Isolation, identification and antimicrobial resistance of \textit{Cronobacter} spp. isolated from various foods in China

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\textbf{A B S T R A C T} \\
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\textit{Cronobacter} spp. are important foodborne pathogens that can cause severe diseases such as meningitis, sepsis, and necrotizing enterocolitis in neonates. In this study, 195 food samples, including cereals, cereal products, powdered infant formula (PIF), infant food formula, herbs, spices, vegetables, and fruits, were analyzed for the presence of \textit{Cronobacter} spp. by culture-based method. The presumptive isolates were further confirmed by targeting the 16S rDNA gene using PCR. Out of 195 samples, 13 samples (6.7\%) were positive for \textit{Cronobacter} species. 12 of 85 cereal and cereal products (14.1\%), and 1 of 22 herbs and spices (4.5\%) were contaminated. In contrast, no \textit{Cronobacter} was detected in commercial powdered infant formula, infant food formula, vegetables, or fruits. Alignment of 16S rRNA gene sequences showed that 13 isolates was most closely related to the genus \textit{Cronobacter}. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis revealed that \textit{Cronobacter sakazakii} was the only \textit{Cronobacter} species isolated from various food samples. The antimicrobial susceptibility of 13 \textit{Cronobacter} isolates was determined by the standard disk diffusion method. All isolated strains, except one resistant to ampicillin, were sensitive or displayed intermediate susceptibility to the 10 antimicrobial agents investigated. No multiple drug resistance was observed. \\
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1. Introduction

The genus \textit{Cronobacter}, a member of the family Enterobacteriaceae, comprises a group of Gram-negative foodborne pathogens that have been implicated as a cause of necrotizing enterocolitis, sepsis, and meningitis in neonates (Bowen & Braden, 2006; Caubilla-Barron et al., 2007; Nazarowec-White & Farber, 1997). This genus consists of seven species: \textit{Cronobacter sakazakii}, \textit{Cronobacter malonaticus}, \textit{Cronobacter muytjensii}, \textit{Cronobacter turicensis}, \textit{Cronobacter dubliniensis}, \textit{Cronobacter condimenti} and \textit{Cronobacter universalis} (Iversen et al., 2008; Joseph et al., 2012). Although the incidence of human infections caused by \textit{Cronobacter} spp. is low, these pathogens have a high mortality rate (more than 40\%) in infected infants, and the survivors often suffer from severe neurological complications (Gallagher & Ball, 1991; Ries, Harms, & Scharf, 1994; Willis & Robinson, 1988). Outbreaks of \textit{Cronobacter} spp. associated with contaminated PIF have been reported in Europe (Arseni, Malamouladas, Koutsia, Xanthou, & Trikka, 1987; Caubilla-Barron et al., 2007) and USA (Himelright et al., 2002).

While infections caused by \textit{Cronobacter} have been epidemiologically linked to the consumption of contaminated PIF (El-Sharoud, El-Din, Ziada, Ahmed, & Klena, 2008; Lampel & Chen, 2009), \textit{Cronobacter} spp. have been frequently isolated from a wide range of foods, such as cereals, meat, herbs, spices, salads, fruits, and vegetables (Jaradat, Ababneh, Saadoun, Samara, & Rashdan, 2009; Shaker, Osaili, Al-Omary, Jaradat, & Al-Zuby, 2007; Wang, Zhu, Xu, & Zhou, 2012; Ye, Wu, Zhou, Dong, & Zhang, 2008). Investigations on the presence of \textit{Cronobacter} in various foods are therefore necessary to aid epidemiological studies.

Although most \textit{Cronobacter} isolates are susceptible to commonly used antimicrobial agents, resistance has been reported in some isolates from food samples (Chon, Song, Kim, Hyeon, & Seo, 2012; Lee, Park, & Chang, 2012; Molloy et al., 2009). Additionally, inappropriate and irrational use of antimicrobial agents in agriculture has increased the emergence of antimicrobial-resistant strains (Girlich et al., 2001). Antimicrobial resistance, especially multiple drug resistance, is a public health problem, because it may cause failure of conventional treatment, resulting in prolonged illness and greater risk of death.

Although the occurrence of \textit{Cronobacter} in food products and environmental samples has been investigated in several countries (El-Sharoud et al., 2009; Hoque et al., 2010; Lee et al., 2012;
Transported to the laboratory in an ice box with ice packs and from different supermarkets. The samples were immediately produced by different food manufacturers and purchased from local supermarkets in Nanjing, China (Table 1). The samples were immediately transported to an ice box with ice packs and examined for the presence of Cronobacter spp. on the day of arrival.

