (optional)	−[a]
Voges-Proskauer (optional)	+[a]
Acid from:	
D-Arabitol	−[a]
D-Sorbitol	−
D-Sucrose	+
α-Methyl-D-glucoside (optional)	+[a]

[a] Most species show this characteristic.

2.2. Method under validation

The method validated is EN ISO 22964:2017, which has major changes from the previous ISO/TS 22964:2006.

The steps of the method are the following:

a) Pre-enrichment in Buffered Peptone Water (BPW) with incubation between 34 °C and 38 °C for 18 h ± 2 h.
b) Selective enrichment in *Cronobacter* Selective Broth (CSB, Iversen et al., 2008b) with incubation at 41.5 °C ± 1 °C for 24 h ± 2 h.
c) Plating out and identification on Chromogenic *Cronobacter* Isolation agar (CCI, Iversen et al., 2008b) with incubation at 41.5 °C ± 1 °C for 24 h ± 2 h.
d) Confirmation of the presence of *Cronobacter* spp. by biochemical tests on five presumptive colonies.

Characteristic colonies growing onto CCI, are selected, purified in a non-selective media and tested. Table 1 shows the confirmation test and expected results.

For the performance of the interlaboratory studies, laboratories also received different lots of commercially available culture media (CSB, ref. CM1121; CCI, ref. CM1122, and vancomycin supplement ref. SR0247E from Oxoid, ThermoFisher Scientific, UK). In order to facilitate the performance and practicability of the confirmation tests, miniaturized galleries (ID 32 E from bioMérieux, France) were used for the identification of colonies. Identification was based on the results obtained with the mandatory tests as described in the standard (Table 1).

2.3. Preparation of ILS samples

Samples for the ILS were prepared by individual inoculation in order to ensure homogeneity at low levels requested for the purpose of this validation. Strains of *Cronobacter* spp. were subcultured in TSB (Tryptone Soy Broth) at 37 °C ± 1 °C for 24 h ± 2 h to obtain fresh cultures that were inoculated by adding 0.1 ml of the appropriate dilution to each sample. To estimate the inoculum levels obtained in the samples, the appropriate dilution of these cultures was plated onto five TSA plates and incubated at 37 °C ± 1 °C for 24 h ± 2 h. Once inoculated, samples were left for 24 h in a laminar flow cabinet with the intention of producing stress in the bacteria by drying conditions. Samples were prepared in 10 g amounts for all the matrices except for the environmental sample, where each sample consisted in an individual swab (Deltalab, Barcelona ref. 300200). More details of the preparation of each matrix are given below.

2.3.1. Infant formula

Commercial milk based first stage infant formula powder that contained bifidobacteria at a level between 1×10^6 CFU/g and

1×10^7 CFU/g was inoculated with *Cronobacter sakazakii* NCTC 8155. Commercial soy based first stage infant formula was inoculated with *Cronobacter sakazakii* WDCM 00214. To act as an interferent, *Enterobacter cloacae* (5 CFU/10 g) was also inoculated at the higher level of contamination for this food item.

2.3.2. Ingredients

Starch and lactose were kindly provided by a company producing infant formula and supplements. Starch was inoculated with *Cronobacter muytjensii* (E-616, Nestlé Pathogen Culture Collection), provided by the Microbial & Molecular Analytics Group, Nestlé Research Center Nestec Ltd. and lactose was inoculated with *Cronobacter turicensis* DSM 18703, For these two types of matrices, the inoculum was placed in a carrier, by mixing it with skimmed milk powder (Scharlab S.L.) before inoculation of the samples. The use of the carrier was necessary because direct inoculation in the matrix was not possible due to the nature of these ingredients (starch form a gel around the inoculum and lactose produce an osmotic stress over the inoculated microorganism).

2.3.3. Environmental sample

Environmental samples were prepared by impregnation of swabs (previously humidified by adding 0.1 ml of 0.9% NaCl solution in water) with a mixture of infant milk powder naturally contaminated with high levels (1×10^6 CFU/g) of aerobic bacteria (*Bacillus* spp.). Swabs were inoculated with *Cronobacter dublinensis* spp. *dublinensis* DSM 18705. Blank samples were contaminated with a mixture of *Franconibacter* (formerly *Enterobacter*) *pulveris* DSM 19144 and *Franconibacter* (formerly *Enterobacter*) *helveticus* DSM 18396, in order to assess specificity.

