Food Control 37 (2014) 109–114

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Isolation, identification and antimicrobial resistance of *Cronobacter* spp. isolated from various foods in China

Yuanhong Li, Qiming Chen, Junfeng Zhao, Hua Jiang, Fengxia Lu, Xiaomei Bie, Zhaoxin Lu*

College of Food Science and Technology, Nanjing Agricultural University, 1 Weigang, Nanjing 210095, PR China

ARTICLE INFO

Article history:
Received 26 April 2013
Received in revised form
1 September 2013
Accepted 14 September 2013

Keywords:
Cronobacter spp.
Isolation
Identification
Antimicrobial resistance

ABSTRACT

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 *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 *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 *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 *Cronobacter*. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis revealed that *Cronobacter sakazakii* was the only *Cronobacter* species isolated from various food samples. The antimicrobial susceptibility of 13 *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.

1. Introduction

The genus *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; NazarowecWhite & Farber, 1997). This genus consists of seven species: *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter muytjensii*, *Cronobacter turicensis*, *Cronobacter dubliniensis*, *Cronobacter condimenti* and *Cronobacter universalis* (Iversen et al., 2008; Joseph et al., 2012). Although the incidence of human infections caused by *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 *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 *Cronobacter* have been epidemiologically linked to the consumption of contaminated PIF (El-Sharoud, El-Din, Ziada, Ahmed, & Klena, 2008; Lampel & Chen, 2009), *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 *Cronobacter* in various foods are therefore necessary to aid epidemiological studies.

Although most *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 *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;

* Corresponding author. Tel./fax: +86 25 84396583.
E-mail address: fmb@njau.edu.cn (Z. Lu).

0956-7135/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodcont.2013.09.017

Terragno et al., 2009), little information is available on the prevalence of these organisms in food products in China. The aim of this study was to investigate the prevalence of *Cronobacter* in various Chinese food products, to identify the isolates by 16S rDNA sequencing and PCR-RFLP analysis, and to check antibiotic resistance patterns of the isolated strains.

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 the laboratory in 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, L-rhamnose, D-sucrose, D-melibiose, amygdaline, dulcitol and methyl-α-D-glucoside.

Table 1
Cronobacter spp. isolated from various food samples by culture-based method and PCR.

Origin	Sample category	No. of samples	Number of positive samples by culture-based method	Number of positive samples by PCR
Cereals and cereal products	Wheat	38	6	6
	Buckwheat	8	1	1
	Corn	12	4	4
	Rice	10	0	0
	Oatmeal	9	0	0
	Barley	8	1	1
Milk powdered	Powdered infant formula (0–6 months)	16	0	0
	Follow-on formular (6–12 months)	11	0	0
	Growing-up milk (12–36 months)	6	0	0
Infant food formula	Biscuit	15	0	0
Spices	Black pepper	5	1	1
	Red pepper	5	0	0
	Ginger powder	4	0	0
	Illicium verum	4	0	0
	Cymous cuminum fruit	4	0	0
Vegetables and fruits	Vegetables	20	0	0
	Fruits	20	0	0
Total		195	13	13

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 Lehner, Tasara, and Stephan (2004) using the primers Esakf (5′-GCTYTGCTGACGAGTGGCGG-3′) and Esakr (5′-ATCTCTGCAGGATTCTCTGG-3′). Because of its high specificity, Esakf/Esakr is the most suitable primer pair for *Cronobacter* detection and identification (Blazkova, Javurkova, Fukal, & Rauch, 2011; Cawthorn, Botha, & Witthuhn, 2008; Lehner 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% (m/v) agarose gel (MDBio, 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. muytjensii* 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) and transformed into competent *Escherichia coli* DH5α cells. Partial sequencing of the 16S rDNA gene was then carried out by Genscript (Nanjing, China) using an ABI 3730 Genetic Analyzer. The resulting sequences were subjected to a GenBank BLASTn search (http://www.ncbi.nlm.nih.gov/BLAST/) to determine potentially homologous sequences in GenBank, and also aligned 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 Witthuhn (2011) was