2. Materials and methods

2.1. Sample collection

One hundred and ninety-five food samples, including cereals, cereal products, PIF, infant food formula, herbs, spices, vegetables, and fruits, were purchased between May 2010 and March 2013 from local supermarkets in Nanjing, China (Table 1). The samples were produced by different food manufacturers and purchased from different supermarkets. The samples were immediately transported to an ice box with ice packs and examined for the presence of Cronobacter spp. on the day of arrival.

2.2. Isolation and identification of Cronobacter spp.

The procedure adopted for the isolation of Cronobacter spp. from various food samples was a modification of the ISO method (ISO, 2006). Briefly, 25 g each sample except powders samples were homogenized with 225 mL of buffered peptone water (BPW; OXOID, Hampshire, UK) and then incubated at 37 °C for 18 h. For powders, 25 g portions per sample were mixed with 225 mL of BPW, and incubated at 37 °C for 18 h as a pre-enrichment step. Subsequently, 1 mL of the BPW suspension was transferred to 10 mL modified lauryl sulfate tryptose broth (OXOID, Hampshire, UK), and after further incubation at 42 °C for 24 h, the broth was streaked on chromogenic Cronobacter isolation agar (CCI; OXOID, Hampshire, UK). The plates were incubated at 42 °C for 24 h. All the suspected isolates, blue-green colonies on CCI, were subjected to the following biochemical tests for identification of the genus Cronobacter: Gram staining, oxidase, catalase, L-Lysine decarboxylase, L-Ornithine decarboxylase, L-Arginine dihydrolase, acid production from malonate, D-sorbitol, D-rihamnose, D-sucrose, D-melibiose, amygdaline, dulcitol and methyl-α-D-glucoside.

2.3. DNA extraction

Presumptive Cronobacter isolates were grown overnight at 37 °C in Luria–Bertani broth. Genomic DNA was extracted using an EZNA Genomic DNA isolation kit (Omega Bio-Tek, Doraville, USA) according to the manufacturer’s instructions. Concentration and purity of DNA samples were estimated by means of a spectrophotometer (GeneQuant 100, GE Healthcare, USA) in order to evaluate the amount and quality of extracted DNA. DNA samples were thereafter stored at −20 °C.

2.4. Confirmation of Cronobacter isolates

PCR confirmation of the presumptive Cronobacter isolates was performed as described in Lechner, Tasara, and Stephan (2004) using the primers Esakf (5’-GCTTGTGAGAGATTGCGG-3’) and Esakr (5’-ACTCTGAGATTCCTTCGTG-3’). Because of its high specificity, Esakf/Esakr is the most suitable primer pair for Cronobacter detection and identification (Blazkova, Javurkova, Fukal, & Rauch, 2011; Cawthon, Botha, & Wittbuhn, 2008; Lechner et al., 2004). The thermal cycle consisted on initial denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 2 min. A final elongation at 72 °C for 7 min completed the programme. After amplification, PCR products were separated by electrophoresis on a 1% (w/v) agarose gel (MBio, QingDao, China) in TAE buffer, followed by staining with ethidium bromide. The gel was then visualized and photographed under a UV transilluminator (Nuoding, Hangzhou, China). Cronobacter type strains (C. sakazakii ATCC 29544 and C. mucyctensii ATCC 51329) were used as positive controls.

2.5. PCR amplification and 16S rDNA gene sequence analysis

The 16S rDNA gene was PCR-amplified in a T-gradient thermocycler (PTC200, Bio-Rad, USA) using Taq DNA polymerase (Fermentas, Shanghai, China) and the universal primers 5’-AGAGTTTG ATCCTGGCTCAG-3’ and 5’-TACCTTGTTACGACTT-3’ (Weisburg, Barns, Pelletier, & Lane, 1991). PCR conditions were 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and elongation at 72 °C for 2 min. A final extension step of 7 min at 72 °C followed the final cycle. PCR products were purified with an EZ-10 Spin Column PCR product purification kit (Sangon, Shanghai, China). The 16S rDNA products were cloned into a pMD19-T vector (Takara, Dalian, China). The 16S rDNA gene sequence analysis was performed using the ClustalX 2.0 multiple alignment program (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997). Phylogenetic analysis was performed using MEGA 4.0. Branch support was evaluated by a bootstrap analysis of 1000 replicates. Phylogenetic trees of 16S rDNA sequences were inferred using the Neighbor-Joining method (Saitou & Nei, 1987).

