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The occurrence and distribution characteristics of *Cronobacter* in diverse cereal kernels, flour, and flour-based products

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ABSTRACT

Cronobacter was positive in cereals at a relatively high rate. In the present study, we investigated the occurrence and characteristics of this pathogen systematically in diverse cereals. All sampled food (N = 467) contained *Cronobacter* with a high positive rate of 54.0%. The enumeration experiment showed the concentration ranged from 0.3 to more than 110 MPN/100 g, and 87.9% of 127 samples were less than 10 MPN/100 g. There were significant differences ($p < 0.05$) in positive rates for *Cronobacter* among cereal kernels (40.2%), cereal flour (66.7%), cereal products made from raw cereal flour (87.6%), and cereal products made from flour (ready-to-eat) (17.4%). The dominant *Cronobacter* species was *C. sakazakii* and *C. dublinensis*, followed by *C. malonaticus* and *C. turicensis*. Two interesting clusters with more than 90% similarities were identified by pulsed-field gel electrophoresis (PFGE). The C1 cluster (four isolates) indicated these strains were derived from a common source and persisted in the food production environment for an extended time. The C2 cluster (six isolates) indicated the pathogen could be transmitted via cereal processing. Our research provided baseline data for *Cronobacter* in diverse cereals and was helpful for understanding *Cronobacter* transmission. The results also indicate that additional control measures should be developed to reduce the risk of infection by these opportunistic pathogenic bacteria.

1. Introduction

Cronobacter was accepted as a new bacterial genus in 2007 (Iversen et al., 2007). It consists of seven species including *C. sakazakii, C. malonaticus, C. turicensis, C. muytjensii, C. dublinensis, C. universalis,* and *C. condimenti* (Iversen et al., 2008; Joseph et al., 2012). This opportunistic foodborne pathogen causes severe systemic infections occasionally in neonates, such as meningitis, septicaemia, and enterocolitis, especially in low birth-weight or immunocompromised infants (Farmer, 2015). The mortality rate for resulting meningitis may be as high as 40%–80% (Nazarowec-White and Farber, 1997; Lai, 2001). The rates of *Cronobacter* infection was 0.66 cases per 100,000 population, with the highest rates occurred among persons > 80 years of age (3.93 cases/100,000 population), followed by persons 70–79 years of age (2.11) and infants (1.81) (Patrick et al., 2014).

Cronobacter infections among infants have been epidemiologically linked to powdered infant formula (PIF) (Hunter and Bean, 2013; Yan et al., 2012). *Cronobacter* contamination of commercial PIF products is always an unavoidable problem (Fei et al., 2018; Parra-Flores et al., 2018). Recalls of PIF as well as cases of severe neonatal infection with *Cronobacter* have still occurred (Caubilla-Barron et al., 2007; Shi et al., 2018; https://sg.theasianparent.com/dumex-mamil-gold-infant-formula-recall). Actually, it is impossible to produce sterile PIF using current methods. In addition to PIF, this microorganism has been isolated from a wide spectrum of common foods and food ingredients (Berthold-Pluta et al., 2017; Brandao et al., 2017; Li et al., 2017; Vasconcellos et al., 2018). It has been confirmed that many *Cronobacter* isolates worldwide originates from plant sources (Chen et al., 2016; Sani and Odeyemi, 2015).

Although great research progress has been achieved, both the source and vehicle of transmission of *Cronobacter* are not always clear. It was previously showed that wheat flour is one likely reservoir and/or transmission route for *Cronobacter* because of the extremely high positive rate (100%) of this pathogen (Lou et al., 2014). Our research indicated that *Cronobacter* was present in different growth stages of rice and wheat, and *Cronobacter* detection rates in food samples notably changed with different processing methods. Moreover, *Cronobacter* in the domestic environment has been related to primary staple foods (Lou et al., 2019).

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Several studies have reported that a number of cereal samples were tested positive for *Cronobacter* spp. (Brandao et al., 2017; Friedemann, 2007; Silva et al., 2019), while another study found that none of the 32 cereal and cereal product samples of were contaminated (Jaradat et al., 2009). This notable discrepancy suggests that *Cronobacter* might occur more frequently in certain kinds of cereals, which should be investigated in further detail. Therefore, the objective of this study was to know the positive rate of *Cronobacter* in a diversity of cereals, including their distribution and characteristics, to thus systematically enrich knowledge about the ecological niche of *Cronobacter* spp. in different cereals.

