Comparison of methods for the microbiological identification and profiling of

***Cronobacter* species from ingredients used in the preparation of infant formula**

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Abstract

Cronobacter spp. (formerly *Enterobacter sakazakii*) can be isolated from a wide range of foods and environments, and its association with neonatal infections has drawn considerable attention from regulatory authorities. The principle route of neonatal infection has been identified as the ingestion of contaminated infant formula. A number of methods have been developed to identify *Cronobacter* spp., however these were before the most recent (2012) taxonomic revision of the genus into seven species. In this study, phenotyping, protein profiling and molecular methods were used to identify *Cronobacter* strains which had been recently isolated from ingredients used in the preparation of infant formula.

Pulsed field gel electrophoresis revealed that different *Cronobacter* strains had been recovered from the same food products. All isolates were identified as *C. sakazakii* according to four genus specific PCR probes and protein profiling using MALDI-TOF analysis. However, 16S rDNA sequence analyses and *fusA* allele sequencing gave more accurate identification: four strains were *C. sakazakii*, one strain was *C. malonaticus* and the remaining strain was *C. universalis*. Multilocus sequence typing showed the strains were different sequence types.

These results demonstrate the presence of different *Cronobacter* species in food ingredients used in the preparation of infant formula, and also the need for molecular identification and profiling methods to be revised according to taxonomic revisions.

1. Introduction

Cronobacter spp. are Gram negative, motile, non-spore forming, peritrichous rods of the *Enterobacteriaceae* family [1,2]. The former use of the name *Enterobacter sakazakii* has been formally replaced by the seven species of the *Cronobacter* genus; *C. sakazakii, C. malonaticus, C. muytjensii, C. turicensis, C. dublinensis, C. universalis,* and *C. condimenti* [3,4,5]. A multilocus sequence typing (MLST) based on the DNA sequence of seven alleles has been established to identify the diversity of the genus and is supported by an open access database (www.pubMLST.org/cronobacter)[6,7]. Phylogenetic analysis, using multilocus sequence analysis (MLSA), predicts that the *Cronobacter* genus split from its closest ancestor in the *Enterobacteriaceae* family approximately 45-68 million years ago (MYA), with *C. sakazakii* further emerging ~15-23 MYA [7].

Although *Cronobacter* infections occur in all age groups, the greatest attention has been given to infections in neonates and infants [8,9,10,11]. To date only three *Cronobacter* species have been isolated from neonatal infections: *C. sakazakii, C. malonaticus,* and *C. turicensis* [2,7]. Of particular significance is the clonal lineage *C. sakazakii* ST4, as this is strongly associated with the neonatal meningitis cases around the world [12]. Contaminated PIF has been associated with many, but not all, neonatal cases of *Cronobacter* infection [11, 13, 14, 15]. The organism can also be found in various foods such as grains, bread, sorghum, rice seeds, herbs, spices, meat, cheese, vegetables, and eggs [16].

A number of methods have been described to identify *Cronobacter* spp. isolates [17]. Many of these are PCR-primer pairs designed to generate a DNA product of a known size, which is interpreted as a positive result without further validation. 16S rDNA sequencing has been used for *Cronobacter* spp., though some studies have reported difficulties distinguishing between *C. sakazakii* and *C. malonaticus* [4]. Subsequently, a MLST scheme has been established for the genus [6,7]. This is based on the sequencing of seven alleles (*atpD, fusA,*

3

glnS, gltB, gyrB, infB, and *ppsA*), giving a 3036 base pair concatenated length which is used for MLSA. This scheme is supported by an online open access database with >390 strain entries; http://www.pubMLST.org/cronobacter. This method was used to support the recent formal recognition of two new *Cronobacter* species: *C. universalis* and *C. condimenti* [5]. MLST also revealed that the majority of isolates from *Cronobacter* infection of neonates leading to meningitis were due to one clonal lineage, *C. sakazakii* ST4 [12]. Recently, Joseph et al. [7] proposed the use of *fusA*, one of the MLST loci, to determine the *Cronobacter* species. It overcomes the ambiguities of microheterogeneties in 16S rDNA sequence analysis.

The objective of this study was to identify *Cronobacter* isolates from food ingredients used in the domestic preparation of infant formula. Due to the recent taxonomic revisions and subjective nature of phenotyping, this study compared the results obtained from a number of molecular and non-molecular methods.