employed. *Cronobacter* type strains *C. sakazakii* ATCC 29544, *C. muytjensii* ATCC 51329, *C. malonaticus* DSM 18702, *C. turicensis* DSM 18703, *C. dubliniensis* DSM 18705, and *C. condimenti* 1 NCTC 9529 were used to determine the sensitivity of the PCR-RFLP protocol. PCR amplification of the RNA polymerase beta-subunit (*rpo*B) gene was performed using the primers CroF2 (5′-TCTCTGGGCGATCTGGATA-3′) and CroR (5′-AGCGGCTTATCAGCGCGCA-3′) (Strydom et al., 2011). The resulting 660-bp amplicon was digested with *Csp*6I (Fermentas, Shanghai, China) and *Hin*P1I (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 (30 μg), gentamicin (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-melibiose, mygdaline, and a methyl-α-D-glucoside; no acid from malonate, D-sorbitol, or dulcitol; negative tests for indole production and L-Lysine decarboxylase, and L-Ornithine decarboxylase and L-Arginine dihydrolase positive. Furthermore, all 13 isolates identified as *Cronobacter* spp. by using conventional bacteriological methods also tested positive using the PCR assay (Table 1).

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 *rpo*B gene were digested with *Csp*6I and *Hin*P1I restriction endonucleases, and the resulting fragments were separated by gel electrophoresis. The predicted sizes of fragment length cleaved by *Csp*6I and/or *Hin*P1I 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 *Csp*6I and *Hin*P1I 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

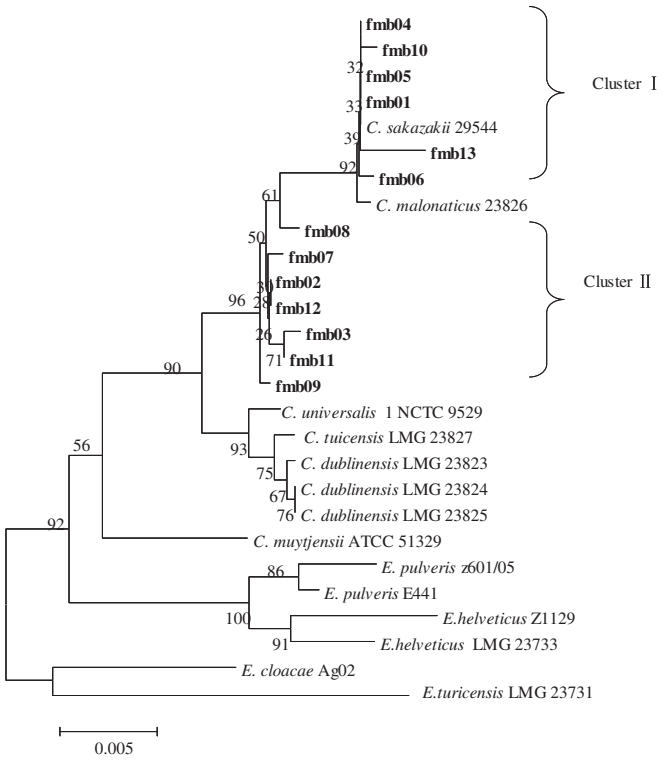

Fig. 1. Neighbor-joining tree of the *Cronobacter* strains and other closely related members of the family *Enterobacteriaceae* based on 16S rDNA gene sequencing. Bootstrap values are indicated above branches and were derived from 1000 replicates. Bar represents 0.005% nucleotide substitutions.

Table 2
RFLP analysis of the *rpo*B gene of the six species of *Cronobacter* type strains. Fragments smaller than 50 bp which were not clearly visible on the gel was not included in the table.