2.4. Homogeneity and stability of the ILS samples

Preliminary tests were performed in the five types of matrices to assess the effect of drying over the inoculum and therefore to estimate the inoculum size needed to provide a fractional recovery in the samples inoculated at the low level.

Homogeneity and stability tests were conducted to characterize the evolution of the inoculated strains at different conditions. The tests were performed by monitoring the population inoculated in each matrix, for up to 14 days, analyzing samples along the time of incubation at 5 °C ± 3 °C. The population of *Cronobacter* spp. was estimated by performing *Enterobacteriaceae* count in VRBG and confirming colonies by MALDI-TOF (Bruker Daltonik Maldi Biotyper/Microflex LT/SH). Temperature abuse conditions were also studied at this stage by analyzing samples that have been kept 24 h at room temperature. In this preliminary study, homogeneity and stability was evaluated by analyzing 10 units per duplicate at three time-intervals (1, 7 and 14 days), with a contamination level between 1×10^2 and 1×10^3 CFU/g. Criteria of acceptance was based on ISO 13528:2005 (Anonymous, 2005), by comparing the between-samples standard deviation with the standard deviation for proficiency assessment (both in Log CFU/g), considered as 0.35.

Studies of stability and homogeneity were also done in each batch distributed for the ILS, after 24 h and after one or two weeks from the date of inoculation. To characterize these parameters, 20 or 10 replicates (depending on the inoculation level) were analysed for the presence of *Cronobacter* spp. using the EN ISO 22964:2017. The analysis of this number of samples was considered enough, as the preliminary work of this study showed good stability of *Cronobacter* spp. The standard deviation between samples from different storage times ranged from 0.09 to 0.16.

2.5. Performance characteristics of the method

The method was characterized by the calculation of the specificity, sensitivity and, when possible, the LOD_{50} derived from the data of the ILS. These parameters were calculated individually for each matrix.

Sensitivity was calculated by the number of samples found positive divided by the number of contaminated samples tested at a given level of contamination. The results are thus dependent on the level of contamination of the sample and this parameter was not calculated for the low level of inoculation when fractional recovery was intentionally obtained. The specificity was calculated by the number of samples found negative divided by the number of blank samples tested.

The LOD_{50} defined as the concentration (CFU/sample) for which the probability of detection is 50%, was calculated by using the spreadsheet by Dr. Cordula Wilrich and Prof. Peter Wilrich PODLOD calculation program (Wilrich and Wilrich, 2009). LOD_{50} was only possible to calculate when fractional recovery was intentionally obtained in the samples. The inoculum size used to calculate the LOD_{50} was assessed by performing MPN counts in five inoculated samples from each lot, using one series of 20 tubes.

3. Results and discussion

3.1. Stability and homogeneity of the ILS samples

According to the results of the preliminary study, strains of *Cronobacter* spp. inoculated in the two types of powdered infant formula and environmental samples were stable during at least 14 days at refrigeration temperatures and withstood the 24 h room temperature challenge (< 0.2 Log CFU/g of standard deviation between samples from different storage times). Stability for the samples of lactose and starch was not acceptable at first, as direct inoculation of the samples produced a rapid drop in the viability of the inoculum (initial counts in these samples dropped from 1×10^2 CFU/g to < 10 CFU/g). After testing different approaches for the inoculation of these two ingredients, the best way to overcome the lack of stability was to inoculate the *Cronobacter* strains in a carrier (skimmed dried milk) before placing the inoculum in these two matrixes. With this inoculation technique, the stability in lactose was still limited to 12 days storage, as it was observed that longer storage periods started to affect the viability of the inoculum, therefore participants were asked to analyze the samples within one week of arrival.