2. Material and Methods

2.1 Materials

Twelve food ingredients were purchased from different local markets in Ankara, Turkey. These were rice flour, rye flour, oat flour, semolina, raw milk, minced meat, fennel, aniseed, camomile, parsley, spinach and broccoli. These are typical raw materials which are used for the domestic preparation of infant formula.

2.2 Methods

2.2.1 Isolation and identification of bacterial samples

Twenty-five grams of each food sample was added to 225 mL Buffered Peptone Water (Merck, Germany). After incubation at 37°C for 24 hours, 10 mL was inoculated into 90 mL *Enterobacteriaceae* Enrichment Broth (Oxoid ThermoFischer, UK) and incubated at 37°C

4

overnight. From the culture suspension, a loop was streaked on chromogenic Brillance *E.*

sakazakii DFI Agar (Oxoid ThermoFischer, UK) and incubated at 37°C for 20 hours.

Presumptive *Cronobacter* colonies (blue-green colouration) were streaked on Tryptone Soya

Agar (Oxoid ThermoFischer, UK) for purity checking before further analysis. Three isolates

were selected from each food sample. The isolates were phenotyped using API 20E and

ID32E (BioMérieux, France) according to the manufacturer's instructions.

2.2.2 Pulsed Field Gel Electrophoresis (PFGE)

PFGE of the isolates was performed following the PulseNet USA protocol for molecular

subtyping of nontyphoidal *Salmonella* serotypes, as previously used for clinical isolates of

Cronobacter spp. [18]. XbaI (Ozyme, France) was used as the restriction enzyme for the

digestion of DNA in the agarose plugs. The gel was run at switch times of 2.2- 63.8 seconds

for 20 hours 6 volts in a CHEF-DR II system (Bio-Rad, CA). PFGE patterns were analyzed

using Bionumerics software (Applied Maths, Belgium).

2.2.3 Protein profiling of Cronobacter strains

MALDI-TOF analyses of strains was by Accugenix (Delawarre, USA) using their Bucker

system and interpreted according to their in-house database.

2.2.4 Polymerase Chain Reaction (PCR) probes for Cronobacter spp. identification

Genomic DNA was prepared with GenElute bacterial DNA kit (Sigma-Aldrich, MO) from 1.5

mL overnight culture of Tryptic Soy Broth (Oxoid ThermoFischer, UK) at 37°C according to

manufacturer's instructions. Each 25 µl amplification reaction mixture comprised ~10 ng

chromosomal DNA, 20 pmol forward and reverse primer (Sigma-Aldrich, UK), 1× PCR

buffer (Promega, UK) containing 1.5 mM $MgCl_2$, 0.8 mM deoxynucleotide triphosphates and

1.25 U Taq (Promega, UK). Four PCR primer pairs were used as given below, using 2.5 U GoTaq Flexi DNA polymerase enzyme (Promega Corporation, WI), 5x Green GoTaq Flexi buffer (Promega Corporation, WI), and a Genius thermocycler (Techne Ltd., UK). Keyser et al. [19] primer pairs Esak2 and Esak3 were used to amplify an 850-bp PCR product the 16S rDNA gene. Lehner et al. [20] primer pairs Esakf and Esakr were used to amplify 929-bp PCR product from the 16S rDNA gene. The *ompA* gene was amplified with primers ESSF and ESSR, resulting in a 469-bp product, as described by Mohan Nair and Venkitanarayanan [21]. The method of Kuhnert et al. [22] used RecN-Es-L and RecN-ES-R to amplify a 1662-bp PCR product. PCR products were visualized on 50mL, 1% agarose gels stained with 5µL ml^{-1} Sybr® Safe DNA gel stain (Invitrogen Corporation, CA).

2.2.5 DNA sequence analysis of 16S rDNA gene and MLST alleles

16S rDNA sequence analysis was performed as previously described by Joseph et al. [5]. Primers and conditions for amplification and sequencing of the MLST alleles *atpD* (390 bp), *fusA* (438 bp), *glnS* (363 bp), *gltB* (507 bp), *gyrB* (402 bp), *infB* (441 bp) and *ppsA* (495 bp) were as described previously [6]. The resultant seven allele sequences for each strain were uploaded to the *Cronobacter* pubMLST database (www.pubMLST.cronobacter) and can be downloaded for independent analysis.