2. Materials and methods

2.1. Sample collection

2.1.1. Food sample collection

In total, 594 cereal and cereal-related samples were collected for our investigation in two different stages. In the first stage, 467 cereal samples, including rice, wheat, and their related products, Job's tears, millet, buckwheat, sorghum, corn, and oat, were collected for testing the presence of *Cronobacter*. In the second stage, 127 related samples were collected for *Cronobacter* isolation, identification, and enumeration. All the samples were purchased from local markets or online shops from October to December 2014 or collected from the land from April to June 2016.

2.2. Cronobacter isolation, identification and enumeration

Cronobacter was isolated from cereals and their related products according to the national food safety standard method for food microbiological examination in China (GB4789.40–2010). Each 100 g sample was added to 900 mL prewarmed buffered peptone water (BPW, Beijing Land Bridge Biotechnology Co. Ltd., China) and then incubated at 37 ± 1 °C for 18 ± 2 h. Next, 1 mL of overnight culture was inoculated into 10 mL of modified lauryl sulfate tryptose (mLST, Beijing Land Bridge Biotechnology Co. Ltd., China) broth with a final concentration of 10 μg/mL vancomycin, followed by further selective cultivation at 44 ± 0.5 °C for 24 ± 2 h. From each enriched sample, a loopful of the culture broth was streaked onto agar-solidified Druggan-Forsythe-Iversen medium (DFI, Oxoid, UK) and incubated at 36 ± 1 °C for 24 ± 2 h. Typical blue-green colonies were selected and presumptively identified using the API ID 32 E system (BioMerieux, France, Ref. 32400) (Iversen et al., 2004). The identity of the strains was further confirmed by PCR (item 2.4). A 3-tube most-probable-number (MPN) procedure was used to enumerate *Cronobacter* cells, using portions of at least 333 g of each sample to ensure that even low levels (≥0.3 MPN/100 g) of the microorganism could be detected and quantified. Amounts of 100 g, 10 g, and 1 g from each sample were weighed in triplicate, and the subsequent procedures were identical to those for *Cronobacter* isolation and identification.

2.3. DNA extraction

All preserved isolates (further investigation was conducted on one isolate of each *Cronobacter*-positive sample) were streaked onto tryptic soy agar (TSA, Beijing Land Bridge Biotechnology Co. Ltd., China) plates and incubated at 36 ± 1 °C for 18 ± 2 h. A single colony of each strain was inoculated into brain heart infusion broth (BHI, Beijing Land Bridge Biotechnology Co. Ltd., China) and grown at 36 ± 1 °C for 18 ± 2 h, after which 2 mL of culture was used to extract genomic DNA with a QIAamp DNA Mini Kit (Qiagen, Germany).

2.4. Polymerase chain reaction (PCR) confirmation of Cronobacter

A triplex PCR system was used to target the 16S rRNA gene, the ITS sequence, and *ompA* gene with an optimized primer concentration ratio of 2:7:3, respectively. Three pairs of primers targeting the ITS sequence (Liu et al., 2006), the *ompA* gene (Mohan Nair and Venkitanarayanan, 2006), and the 16S rRNA gene (Hassan et al., 2007) were synthesized by Sangon Biotech, Shanghai. A TaKaRa Ex Taq® PCR kit (catalog number: RR001A) was used for PCR, which was performed in 50μL reaction mixtures containing 5 μL 10 × PCR buffer with $MgCl_2$, 4 μL dNTP (2.5 mmol/L), 0.3 μL Ex Taq polymerase (5 U/μL), 2 μL primers (20 μmol/L), and 2 μL DNA template (20 ng/μL). Amplification was carried out as follows: an initial denaturation at 94 °C for 5 min, followed by 30 amplification cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C, and a final extension of 8 min at 72 °C. Subsequently, 5 μL of each PCR product was separated on a 1.5% agarose gel in 1 × Tris/Borate/EDTA (TBE) buffer. After staining with GelRed™ Nucleic Acid Gel Stain (Biotium, USA, catalog Number: 41003) and visualization by ultraviolet (UV) illumination, the gels were photographed and interpreted visually. When all three gene markers tested positive, the strain was judged positive; if only one or two gene markers tested positive, 16S rDNA sequencing was performed for confirmation. The DNA extracted from *Cronobacter* ATCC 25944 and sterilized water were used as positive and negative controls, respectively.