Homogeneity and stability of each batch of samples prepared for the first ILS were in agreement with the expected results, taking into account that samples for the low level of contamination were intentionally inoculated to obtain fractional recovery. The percentage of positives for inoculated samples at the low level of inoculation were 80% for the milk based powdered infant formula and 70% for the soy based powdered infant formula. For the higher level of inoculation and blank samples, the percentage of agreement with the expected result was 100% for these matrices.

For the second ILS, homogeneity for all samples was in agreement with the expected results. Fractional recovery was achieved only for starch, where 70% of samples at the low level of contamination were positive and 100% for the high level of contamination. The recovery of environmental samples and lactose at both levels of inoculation was 100% after preparation, but decreased for the lactose to 50% after 14 days of preparation. This result confirmed the low stability of the inoculum in this matrix, that was also found in the preliminary studies.

3.2. Performance characteristics of the method

The ILS were organized in two separate deliveries. For the first ILS launched in October 2013, 17 laboratories participated in the analysis of milk based powdered infant formula and 16 laboratories participated in the analysis of soy based powdered infant formula and returned the results. For both types of foods, two laboratories were discarded for technical reasons. One laboratory reported problems with the preparation of the isolation media (CCI). The other laboratory was unable

Table 2
Summary of participant results for the first round of the ILS. The results indicate the number of positive results for each set of 8 samples at the different inoculation levels (B negative; L low level; H high level).

Level	Laboratory code																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Milk based powdered infant formula																	
B	0	0	0	0	0	0	0	NR	1	0	0	0	0	NR	0	0	0
L	4	4	4	4	2	6	6	NR	6	5	3	3	6	NR	3	3	5
H	8	8	8	8	8	8	8	NR	8	8	8	8	8	NR	8	8	8
Soy based powdered infant formula																	
B	1	0	2	0	0	0	NT	NR	0	1	0	0	0	NR	0	0	0
L	5	7	7	8	5	6	NT	NR	5	8	6	7	8	NR	5	7	6
H[a]	4	3	6	7	7	6	NT	NR	4	8	7	6	5	NR	6	3	8

[a] Together with *Cronobacter sakazakii*, *Enterobacter cloacae* was inoculated at a level of 5 cfu/10 g at the higher level of contamination. NR, data not retained due to technical problems. NT, not tested.

to analyze the samples within the established time, so stability of the samples could not be assured. Three results from blank level of the soy based infant formula were not retained due to inconsistency with the primary data (the reported result was not in agreement with the raw data provided by the participant). Table 2 shows the results of the participants for this round.

For the second ILS that was launched in November 2013, 16 laboratories participated in the analysis of lactose, 15 laboratories participated in the analysis of environmental samples and 14 in the analysis of starch.

Data from the 16 laboratories that analysed lactose were retained. However, data from 5 laboratories analyzing environmental samples was discarded for inconsistency in the primary data and the delivered result. For the analysis of starch, data from two laboratories was not retained, one laboratory was discarded for reporting inconsistency in results and another for not doing the dilution requested in the instructions (Table 3).

Tables 4 to 8 show the parameters calculated for the five matrices used in the validation, depending on the inoculum level, type of matrix and the presence of interfering microbiota.

Sensitivity of the method was only calculated when non-fractional recovery was expected in the samples, this was the case of the high level of contamination, but not for the low level of contamination, in which three of the five matrices were inoculated with levels of contamination that produced fractional recovery. This level of contamination was intended in order to calculate the LOD_{50}. Sensitivity calculated in milk based powered infant formula, lactose and environmental samples for the high level of contamination ranged between 99 and 100%. These sensitivity values for the high level of inoculation are similar to the values obtained in other validation studies for standards such as ISO 6579-1:2017 for *Salmonella* spp. detection (Anonymous, 2017b) and ISO 11290-1:2017 for *Listeria monocytogenes* detection (Anonymous, 2017c). However, the sensitivity of the method was considerably lower (71%), for samples that were also inoculated with *Enterobacter cloacae* (soy based powered infant formula at high level contamination). This microorganism grew vigorously in the selective broth CSB, which includes only vancomycin to inhibit Gram-positive organisms, without intentional inhibition of bacteria such as *Enterobacteriaceae*. This is due to the fact that the majority of *Cronobacter* spp. are more sensitive to antibiotics than other background microbiota including species of *Enterobacter* (Druggan and Iversen, 2009). *E. cloacae* is able to grow at 41.5 °C producing non-characteristic colonies on CCI that may partially mask the growth of *Cronobacter* spp. Only laboratories able to distinguish typical colonies in the mixed culture were able to obtain good results. After these findings, extra samples were analysed by the organizer laboratory in order to compare CSB and CCI agar with the media prescribed in ISO/TS 24964:2006: mLST broth and ESIA. However,