2.2.6 Multi-Locus Sequence Typing (MLST) and phylogenetic analysis

DNA sequences were independently aligned with corresponding sequences from strains representing all species of the genus *Cronobacter* using MEGA5 [23]. Stability of the relationships was assessed by the bootstrap method (1000 replicates). The phylogenetic trees for *fusA* and all seven loci were constructed using existing sequences downloaded from the open access pubMLST database [7].

3. Results

3.1 Isolation of Cronobacter spp. from food samples.

Cronobacter strains were isolated from four out of 12 food ingredients; rice flour, rye flour, oat flour and fennel. No *Cronobacter* spp. were obtained from semolina, raw milk, minced meat, camomile, parsley, spinach or broccoli samples.

3.2 PFGE analyses

Since multiple presumptive *Cronobacter* colonies were picked from the *Cronobacter* primary isolation plates for each food sample, PFGE was used to ensure only unique strains were used for further detailed studies. Consequently from 12 initial isolates, only six were selected for further identification and analysis. Of the six strains, two were from oat flour (1437 and 1438), two from rye flour (1435 and 1436), and one each from rice flour (1432) and fennel seeds (1434).

3.3 Isolate identification according to phenotyping

Using two commercially available phenotyping kits, API20E and ID32E, the six strains were identified as *Enterobacter sakazakii*; Table 1. The BioMerieux online database does not recognize the *Cronobacter* genus, and therefore no further identification to species level was possible.

3.4 Isolate identification according to protein profiling

All six isolates were identified as *C. sakazakii* according to MALDI-TOF analysis.

3.5 Isolate identification according to molecular methods

The six selected strains produced PCR products with four PCR primer sets for *Cronobacter* spp.; Table 1. The 16S rDNA sequence based phylogenetic tree revealed the strains were from different *Cronobacter* species (results not shown). The rice flour isolate (1432) clustered with *C. malonaticus*, one rye flour isolate (1435) clustered with *C. universalis*. The other four strains (1436, 1437, 1438, 1434) were in the *C. sakazakii* cluster. This agreed with the identification based on *fusA* sequence analysis as shown in Figure 1. The corresponding phylogenetic tree, based on the concatenation of the seven allele sequences, is shown in Figure 2.

3.6 Genotyping using MLST analyses

The sequence types for the six *Cronobacter* isolates are shown in Table 1. The two *C. sakazakii* strains from oat flour were different sequence types (ST1 and ST52) and had 2% (66/3036) nucleotide differences across in all seven loci.

4. Discussion

Cronobacter was recovered from 4/12 food ingredients (rice flour, rye flour, oat flour and fennel) used in the domestic preparation of infant formula. The occurrence of *Cronobacter* in plant-based food ingredients has been reported many times already [24,25,26], and probably reflects the normal habitat for the bacterium [27]. Flour is used for starch production which is a common food ingredient especially in infant formula, and is a recognized source of *Cronobacter* spp. [9].

All isolates were identified as '*Enterobacter sakazakii*' via API 20E and ID 32E. It is surprising that these databases have not been updated with the post-2007 taxonomic revisions and do not recognize the *Cronobacter* genus. Despite the ease of use in routine laboratories,

8

phenotyping using such commercial kits has limited value for investigative studies due to the subjective nature of their results.

The PCR-primer pairs all gave PCR-products of the expected sizes for the six isolates, indicating they were *Cronobacter* spp., but these do not identify the particular species. The presumptive *Cronobacter* isolates were identified and speciated using 16S rDNA and *fusA* sequence analysis, with agreement between the two methods. There were four strains of *C. sakazakii*, and one each of *C. malonaticus* and *C. universalis*. The rye flour strain 1435 was used in the recent formal description of *Cronobacter universalis* sp. nov. as a newly recognized species of *Cronobacter* [5].

The recovery of different *Cronobacter* strains, even different species, from the same food ingredient sample shows the importance of picking multiple colonies from primary isolation plates. Rye flour contained two *Cronobacter* species, *C. sakazakii* strain 1436 and *C. universalis* strain 1435. Oat flour contained two different sequence types of *C. sakazakii*, which were ST1 and ST52 (strains 1437 and 1438) which differed in each of the seven sequenced loci and therefore are unlikely to be recent variants. These strains were not distinguishable according to colony morphologies, or API20E and ID32E phenotyping. The recovery of *C. sakazakii* ST1 from oat flour is of note since this sequence type had been associated with severe neonatal meningitis infection in US [14].