2.6. PCR-RFLP

To identify all isolates at the species level, a PCR-RFLP protocol modified from that of Strydom, Cameron, and Wittbuhn (2011) was

<table>
<thead>
<tr>
<th>Origin</th>
<th>Sample category</th>
<th>No. of samples</th>
<th>Number of positive samples by culture-based method</th>
<th>Number of positive samples by PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cereals and cereal</td>
<td>Wheat</td>
<td>38</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>products</td>
<td>Buckwheat</td>
<td>8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Corn</td>
<td>12</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Rice</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Oatmeal</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Milk powdered</td>
<td>Powdered infant</td>
<td>16</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>formula (0–6 months)</td>
<td>Follow-on formul</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Growing-up milk</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Biscuit</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Infant food formula</td>
<td>Black pepper</td>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Red pepper</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Ginger powder</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Ilicium verum</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Cymous cumum</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vegetables and fruits</td>
<td>Vegetables</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Fruits</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
| Total                   | 195                 | 13             | 13                                                 |}
employed. Cronobacter type strains C. sakazakii ATCC 29544, C. mutiliensii ATCC 51329, C. malonaticus DSM 18702, C. turicensis DSM 18703, C. dublieniensis DSM 18705, and C. condimenti I NCTC 9529 were used to determine the sensitivity of the PCR-RFLP protocol. PCR amplification of the RNA polymerase beta-subunit (rpoB) gene was performed using the primers CroF2 (5’-TTCTGCGCCATCTGGATA-3’) and CroR (5’-AGCGCTTATCCGCGGCA-3’) (Strydom et al., 2011). The resulting 660-bp amplicon was digested with Csp61 (Fermentas, Shanghai, China) and HinP11 (Fermentas, Shanghai, China) endonucleases and analyzed by 5% agarose gel electrophoresis.

2.7. Antimicrobial susceptibility testing

All isolates were tested using the standard Kirby–Bauer disk diffusion method on Mueller–Hinton agar, as described by the National Committee for Clinical and Laboratory Standards (CLSI, 2010). The antimicrobial agents tested and their corresponding concentrations were as follows: ampicillin (10 μg), nalidixic acid (30 μg), cephalothin (10 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), tetracycline (5 μg), amikacin (30 μg), trimethoprim (5 μg), and trimethoprim–sulfamethoxazole (1.25 μg/23.75 μg). After incubation at 37 °C for 24 h, Cronobacter isolate susceptibility to each antimicrobial agent was measured and the results were interpreted in accordance with criteria provided by CLSI, 2010. E. coli ATCC 25922 was used as reference strain in antimicrobial susceptibility testing.

3. Results

3.1. Isolation and identification of Cronobacter spp.

In the present study, 195 samples of various food products were tested for the presence of Cronobacter (Table 1). In total, 13 of the 195 samples (6.7%) were positive for Cronobacter spp. The highest prevalence of Cronobacter was found in cereals and cereal products (14.1%), followed by herb and spice samples (4.5%). Samples of PIF, infant food formula, vegetables, and fruits were free of Cronobacter contamination.

The colonies that were small, convex, green—blue colonies on CCI agar plates, were considered as Cronobacter in this study. All the suspected isolates were Gram negative, oxidase negative and catalase positive. The distinctive biochemical tests of 13 Cronobacter isolates were as follows: acid from L-rhamnose, D-sucrose, D-catalase positive. The distinctive biochemical tests of 13 suspected isolates were Gram negative, oxidase negative and CCI agar plates, were considered as contamination.

3.2. 16S rRNA gene sequencing

The nearly full-length 16S rDNA (~1.5 kb) sequences were amplified from the isolated strains (fmb01–fmb13) and submitted to GenBank with the accession numbers JF330127 to JF330139. Blast searches based on 16S rDNA gene sequences showed that the 13 Cronobacter isolates had high sequence identity (>90%) with orthologues from the Enterobacteriaceae family, and highest sequence identity (>99%) with members of the genus Cronobacter. Sequence alignment indicated that the 16S rDNA sequences of the 13 isolates were 99%–100% identical to those of type strains C. sakazakii ATCC 29544 (EF088379) and C. malonaticus ATCC 51329 (AY752937). Consequently, although these isolates could be characterized as members of the genus Cronobacter using 16S rDNA gene sequence analysis, they could not be identified to the species level. In addition, phylogenetic analysis showed that all 13 presumptive isolates belonged to the genus Cronobacter and could be classified into at least two phylogenetic clusters, which might be related to the genetic diversity of C. sakazakii in ingredients of food samples (Fig. 1).