Species	Restriction enzymes and fragments (bp)		
	*Csp*6I	*Hin*P1I	*Csp*6I and *Hin*P1I
C. sakazakii ATCC 29544	325, 295	224, 187, 142, 88	187, 142, 112, 106, 79
C. muytjensii ATCC 51329	325, 212, 124	202, 189, 100, 96	189, 167, 96, 87
C. malonaticus DSM 18702	325, 221, 83	321, 196, 142	196, 167, 142, 83
C. turicensis DSM 18703	364, 212, 83	312, 187, 142	202, 187, 142, 83
C. condimenti NCTC 9529	315, 222, 83	312, 168, 148	168, 167, 148, 83
C. dubliniensis DSM 18705	325, 212, 122	299, 202, 142	212, 167, 142, 87

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, cephalothin, 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

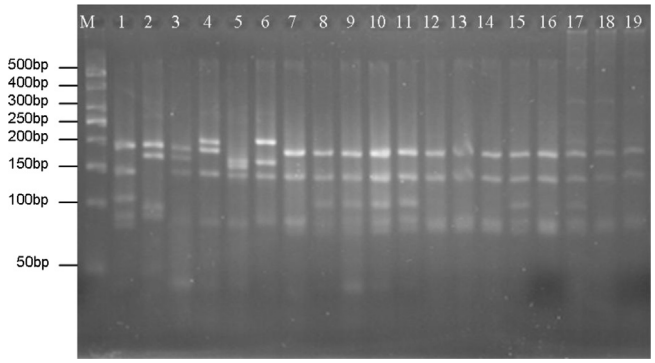

Fig. 2. RFLP patterns of PCR products from the *rpo*B gene of 6 *Cronobacter* type strains and 13 *Cronobacter* isolates after digestion with a combination of *Csp*6I and *Hin*P1I. Lane M: 50 bp DNA ladder; lane 1: *C. sakazakii* ATCC 29544; lane 2: ATCC 51329; lane 3: *C. malonaticus* DSM 18702; lane 4: *C. turicensis* DSM 18703; lane 5: *C. universalis* NCTC 9529; lane 6: *C. dubliniensis* DSM 18705; lanes 7 to 19: *Cronobacter* isolates from fmb01 to fmb13.

Table 3
Susceptibility rates of antimicrobial agents tested against *Cronobacter* isolates from food samples ($n = 13$).

Antibiotics	No. of isolates (%)		
	Resistant	Intermediate	Susceptible
Ampicillin	1 (7.7)	1 (7.7)	11 (84.6)
Nalidixic	0 (0)	0 (0)	13 (100)
Cephalothin	0 (0)	2 (15.4)	11 (84.6)
Gentamicin	0 (0)	0 (0)	13 (100)
Chloramphenicol	0 (0)	1 (7.7)	12 (92.3)
Ciprofloxacin	0 (0)	1 (7.7)	12 (92.3)
Tetracycline	0 (0)	0 (0)	13 (100)
Amikacin	0 (0)	0 (0)	13 (100)
Trimethoprim	0 (0)	0 (0)	13 (100)
Trimethoprim–sulfamethoxazole	0 (0)	0 (0)	13 (100)

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 has 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 be 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

(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 formular 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 resistance 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 formular 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 formular, hospital neonatal units and kitchens where preparation of infant feeds for neonates is carried out.

Acknowledgments

This work was supported by the National Twelfth Five-Year Research Program of China (2012BAK08807), the Science and Technology Support Program of Jiangsu Province (BE2011782), the Graduate Student Research Innovation Program of Jiangsu Province (CX2211-0657), and a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

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.

References

Arseni, A., Malamouladas, E., Koutsia, C., Xanthou, M., & Trikka, E. (1987). Outbreak of colonization of neonates with *Enterobacter-Sakazakii*. *Journal of Hospital Infection, 9*(2), 143–150.