Our results demonstrate that different identifications can be obtained for the same strain of *Cronobacter*. Due to recent taxonomic revisions [7], various phenotypic and genotypic methods used for *Cronobacter* identification may require re-evaluation to determine their accuracy. Although commercial phenotyping kits are commonly preferred by food and clinical laboratories, molecular identification methods such as 16S rDNA, *fusA* and

MLST are much more reliable. The use of *fusA* and MLST sequence analysis has the added

advantage of an open access, curated online database; http://www.pubMLST.org/cronobacter.

Acknowledgements

References

[1] Kucerova, E., Clifton, S.W., Xia, X.Q., Long, F., Porwollik, S., Fulton, L., Fronick, C., Minx, P., Kyung, K., Warren, W., Fulton, R., Feng, D., Wollam, A., Shah, N., Bhonagiri, V., Nash, W.E., Hallsworth-Pepin, K., Wilson, R.K., McClelland, M., Forsythe, S.J., 2010. Genome sequence of *Cronobacter sakazakii* BAA-894 and comparative genomic hybridization analysis with other *Cronobacter* species. PLoS ONE 5:e9556.

[2] Kucerova, E., Joseph, S., Forsythe, S., 2011. The *Cronobacter* genus: ubiquity and diversity. Qual. Assur. Safety Crops & Foods. 3, 104-122.

[3] Iversen, C., Lehner, A., Mullane, N., Bidlas, E., Cleenwerck, I., Marugg, J., Fanning, S., Stephan, R., Joosten, H., 2007. The taxonomy of *Enterobacter sakazakii*: proposal of a new genus *Cronobacter* gen. nov. and descriptions of *Cronobacter sakazakii* comb. nov. *Cronobacter sakazakii* subsp. *sakazakii*, comb. nov., *Cronobacter sakazakii* subsp. *malonaticus* subsp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov. and *Cronobacter* genomospecies

1. BMC Evol. Biol. 7, 64.

[4] Iversen, C., Mullane, N., McCardell, B., *et al.* 2008. *Cronobacter* gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii.* Intl. J. Syst. Evol. Microbiol. 58, 1442–1447.

[5] Joseph, S., Cetinkaya, E., Drahovska, H., Levican, A., Figueras, M. and Forsythe, S.J., 2012a. *Cronobacter condimenti*, sp. nov., isolated from spiced meat and *Cronobacter universalis* sp. nov., a novel species designation for *Cronobacter* sp. genomospecies 1, recovered from a leg infection, water, and food ingredients. Intl. J. System. Evol. Microbiol. 62, 1277-1283.

[6] Baldwin, A., Loughlin, M., Caubilla-Barron, J., Kucerova, E., Manning, G., Dowson, C., Forsythe, S., 2009. Multilocus sequence typing of *Cronobacter sakazakii* and *Cronobacter malonaticus* reveals stable clonal structures with clinical significance which do not correlate with biotypes. BMC Microbiol. 9, 223.

[7] Joseph, S., Sonbol, H., Hariri, S., Desai, P., McClelland, M., Forsythe, S.J., 2012b. Diversity of the *Cronobacter* genus as revealed by multi locus sequence typing. J. Clin. Microbiol. 50, 3031-3039.

[8] Lai, K.K. 2001. *Enterobacter sakazakii* infections among neonates, infants, children, and adults. Medicine 80, 113–122.

[9] FAO/WHO, 2004. Workshop on *Enterobacter sakazakii* and other microorganisms in powdered infant formula, Geneva, 2–5 February 2004. Available at http://www.who.int/foodsafety/micro/jemra/meetings/feb2004/en/index.html.

[10] FAO/WHO, 2008. *Enterobacter sakazakii* (*Cronobacter* spp.) in powdered follow-up

formulae. Microbiological Risk Assessment Series No. 15, Washington. Available at

http://www.who.int/foodsafety/publications/micro/mra_followup/en/

[11] Centers for Disease Control and Prevention, 2011. CDC Update: investigation

of *Cronobacter* infections among infants in the United States.

http://www.cdc.gov/foodsafety/diseases/cronobacter/2011-investigation/.