3.3. PCR-RFLP

In the present study, a modified PCR-RFLP genotyping protocol was performed to identify the Cronobacter isolates, and six Cronobacter species type strains were used as reference strains. PCR products of the rpoB gene were digested with Csp61 and HinP11 restriction endonucleases, and the resulting fragments were separated by gel electrophoresis. The predicted sizes of fragment length cleaved by Csp61 and/or HinP11 endonuclease are presented in Table 2. Interestingly, it has been found that the profiles for C. sakazakii type strains ATCC 29544 was inconsistent with that of C. sakazakii type strains DSM 4485. Digestion of C. sakazakii ATCC 29544 with Csp61 and HinP11 resulted in fragments of 187, 142, 112, 106 and 79 in the present study, whereas C. sakazakii DSM 4485 showed a profile with fragments of 190, 140, 87 and 78 bp (Strydom et al., 2011). After excluding from comparison any fragments smaller than 50 bp that were not clearly visible on the gel, all 13 Cronobacter isolates were found to have profiles identical to that of C. sakazakii type strains ATCC 29544 or DSM 4485, and were therefore classified as C. sakazakii (Fig. 2).

3.4. Antimicrobial susceptibility

Antimicrobial susceptibilities of the 13 Cronobacter strains isolated from food samples were examined using the standard disk diffusion method. The resistance pattern of the Cronobacter isolates

![Fig. 1. Neighbor-joining tree of the Cronobacter strains and other closely related members of the family Enterobacteriaceae based on 16S rDNA gene sequencing.](image-url)
to the 10 antimicrobial agents tested in this study is shown in Table 3. Antibiotic resistance profiling indicated that all 13 isolates were sensitive or displayed intermediate susceptibility to 9 of the 10 antimicrobial agents investigated; 1 of 13 (7.7%) isolates was ampicillin-resistant. Intermediate resistance to ampicillin, cephalexin, chloramphenicol, and ciprofloxacin was observed. All Cronobacter isolates were susceptible to nalidixic acid, gentamicin, tetracycline, trimethoprim, and trimethoprim–sulfamethoxazole. No multiple drug resistance was observed.

4. Discussion

In the past few years, several reports have been published on the prevalence of various Cronobacter strains in food samples based on phenotype and genotype. Very little information is available, however, on the prevalence of Cronobacter in China. In the present study, 195 food samples collected from Nanjing, China were investigated for the presence of Cronobacter. Cronobacter isolates were characterized using genus-specific PCR, 16S rDNA sequencing, and PCR-RFLP analysis, and their antimicrobial susceptibilities were examined by the standard disk diffusion method.

Cronobacter spp. have been frequently isolated from the environment (Kandhai, Reij, Gorris, Guillaume-Gentil, & van Schothorst, 2004; Reich, Konig, von Wiese, & Klein, 2010), clinics (Lai, 2001), and various food products such as powdered infant formula, cereals, meat products, fruits, and vegetables (Jaradat et al., 2009; Shaker et al., 2007; Wang et al., 2012; Ye et al., 2008). In our study, 12 of 85 cereal and cereal product samples (14.1%) and 1 of 22 (4.5%) herb and spice samples were positive for Cronobacter. Interestingly, a previous study did not detect Cronobacter in any cereal or cereal products but found a significantly higher prevalence (38.8%) in herbs and spices (Jaradat et al., 2009). In another study, Cronobacter was detected in 5.5% of cereal products, including starch, ground rice, semolina, bread crumbs, oats, and flour using a traditional culture method (Gurtler, Kornacki, & Beuchat, 2005). More recent studies have also found that Cronobacter could be isolated from cereal and cereal products (Lee et al., 2012). High Cronobacter prevalence in cereal products might be due to its ubiquitous presence in the natural environment or contamination in the production and processing environment.