Blazkova, M., Javurkova, B., Fukal, L., & Rauch, P. (2011). Immunochromatographic strip test for detection of genus *Cronobacter*. *Biosensors & Bioelectronics, 26*(6), 2828–2834.

Bowen, A. B., & Braden, C. R. (2006). Invasive *Enterobacter sakazakii* disease in infants. *Emerging Infectious Diseases, 12*(8), 1185–1189.

Caubilla-Barron, J., Hurrell, E., Townsend, S., Cheetham, P., Loc-Carrillo, C., Fayet, O., et al. (2007). Genotypic and phenotypic analysis of *Enterobacter sakazakii* strains from an outbreak resulting in fatalities in a neonatal intensive care unit in France. *Journal of Clinical Microbiology, 45*(12), 3979–3985.

Cawthorn, D. M., Botha, S., & Witthuhn, R. C. (2008). Evaluation of different methods for the detection and identification of *Enterobacter sakazakii* isolated from South African infant formula milks and the processing environment. *International Journal of Food Microbiology, 127*(1–2), 129–138.

Chap, J., Jackson, P., Siqueira, R., Gaspar, N., Quintas, C., Park, J., et al. (2009). International survey of *Cronobacter sakazakii* and other *Cronobacter* spp. in follow up formulas and infant foods. *International Journal of Food Microbiology, 136*(2), 185–188.

Chon, J. W., Song, K. Y., Kim, S. Y., Hyeon, J. Y., & Seo, K. H. (2012). Isolation and characterization of *Cronobacter* from desiccated foods in Korea. *Journal of Food Science, 77*(7), M354–M358.

Clinical and Laboratory Standards Institute. (2010). *Performance standards for antimicrobial susceptibility testing*, 20th informational supplement. CLSI document M100-S20. Wayne, Pa. USA: Clinical and Laboratory Standards Institute.

El-Sharoud, W. M., El-Din, M. Z., Ziada, D. M., Ahmed, S. F., & Klena, J. D. (2008). Surveillance and genotyping of *Enterobacter sakazakii* suggest its potential transmission from milk powder into imitation recombined soft cheese. *Journal of Applied Microbiology, 105*(2), 559–566.

El-Sharoud, W. M., O'Brien, S., Negredo, C., Iversen, C., Fanning, S., & Healy, B. (2009). Characterization of *Cronobacter* recovered from dried milk and related products. *BMC Microbiology, 9*.

Gallagher, P. G., & Ball, W. S. (1991). Cerebral infarctions due to CNS infection with *Enterobacter-Sakazakii*. *Pediatric Radiology, 21*(2), 135–136.

Girlich, D., Poirel, L., Leelaporn, A., Karim, A., Tribuddharat, C., Fennewald, M., et al. (2001). Molecular epidemiology of the integron-located VEB-1 extended-spectrum beta-lactamase in nosocomial enterobacterial isolates in Bangkok, Thailand. *Journal of Clinical Microbiology, 39*(1), 175–182.

Gurtler, J. B., Kornacki, J. L., & Beuchat, L. R. (2005). *Enterobacter sakazakii*: a coliform of increased concern to infant health. *International Journal of Food Microbiology, 104*(1), 1–34.

Himelright, I., Harris, E., Lorch, V., Anderson, M., Jones, T., Craig, A., et al. (2002). *Enterobacter sakazakii* infections associated with the use of powdered infant formula – Tennessee, 2001. *JAMA-Journal of the American Medical Association, 287*(17), 2204–2205 (Reprinted from MMWR, vol. 51, pg 297–300, 2002).

Hoque, A., Ahmed, T., Shahidullah, M., Hossain, A., Mannan, A., Noor, K., et al. (2010). Isolation and molecular identification of *Cronobacter* spp. from powdered infant formula (PIF) in Bangladesh. *International Journal of Food Microbiology, 142*(3), 375–378.