[12] Joseph, S., Forsythe, S.J., 2011. Predominance of *Cronobacter sakazakii* sequence type 4

in neonatal infections. Emerg. Infect. Dis. 17, 1713-1715.

[13] van Acker, J., de Smet F., Muyldermans G., Bougatef A., Naessens

A., Lauwers S. 2001. Outbreak of necrotizing enterocolitis associated with

Enterobacter sakazakii in powdered milk formula. J. Clin. Microbiol. 39, 293–297.

[14] Himelright, I., Harris, E., Lorch, V., Anderson, M., 2002. *Enterobacter sakazakii*

infections associated with the use of powdered infant formula -Tennessee, 2001. J.

Am. Med. Assoc. 287, 2204–2205.

[15] Bowen, A.B., Braden, C.R., 2006. Invasive *Enterobacter sakazakii* disease in infants.

Emerg. Infect. Dis. 12, 1185-1189.

[16] Friedemann, M., 2007. *Enterobacter sakazakii* in food and beverages (other than infant

formula and milk powder). Int J Food Microbiol. 116,1-10.

[17] Fanning, S., Forsythe, S., 2008. Isolation and identification of *Enterobacter sakazakii*,

pp.27-60. In 'Emerging issue in food safety: *Enterobacter sakazakii* '. Eds. J.M. Farber

& S.J. Forsythe. ASM Press.

[18] Caubilla-Barron, J., Hurrell, E., Townsend, S., Cheetham, P., Loc-Carrillo, C., Fayet, O.,

Prere, M. -F., Forsythe, S.J., 2007. Genotypic and phenotypic analysis

of *Enterobacter sakazakii* strains from an outbreak resulting in fatalities in a neonatal

intensive care unit in France. J. Clin. Microbiol. 45, 3979-3985.

[19] Keyser, M., Witthuhn, R.C., Ronquest, L.C., Britz, T.J., 2003. Treatment of winery

effluent with upflow anaerobic sludge blanket (UASB)-granular sludges enriched with

Enterobacter sakazakii. Biotechnol. Lett. 25, 1893–1898.

[20] Lehner, A., Tasara, T., Stephan, R., 2004. 16S rRNA gene based analysis of

Enterobacter sakazakii strains from different sources and development of a PCR assay

for identification. BMC Microbiol. 4, 43.

[21] Mohan Nair, M. K., Venkitanarayanan, K.S, 2006. Cloning and sequencing of the *ompA*

gene of *Enterobacter sakazakii* and development of an *ompA*-targeted PCR for rapid

detection of *Enterobacter sakazakii* in infant formula. Appl. Environ. Microbiol.

72, 2539–2546.

[22] Kuhnert, P., Korczak, B.M., Stephan, R., Joosten, H., Iversen, C., 2009. Phylogeny and

prediction of genetic similarity of *Cronobacter* and related taxa by multilocus

sequence analysis (MLSA). Intl. J. Food Microbiol. 136, 152-158.

[23] Tamura, K., Peterson, D., Peterson, N., Stecher G., Nei, M., Kumar, S., 2011. MEGA5:

Molecular evolutionary genetics analysis using maximum likelihood, evolutionary

distance, and maximum parsimony methods. Mol. Bio. Evol. 28, 2731-2739.

[24] Nazarowec-White, M., Farber, J.M., 1999. Phenotypic and genotypic typing of food and

clinical isolates of *Enterobacter sakazakii*. J. Med. Microbiol. 48, 559-567.

[25] Iversen, C., Forsythe, S.J., 2004. Isolation of *Enterobacter sakazakii* and other

Enterobacteriaceae from powdered infant formula milk and related products. Food

Microbiol. 21, 771-776.

[26] Turcovský, I., Kuniková, K., Drahovská, H., Kaclíková, E., 2011. Biochemical and

molecular characterization of *Cronobacter* spp. (formerly *Enterobacter sakazakii*)

isolated from foods. Antonie van Leeuwenhoek 99, 257–269.

[27] Iversen, C., Forsythe, S.J., 2003. Risk profile of *Enterobacter sakazakii*, an emergent

pathogen associated with infant milk formula. Trends Food Sci. Technol. 14, 443–454.