2.5. Cronobacter species analysis

Genomic DNA was extracted as item 2.3. Primers were synthesized by Sangon Biotech (Shanghai, China) according to the *Cronobacter* PubMLST website (http://pubmlst.org/*Cronobacter*). A TaKaRa Ex Taq® PCR kit (catalog number: RR001A) and the same PCR system (with 5 μmol/L primers and 2 μL 20 ng/μL DNA template) were used as Item 2.4. Thermal cycling was carried out using an initial denaturation step of 96 °C for 1 min, followed by 30 cycles of denaturation at 96 °C for 1 min, annealing at 58 °C for 1 min, and elongation 72 °C for 2 min. Amplicons were sequenced by the Sangon Biotech(Shanghai) company. The *fusA* sequences were queried in the online MLST *Cronobacter* database (http://pubmlst.org/*Cronobacter*) to identify the allele number and species.

2.6. Determination of molecular subtypes by pulsed-field gel electrophoresis (PFGE) and band analysis

Cronobacter isolate subtypes were determined by PFGE using previously described methods (Mullane et al., 2007), and PFGE analysis was performed on all 310 bacterial isolates. The resulting gel was then stained with GelRed™ Nucleic Acid Gel Stain (Biotium, USA, catalog number: 41003) for 30 min and destained with 500 mL 18.2 M Ω.cm water for a further 30 min. Tagged Image File Format (TIFF) images were then acquired and uploaded to BioNumerics version 6.6 (AppliedMaths, Saint-Martens-Latem, Belgium) for analysis using the DICE coefficient and unweighted pair group method with arithmetic mean (UPGMA). Both the optimization and band-matching tolerance were 1.5%. When comparing the DNA fingerprint patterns, a cut-off value of 90% similarity was applied. *Salmonella* strain H9812 was used as a molecular weight marker.

2.7. Data analysis

All data were analysed using a Chi-square test with Excel 2010 and SPSS Version 19.0 (SPSS. Inc., Standard Version).

3. Results and discussion

In the first stage, our evaluation of the presence of *Cronobacter* in a wide range of cereal products (N = 467) revealed that 54.0% (252/467) of the samples contained the bacterium (Table 1). Here, a triplex PCR targeting 16S rDNA, the ITS sequence and *ompA* gene was used as previously described (Lou et al., 2014) to achieve high accuracy of

Table 1
Categories tested for the presence of *Cronobacter* and the quantity of the confirmed *Cronobacter* isolates.

Origin of sample (food categories)	Number of samples analysed	Number of positive samples (*Cronobacter* isolates)	% of positive samples in the category
Rice or related products	153	65	42.5
Wheat or related products	100	87	87.0
Corn	33	10	30.3
Sorghum	18	3	16.7
Millet	48	30	62.5
Oats	41	13	31.7
Job's tears	40	18	45.0
Buckwheat	34	26	76.5
Total	467	252	54.0

Cronobacter identification, and all of the 252 isolates tested positive for all three gene markers. There were 65 positive tests out of 153 samples (42.5%) of rice or rice-related food products. For wheat or wheat-related products, *Cronobacter* was detected in 87 of 100 samples (87.0%). Notably, 10 of the 33 samples (30.3%) of corn and three of the 18 samples (16.7%) of sorghum contained the bacterium. For the food categories of millet, oats and Job's tears, the positive rate of *Cronobacter* was 62.5% (30 of 48 samples), 31.7% (13 of 41 samples) and 45.0% (18 of 40 samples), respectively. Moreover, a very high positive rate (76.5%) was found in buckwheat products, for which 26 of 34 samples tested positive. These results show that all the diverse cereal categories sampled contained *Cronobacter* and suggested that cereal-related products would add potential risk during the stages of food consumption. It is not only necessary to efficiently eliminate this pathogen from these products, but also work in communication of the risk to the population including parents and baby careers. Here, we used mLST broth (incubation at 44 °C) according to the standard method for food microbiological examination in China. Since the mLST is known to miss some strains unable to grow in this medium, and 1% of *Cronobacter* strains do not grow at or above 44 °C, including the *C. sakazakii* type strain ATCC 29544T (Nazarowec-White and Farber, 1997; Iversen et al., 2007), the positive rate of *Cronobacter* spp. would be underestimated a little.