International Organization for Standardization. (2006). *Milk and milk products – Detection of Enterobacter sakazakii ISO/TS 22964: 2006*. Geneva: International Organization for Standardization.

Iversen, C., Lehner, A., Mullane, N., Bidlas, E., Cleenwerck, I., Marugg, J., et al. (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 I. *BMC Evolutionary Biology, 7*.

Iversen, C., Mullane, N., McCardel, B., Tal, B. D., Lehner, A., Fannin, S., et al. (2008). *Cronobacter* gen nov, a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen nov, comb nov,

Cronobacter malonaticus sp nov, *Cronobacter turicensis* sp nov, *Cronobacter muytjensii* sp nov, *Cronobacter dublinensis* sp nov, *Cronobacter* genomospecies 1, and of three subspecies, *Cronobacter dublinensis* subsp *dublinensis* subsp nov, *Cronobacter dublinensis* subsp *lausannensis* subsp nov and *Cronobacter dublinensis* subsp *lactaridi* subsp nov. *International Journal of Systematic and Evolutionary Microbiology, 58*, 1442–1447.

Jaradat, Z. W., Ababneh, Q. O., Saadoun, I. M., Samara, N. A., & Rashdan, A. M. (2009). Isolation of *Cronobacter* spp. (formerly *Enterobacter sakazakii*) from infant food, herbs and environmental samples and the subsequent identification and confirmation of the isolates using biochemical, chromogenic assays, PCR and 16S rRNA sequencing. *BMC Microbiology, 9*.

Joseph, S., Cetinkaya, E., Drahovska, H., Levican, A., Figueras, M. J., & Forsythe, S. J. (2012). *Cronobacter condimenti* sp nov., isolated from spiced meat, and *Cronobacter universalis* sp nov., a species designation for *Cronobacter* sp genomospecies 1, recovered from a leg infection, water and food ingredients. *International Journal of Systematic and Evolutionary Microbiology, 62*, 1277–1283.

Kandhai, M. C., Reij, M. W., Gorris, L. G. M., Guillaume-Gentil, O., & van Schothorst, M. (2004). Occurrence of *Enterobacter sakazakii* in food production environments and households. *Lancet, 363*(9402), 39–40.

Kim, K., Jang, S. S., Kim, S. K., Park, J. H., Heu, S., & Ryu, S. (2008). Prevalence and genetic diversity of *Enterobacter sakazakii* in ingredients of infant foods. *International Journal of Food Microbiology, 122*(1–2), 196–203.

Kim, J. B., Park, Y. B., Kang, S. H., Lee, M. J., Kim, K. C., Jeong, H. R., et al. (2011). Prevalence, genetic diversity, and antibiotic susceptibility of *Cronobacter* spp. (*Enterobacter sakazakii*) isolated from Sunshik, its ingredients and soils. *Food Science and Biotechnology, 20*(4), 941–948.

Kucerova, E., Clifton, S. W., Xia, X. Q., Long, F., Porwollik, S., Fulton, L., et al. (2010). Genome sequence of *Cronobacter sakazakii* BAA-894 and comparative genomic hybridization analysis with other *Cronobacter* species. *Plos One, 5*(3), A51–A60.

Lai, K. K. (2001). *Enterobacter sakazakii* infections among neonates, infants, children, and adults – case reports and a review of the literature. *Medicine, 80*(2), 113–122.

Lampel, K. A., & Chen, Y. (2009). Method for the isolation and detection of *Enterobacter sakazakii (Cronobacter)* from powdered infant formula. *International Journal of Food Microbiology, 136*(2), 179–184.

Lee, Y. D., Park, J. H., & Chang, H. (2012). Detection, antibiotic susceptibility and biofilm formation of *Cronobacter* spp. from various foods in Korea. *Food Control, 24*(1–2), 225–230.