Figure 1. Maximum-Likelihood tree of the *fusA* alleles (438 bp) of the *Cronobacter* MLST dataset and the six *Cronobacter* strains isolated from infant formula ingredients. The numbers at the end of each branch indicate the allelic profiles. The tree is drawn to scale using MEGA5, with 1000 bootstrap replicates.
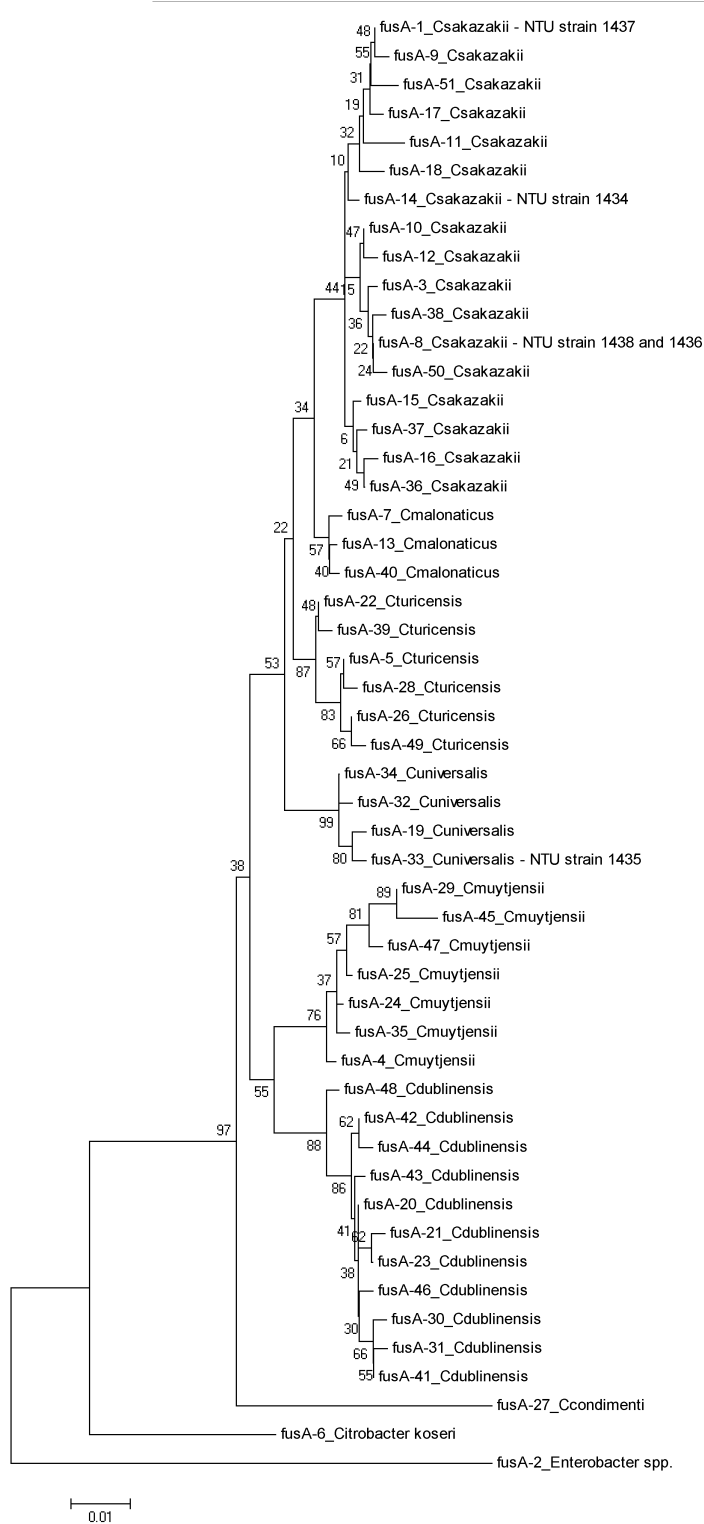
Figure 2. Maximum Likelihood tree based on the concatenated sequences (3036 bp) of the 7 MLST loci for the six *Cronobacter* isolates and representative species strains in the genus *Cronobacter*. The STs and the corresponding species are indicated at the tip of each branch. The tree is drawn to scale using MEGA5, with 1000 bootstrap replicates.
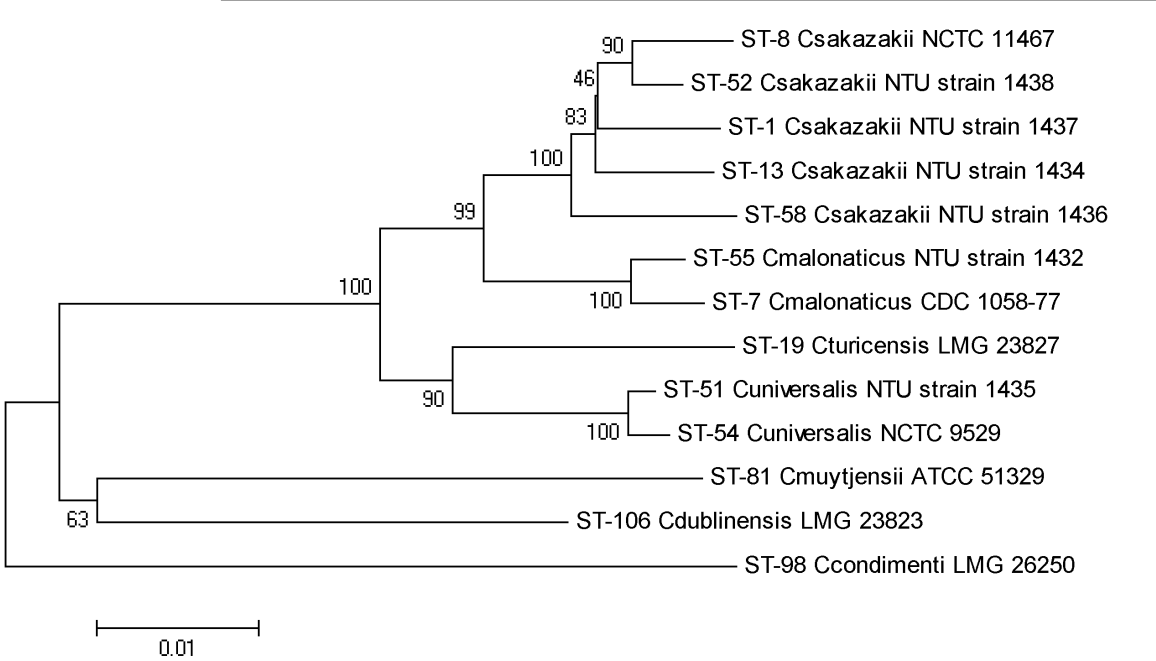
Table 1. Summary of identification and profiles for *Cronobacter* isolates from infant formula ingredients