Since all of the food categories of cereal samples that we tested contained *Cronobacter*, we then collected 127 more samples in the second stage for enumerating these bacteria in different cereal categories to make the results more accurate. The presence of *Cronobacter* was confirmed in 58 of 127 samples (45.7%). Among cereal products, 16 of 36 rice or rice-related products (44.4%) tested positive for *Cronobacter*, along with eight of 26 samples (30.8%) of wheat or wheat-related products (no wheat flour samples), and four of eight sorghum samples (50.0%) were positive. The rates of positive tests in millet, Job's tears, and buckwheat samples were 25.0% (4 of 16), 66.7% (6 of 9) and 40.0% (8 of 20), respectively. Among samples of corn (6 of 6) and oats (6 of 6), the rate of *Cronobacter*-positive samples was 100% (Table 2). Finally, the concentration of *Cronobacter* in these samples ranged from 0.3 to > 110 MPN/100 g, and the medium concentration of *Cronobacter* in all these food categories was 0.92 MPN/100 g. The quantitative analysis indicated that the *Cronobacter* concentration within 87.9% of samples was under 10 MPN/100 g, while two samples (one millet and one buckwheat) displayed more than 100 MPN/100 g.

Cereals are often used as staple foods, and different cereal grains have different styles for eating according to local culture and acquired tastes. Rice is most commonly cooked with water and then eaten, and rice flour is often made into different kind of flash-frozen foods. In contrast, wheat is usually milled into flour, which is then used to prepare foods such as steamed buns, noodles, dumplings, bread and flash-frozen foods. Whole-grain wheat is typically not cooked alone. Our previous research indicated that *Cronobacter* detection rates in rice- and wheat-related samples notably changed with different processing methods. Here, we combined all the cereal samples detected (Tables 1 and 2) and divided them into four groups according to their different processing methods. These groups were cereal kernels, cereal flour, cereal products made from raw cereal flour and cereal products made from cereal flour (ready to eat). The positive rates of *Cronobacter* ranged from 16.7% to 53.3%, with an average positive rate of 40.2% in cereal kernel samples. In cereal flour samples, the positive rate ranged from 0.0% to 100.0%, with an average positive rate of 66.7%. As for the food category of cereal products made from raw cereal flour, the positive test rate of the bacteria ranged from 10.0% to 100.0%, with an average positive rate of 87.6%. Finally, the bacterium was isolated from 17.4% of cereal products made from cereal flour (ready to eat). Further analysis showed significant differences in the positive rates of *Cronobacter* among these four groups ($p < 0.05$). With the difference in procedures for handling raw material, the presence of *Cronobacter* notably changed. When cereal kernels became cereal flour and related products after processing, the positive rate of samples quickly rose due to the potential cross-contamination. When the products were treated with heat, most of the bacteria disappeared. The highest rate of positive samples (100.0%) was found in wheat flour, wheat flour-based noodles and dumpling (quick-frozen products), followed by very famous Chinese traditional foods, which were quick-frozen products of rice flour called Tang-yuan (94.7%), and rice flour (not infant nutrition rice flour) (90.1%). The positive rates of *Cronobacter* in the flour of corn-, millet-, and buckwheat flour-based noodles were also all above 80% (Table 3). However, both infant nutrition rice flour and rice noodles showed relatively low positive rates for *Cronobacter* (0.0% and 10.0%, respectively) versus the average level in their own complete categories because most of the processing for these foods in China involves a steaming or sterilization step (Li et al., 2015). The results were in exact accordance with our former results (Lou et al., 2019) and again confirmed the hypothesis that raw material processing might be the critical factor for the presence of *Cronobacter* in different cereals.

Table 2
Enumeration of confirmed *Cronobacter* in diverse cereal samples.

Origin of sample (food categories)	Number of samples analysed	Number of *Cronobacter* isolates	% of positive samples in the category	Enumeration of *Cronobacter* (MPN/100 g)		
				Median	Minimum	Maximum
Rice or related products	36	16	44.4	0.92	0.36	9.3
Wheat or related products (no wheat flour)	26	8	30.8	0.62	0.3	9.3
Corn	6	6	100.0	1.5	0.74	> 110
Sorghum	8	4	50.0	0.72	0.72	0.72
Millet	16	4	25.0	7.5	4.3	24
Oats	6	6	100.0	0.36	0.3	0.36
Job's tears	9	6	66.7	1.1	0.92	1.6
Buckwheat	20	8	40.0	2.3	0.92	110
Total	127	58	45.7	0.92	0.3	> 110

Table 3
Categories of cereal-related samples tested for the presence of *Cronobacter* from raw material to products.