Although many authors have described Cronobacter infections arising from PIF consumption (Lai, 2001; Lampel & Chen, 2009), we did not detect Cronobacter in any such samples. Similarly, Cronobacter was not isolated from any follow-up formula or infant food products in a coordinated survey for its presence in Brazil, Korea, and Malaysia (Chap et al., 2009). Lee et al. (2012) isolated strains of Cronobacter from 19 of 128 vegetables (14.8%). In another study, Chon et al. (2012) studied the prevalence of Cronobacter collected from retailers in Seoul, South Korea. Of 10 vegetables, 30% were contaminated. In our study, no Cronobacter spp. were isolated from fruit or vegetable samples. The absence of Cronobacter in the present study may be due to the smaller number of samples analyzed or recent improvements in food industry hygiene levels.

A microbiological analysis performed by Reich et al. (2010) in a PIF processing environment revealed that environment was correlated with Cronobacter contamination in the final products, implying that the processing environment may be a contamination source. Moreover, post-processing contamination of Cronobacter is also an important consideration and should not be overlooked. Several studies have reported that Cronobacter contamination during post process (Mullane, Healy, et al., 2008; Mullane, Whyte, Wall, Quinn, & Fanning, 2007). Because infections caused by Cronobacter may result in serious illness or death, more hygienic processes, such as Hazard Analysis and Critical Control Point (HACCP) and Good Manufacturing Practice (GMP), are therefore necessary to control these foodborne pathogens in the food processing industry.

Species identification of members of the genus Cronobacter is of great importance for epidemiological studies. Previous studies indicated that Cronobacter exhibited differences on their antibiotic resistance and virulence (Kucerova et al., 2010; Lai, 2001), and therefore, further studies are necessary to identify the Cronobacter strains at species level. Several techniques for the species identification of Cronobacter spp. have been developed, including random amplified polymorphic DNA (RAPD), pulsed-field gel electrophoresis (PFGE), ribotyping, amplified fragment length polymorphisms (AFLP), multiple-locus variable-number tandem repeat analysis.

![Image](https://via.placeholder.com/150)

**Table 2**

<table>
<thead>
<tr>
<th>Species</th>
<th>Restriction enzymes and fragments (bp)</th>
<th>Csp6I</th>
<th>HinP1I</th>
<th>Csp6I and HinP1I</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. sakazakii ATCC 29544</td>
<td>325, 295</td>
<td>224</td>
<td>187</td>
<td>142, 88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>187</td>
<td>142, 112, 106, 79</td>
<td></td>
</tr>
<tr>
<td>C. muytjensis ATCC 51329</td>
<td>325, 212, 124</td>
<td>202</td>
<td>189, 100, 96, 87</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>189</td>
<td>167, 96, 87</td>
<td></td>
</tr>
<tr>
<td>C. malonaticus DSM 18702</td>
<td>325, 221, 83</td>
<td>321</td>
<td>196, 142, 83</td>
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<td></td>
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<td>196</td>
<td>167, 142, 83</td>
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<tr>
<td>C. turicensis DSM 18703</td>
<td>364, 212, 83</td>
<td>312</td>
<td>187, 142, 83</td>
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<td></td>
<td></td>
<td>202</td>
<td>187, 142, 83</td>
<td></td>
</tr>
<tr>
<td>C. condimenti NCTC 9529</td>
<td>315, 222, 83</td>
<td>312</td>
<td>168, 148, 83</td>
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<tr>
<td></td>
<td></td>
<td>168</td>
<td>167, 148, 83</td>
<td></td>
</tr>
<tr>
<td>C. dubliniensis DSM 18705</td>
<td>325, 212, 122</td>
<td>299</td>
<td>202, 142</td>
<td>212, 167, 142, 87</td>
</tr>
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</table>