Table 3
Summary of participant results for the second round of the ILS. The results indicate the number of positive results for each set of 8 samples at the different inoculation levels (B negative; L low level; H high level).

Level	Laboratory code															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Lactose																
B	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0
L	4	7	7	3	4	4	4	6	7	7	6	6	7	4	5	5
H	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Starch																
B	0	NR	0	0	0	NR	NT	0	0	0	0	0	0	0	0	NT
L	6	NR	5	6	8	NR	NT	4	7	5	7	7	6	8	6	NT
H	5	NR	4	5	6	NR	NT	5	6	5	5	6	5	8	2	NT
Environmental sample																
B	0	NR	0	0	NR	0	NR	0	0	NR	0	0	0	0	NR	NT
L	8	NR	8	5	NR	7	NR	8	8	NR	8	6	7	8	NR	NT
H	8	NR	8	8	NR	8	NR	8	8	NR	8	8	7	8	NR	NT

NR, data not retained due to technical problems. NT, not tested.

Table 4
Method performance obtained with milk based powdered infant food formula.

	Blank	Low inoculation level	High inoculation level
		4 cfu/10 g	55 cfu/10 g
Number of participating collaborators	17	17	17
Number of collaborators retained after evaluation of the data	15	15	15
Number of samples per lab	8	8	8
Number of samples retained after evaluation of the data	120	88[a]	120
Sample size (g)	10	10	10
Sensitivity %	–	–	100
Specificity %	99	–	–
LOD_{50}, (95% confidence interval) cfu/sample of 10 g	–	1.1 (0.8–1.4)	

[a] Data from 4 laboratories were not included in the calculation of LOD_{50} following the instructions of the PODLOD calculation program (Wilrich and Wilrich, 2009).

Table 5
Method performance obtained with soy based powdered infant food formula.

	Blank	Low inoculation level	High inoculation level
		4 cfu/10 g	50 cfu/10 g[a]
Number of participating collaborators	16	16	16
Number of collaborators retained after evaluation of the data	14	14	14
Number of samples per lab	8	8	8
Number of samples retained after evaluation of the data	109	112	112
Sample size (g)	10	10	10
Sensitivity %	–	–	71
Specificity %	99	–	–
LOD_{50}, (95% confidence interval) cfu/sample of 10 g	–	0.83 (0.66–1.05)	

[a] High inoculation samples also contained *Enterobacter cloacae*, at a concentration of 5 cfu/10 g.

Table 6
Method performance obtained with lactose.

	Blank	Low inoculation level	High inoculation level
		27 cfu/10 g	65 cfu/10 g
Number of participating collaborators	16	16	16
Number of collaborators retained after evaluation of the data	16	16	16
Number of samples per lab	8	8	8
Number of samples retained after evaluation of the data	128	128	128
Sample size (g)	10	10	10
Sensitivity %	–	67[a]	100
Specificity %	99	–	–
LOD_{50}, (95% confidence interval) cfu/sample of 10 g	–	ND	

ND, not determined.

[a] The low sensitivity of *Cronobacter* spp. detection at this level was likely due to loss of viability of the inoculated bacteria observed after the preparation of the samples.

Table 7
Method performance obtained with starch.

	Blank	Low inoculation level	High inoculation level
		11 cfu/10 g	19 cfu/10 g
Number of participating collaborators	14	14	14
Number of collaborators retained after evaluation of the data	12	12	12
Number of samples per lab	8	8	8
Number of samples retained after evaluation of the data	96	96	96
Sample size (g)	10	10	10
Sensitivity %	–	–	65[a]
Specificity %	100	–	–
LOD_{50}, (95% confidence interval) cfu/sample of 10 g	–	0.94 (0.73–1.21)	

[a] The low sensitivity of *Cronobacter* spp. detection at this level can be due to obtaining a lower inoculum level than expected in these samples.