Lee, Y. D., Ryu, T. W., Chang, H. I., & Park, J. H. (2010). Identification and classification of *Cronobacter* spp. isolated from powdered food in Korea. *Journal of Microbiology and Biotechnology, 20*(4), 757–762.

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 Microbiology, 4*.

Molloy, C., Cagney, C., O'Brien, S., Iversen, C., Fanning, S., & Duffy, G. (2009). Surveillance and characterisation by pulsed-field gel electrophoresis of *Cronobacter* spp. in farming and domestic environments, food production animals and retail foods. *International Journal of Food Microbiology, 136*(2), 198–203.

Mullane, N., Healy, B., Meade, J., Whyte, P., Wall, P. G., & Fanning, S. (2008). Dissemination of *Cronobacter* spp. (*Enterobacter sakazakii*) in a powdered milk protein manufacturing facility. *Applied and Environmental Microbiology, 74*(19), 5913–5917.

Mullane, N. R., Ryan, M., Iversen, C., Murphy, M., O'Gaora, P., Quinn, T., et al. (2008). Development of multiple-locus variable-number tandem-repeat analysis for the molecular subtyping of *Enterobacter sakazakii*. *Applied and Environmental Microbiology, 74*(4), 1223–1231.

Mullane, N. R., Whyte, P., Wall, P. G., Quinn, T., & Fanning, S. (2007). Application of pulsed-field gel electrophoresis to characterise and trace the prevalence of *Enterobacter sakazakii* in an infant formula processing facility. *International Journal of Food Microbiology, 116*(1), 73–81.

NazarowecWhite, M., & Farber, J. M. (1997). *Enterobacter sakazakii*: a review. *International Journal of Food Microbiology, 34*(2), 103–113.

Reich, F., Konig, R., von Wiese, W., & Klein, G. (2010). Prevalence of *Cronobacter* spp. in a powdered infant formula processing environment. *International Journal of Food Microbiology, 140*(2–3), 214–217.

Ries, M., Harms, D., & Scharf, J. (1994). Multiple cerebral infarcts with resulting multicystic encephalomalacia in a premature infant with *Enterobacter sakazakii* meningitis. *Klinische Padiatrie, 206*(3), 184–186.

Saitou, N., & Nei, M. (1987). The neighbor-joining method – a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution, 4*(4), 406–425.

Shaker, R., Osaili, T., Al-Omary, W., Jaradat, Z., & Al-Zuby, M. (2007). Isolation of *Enterobacter sakazakii* and other *Enterobacter* sp from food and food production environments. *Food Control, 18*(10), 1241–1245.

Strydom, A., Cameron, M., & Witthuhn, R. C. (2011). PCR-RFLP analysis of the *rpoB* gene to distinguish the five species of *Cronobacter*. *Food Microbiology, 28*(8), 1472–1477.

Terragno, R., Salve, A., Pichel, M., Epszteyn, S., Brengi, S., & Binsztein, N. (2009). Characterization and subtyping of *Cronobacter* spp. from imported powdered infant formulae in Argentina. *International Journal of Food Microbiology, 136*(2), 193–197.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research, 25*(24), 4876–4882.

Wang, X., Zhu, C. Q., Xu, X. L., & Zhou, G. H. (2012). Real-time PCR with internal amplification control for the detection of *Cronobacter* spp. (*Enterobacter sakazakii*) in food samples. *Food Control, 25*(1), 144–149.

Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16s ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology, 173*(2), 697–703.

Willis, J., & Robinson, J. E. (1988). *Enterobacter-Sakazakii* meningitis in neonates. *Pediatric Infectious Disease Journal, 7*(3), 196–199.

Ye, Y. W., Wu, Q. P., Zhou, Y. H., Dong, X. H., & Zhang, J. M. (2008). Analysis of major band of *Enterobacter sakazakii* by ERIC-PCR and development of a species-specific PCR for detection of *Ent. sakazakii* in dry food samples. *Journal of Microbiological Methods, 75*(3), 392–397.