**Table 3**

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>No. of isolates (%)</th>
<th>Resistant</th>
<th>Intermediate</th>
<th>Susceptible</th>
</tr>
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<tbody>
<tr>
<td>Amoxicillin</td>
<td></td>
<td>1 (7.7)</td>
<td>1 (7.7)</td>
<td>11 (84.6)</td>
</tr>
<tr>
<td>Nalidixic</td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>13 (100)</td>
</tr>
<tr>
<td>Cephalothine</td>
<td></td>
<td>0 (0)</td>
<td>2 (15.4)</td>
<td>11 (84.6)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>13 (100)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td></td>
<td>0 (0)</td>
<td>1 (7.7)</td>
<td>12 (92.3)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td></td>
<td>0 (0)</td>
<td>1 (7.7)</td>
<td>12 (92.3)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>13 (100)</td>
</tr>
<tr>
<td>Amikacin</td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>13 (100)</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>13 (100)</td>
</tr>
<tr>
<td>Trimethoprim–sulfamethoxazole</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>13 (100)</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 2.** RFLP patterns of PCR products from the rpoB gene of 6 Cronobacter type strains and 13 Cronobacter isolates after digestion with a combination of Csp6I and HinP1I. Lane M: 50 bp DNA ladder; lane 1: C. sakazakii ATCC 29544; lane 2: ATCC 51329; lane 3: C. malonaticus DSM 18702; lane 4: C. turicensis DSM 18703; lane 5: C. universalis NCTC 9529; lane 6: C. dubliniensis DSM 18705; lanes 7 to 19: Cronobacter isolates from fnb01 to fnb13.
(MLVA) and, more recently, PCR-RFLP (Iversen et al., 2007; Kim et al., 2011; Mullane, Ryan, et al., 2008; Strydom et al., 2011). In the present study, a modified PCR-RFLP protocol was carried out to identify Cronobacter isolates recovered from various food samples. All 13 Cronobacter isolates recovered from food samples were identified as C. sakazakii by PCR-RFLP analysis in our study (Fig. 2). Studies in other countries have also reported C. sakazakii as the predominant Cronobacter species recovered from different sample sources. For instance, Cronobacter were isolated from 41 out of 86 Saengsik samples, and 7 out of 102 infant formula samples in South Korea; of the 48 Cronobacter strains isolated, 42 (87.5%) of these strains were identified as C. sakazakii (Lee, Ryu, Chang, & Park, 2010). Cronobacter were isolated from 33 of 518 samples from farming and domestic environments, food production animals and retail foods in Ireland; of the 33 Cronobacter strains isolated, 21 (63.6%) of these strains were C. sakazakii (Molloy et al., 2009). In South Africa, a total of 28 Cronobacter strains were isolated from various food and environmental sources, and 26 isolates (92.8%) were C. sakazakii (Strydom et al., 2011). The absence of other Cronobacter species in the present study may be due to the smaller number of samples analyzed, the source of samples, and the fact that C. sakazakii is the dominant species in terms of isolation frequency.

Cronobacter strains were tested for susceptibility to 10 antimicrobial agents as recommended by the CLSI for the testing of Enterobacteriaceae. In the present study, most Cronobacter isolates were sensitive or displayed intermediate susceptibility to 9 of 10 antimicrobial agents investigated, whereas only one isolate (7.7%) was resistant to ampicillin. Similarly, previous studies performed by Kim et al. (2008) reported 31.6% of isolated strains was resistant to ampicillin. Although the occurrence of cephalothin resistance in Cronobacter isolated from different sources is common (Chon et al., 2012; Kim et al., 2008; Molloy et al., 2009), cephalothin-resistant strains were not detected in this study. El-Sharoud et al. (2009) investigated the antibiotic resistance of Cronobacter spp. recovered from dried milk and related products in Ireland, and reported one Cronobacter isolate resistant to trimethoprim and another to neomycin. These results differ from those of our study, in which all isolates were susceptible to both trimethoprim and trimethoprim in combination with sulfamethoxazole. Lee et al. (2012) reported that two Cronobacter strains (3.4%) isolated from food samples were resistant to chloramphenicol. In contrast, only one strain in our study showed intermediate susceptibility to chloramphenicol. It has been reported that infections caused by Cronobacter species can be successfully treated with ampicillin-gentamicin or ampicillin-chloramphenicol during therapy (Lai, 2001). More attention should be paid to infections caused by Cronobacter species, however, because abuse and misuse of antimicrobial agents may lead to the emergence of antimicrobial-resistant bacteria, a serious public health problem.

In conclusion, we investigated the presence of Cronobacter spp. in various food samples and used molecular techniques to identify and further classify the isolates. C. sakazakii was the only Cronobacter species isolated from various food samples and most isolates showed no resistance to commonly used antibiotics. The presence of these foodborne pathogens in 1st stage infant formula for infants under 6 months of age is of particular because they can cause severe disease in neonates. Infections have been reported in the elderly and in immunocompromised adults, however these have not been linked to ingestion of food and infections are less severe. To reduce the risk of Cronobacter infections, strict hygienic guidelines to control these foodborne pathogens is needed especially in production facilities for 1st stage infant formula, hospital neonatal units and kitchens where preparation of infant feeds for neonates is carried out.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.foodcont.2013.09.017.

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