Table 8
Method performance obtained with environmental samples (swabs).

	Blank	Low inoculation level	High inoculation level
		28 cfu/item	95 cfu/item
Number of participating collaborators	15	15	15
Number of collaborators retained after evaluation of the data	10	10	10
Number of samples per lab	8	8	8
Number of samples retained after evaluation of the data	80	80	80
Sample type (item)	Swab	Swab	Swab
Sensitivity %	–	91	99
Specificity %	100	–	–
LOD_{50}, (95% confidence interval) cfu/sample of 10 g	–	ND	

ND, not detected.

there was not any improvement found in the recovery of *Cronobacter* spp. by using these media. When these results were discussed with TAG 14 members, some of the members had acknowledged the fact that *E. cloacae* can also interfere with the detection of *Cronobacter* spp. when using ISO/TS 24964:2006 method.

Sensitivity obtained for the high level of inoculation for starch samples was also lower than expected (65%). The reason for this value could be due to obtaining a lower inoculum level than expected in these samples, probably due to a dilution error in the preparation of the inoculum. The high level of contamination normally aims to be ten times higher than the low inoculation level, and in this case was less than double (19 CFU/10 g).

The inoculation of the lactose to the low level of contamination did not produce fractional recovery when assessed by the organizer laboratory, before sending the samples to the participants. However, the results obtained by the participants produced a considerable percentage of negative results at this level and the overall sensitivity obtained with this matrix was 67%. It is suspected that the stability of the inoculum, assessed in the preliminary studies for this matrix, could have been worse than expected in the samples analysed by the participants, therefore influencing the results. *Cronobacter* desiccation stress can reduce population on average 3.3 log CFU/g (Margot et al., 2016). Moreover, desiccation stress produced by lactose was found more harmful for several microorganisms than desiccation produced by milk powder (Lindner and Lindhart, 2017).

Specificity calculated with the blank samples was similar in all cases, varying from 99 to 100%, with some false positive results obtained in the two types of powdered milk and lactose. The most probable cause for these false positive results is the cross-contamination of the samples, either at the organizer's level or at the participating laboratories' level. These specificity values are similar to the values obtained in other validation studies for standards such as EN ISO 11290-1:2017 for *Listeria monocytogenes* detection and EN ISO 10273:2017 for pathogenic *Yersinia enterocolitica* (Anonymous, 2017d).

The LOD_{50} was calculated for three matrices, the two infant formulas and the starch. Values ranged between 0.8 and 1.1 CFU/10 g, very similar to the LOD_{50} values (between 0.8 and 1.2 CFU) obtained in similar food items (milk and soy infant formula, swabs) with the previous ISO/TS 22964:2006 conducted by a single laboratory validation for alternative methods (AFNOR Validation). For the environmental samples and lactose, it was not possible to calculate this parameter because the low level of inoculation was too high for this purpose, not producing a fractional recovery.

4. Conclusion

The new method EN ISO 22964:2017 for the detection of *Cronobacter* spp. has been successfully validated in five different matrices, including ingredients and environmental samples.

Performance characteristics calculated for the method in the different matrices and background microbiota were generally good, obtaining high values for the sensitivity and specificity of the method in most cases and an adequate LOD_{50} for the three matrices where this parameter was calculated. Nevertheless, the influence of several factors was observed in some of the result. Sensitivity of the method was affected by the presence of interfering microbiota, such as *Enterobacter cloacae*. However, it was not affected by the presence of bifidobacteria and *Bacillus* spp. *Enterobacter cloacae* grows in the isolation agar producing non-characteristics colonies, but can become predominant making it difficult to detect *Cronobacter* colonies. As a consequence of these results, a note is included in the new standard to make users aware of this fact. Regarding the effect of the matrix, some ingredients such lactose can have an inhibitory effect on the inoculated *Cronobacter*, affecting artificially the result obtained for the sensitivity in this matrix at the low level of contamination.

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