Strain number	Source	API20E profile	API 20E ID (%)	ID32E profile	ID32E ID (%)	PCR-probes[a]	MALDI-TOF	16S rDNA sequence	*fusA* profile	MLST sequence type
1437	Oat flour	3305173	*E. sakazakii* (51.1)	34276763010	*E. sakazakii* (99.9)	*Cronobacter* spp.	*C. sakazakii*	*C. sakazakii*	*C. sakazakii*	1
1438	Oat flour	1304373	*E. sakazakii* (59.6%)	14234767010	*E. sakazakii* (99.9)	*Cronobacter* spp.	*C. sakazakii*	*C. sakazakii*	*C. sakazakii*	52
1434	Fennel	3305373	*E. sakazakii* (98.4%)	34276367210	*E. sakazakii* (99.9)	*Cronobacter* spp.	*C. sakazakii*	*C. sakazakii*	*C. sakazakii*	13
1436	Rye flour	3305373	*E. sakazakii* (98.4)	34276763011	*E. sakazakii* (99.9)	*Cronobacter* spp.	*C. sakazakii*	*C. sakazakii*	*C. sakazakii*	58
1435	Rye flour	3304373	*E. sakazakii* (97.3)	34356767010	*E. sakazakii*	*Cronobacter* spp.	*C. sakazakii*	*C. universalis*	*C. universalis*	51
1432	Rice	3325571	*E. cloacae*	34274767251	*E. sakazakii*	*Cronobacter*	*C. sakazakii*	*C. malonaticus*	*C. malonaticus*	55

flour	(95.1)	(99.9)	spp.

a Same result obtained for four PCR-probes; Keyser et al. (2003)[23], Lehner et al. (2004)[24], Mohan Nair and Venkitanarayanan (2006) [25] and Kuhnert et al. (2009) [26].