Origin of sample (food categories)	Number of samples analysed	Number of positive samples (%)	Number of *Cronobacter* species			
			C. sak	*C. mal*	*C. dubl*	*C. turi*
Cereal kernels						
Rice (brown rice and white rice)	118*	49/118 (41.5%)	30	3	13	2
Wheat	15	8/15 (53.3%)	5	1	2	0
Corn	24	4/24 (16.7%)	3	1	0	0
Sorghum	26	7/26 (26.9%)	5	0	2	0
Millet	52	24/52 (46.2%)	14	1	8	0
Oats	31	11/31 (35.5%)	10	1	0	0
Job's tears	36	19/36 (52.8%)	13	3	2	1
Buckwheat	21	8/21 (38.1%)	5	2	0	1
Total	323	130/323 (40.2%)	85	12	27	4
Cereal flour						
Rice (not infant)	11*	10/11 (90.1%)	10	0	0	0
Infant nutrition rice flour	14*	0/14 (0.0%)	0	0	0	0
Wheat	21	21/21 (100.0%)	14	2	5	0
Corn	15	12/15 (80.0%)	8	1	1	2
Millet	12	10/12 (83.3%)	6	1	3	0
Oats	16	8/16 (50.0%)	8	0	0	0
Job's tears	13	5/13 (38.5%)	4	1	0	0
Buckwheat	18	14/18 (77.8%)	7	2	4	1
Total	120	80/120 (66.7%)	57	7	13	3
Products made from raw cereal flour						
Rice noodles (made via heating)	10	1/10 (10.0%)	1	0	0	0
Tangyuan (rice flour-based, flash-frozen food)	19	18/19 (94.7%)	14	4	0	0
Wheat flour-based noodles	30	30/30 (100.0%)	25	2	3	0
Dumpling (wheat flour-based, flash-frozen food)	31	31/31 (100.0%)	19	4	7	0
Buckwheat flour-based noodles	15	12/15 (80.0%)	8	1	2	1
Total	105	92/105 (87.6%)	67	11	12	1
Products made from cereal flour (ready to eat)						
Rice	17*	3/17 (11.8%)	2	0	1	0
Wheat	29	5/29 (17.2%)	3	2	0	0
Total	46	8/46 (17.4%)	5	2	1	0

(Note: All the samples collected in the categories of rice flour, rice noodles, infant nutrition rice flour and flash-frozen products of rice flour were made from white rice according to Chinese tradition. Number of *Cronobacter* species identified as *C. sak, C. mal, C. dubl* and *C. turi* was totally 307, and 3 isolates were other species not contained in the database with the fusA allele number of 71,75 and 204. Data with* was published in Lou et al., 2019).

Cronobacter species were identified using *fusA* allele sequencing. *C. sakazakii* was identified among 214 (69.0%) of the 310 isolates, thus constituting the most dominant species. 53 isolates (17.1%) were identified as *C. dublinensis*,32 isolates (10.3%) were identified as *C. malonaticus,* and 8 isolates (2.6%) were *C. turicensis*. 3 other *Cronobacter* species were identified with the *fusA* allele number of 71, 75 and 204. (Table 3). Further analysis showed no significant difference in *Cronobacter* species distribution among cereal kernels, cereal flour, cereal products made from raw cereal flour and cereal products made from cereal flour (ready to eat) ($p > 0.05$). Moreover, we selected 60 of these isolates for high-throughput sequencing (Illumina HiSeq, PE150), and the species identification consisted of *fusA* allele sequencing (data not shown).

We further subtyped all of these *Cronobacter* isolates by PFGE subtyping analysis and obtained 306 effective fingerprints with a very high degree of genetic diversity. Two interesting clusters (with similarities above 90%) were identified in the food categories of rice kernels, rice flour, products made from raw rice flour and products made from rice flour (ready to eat) (Fig. 1). The pulsotype-designated cluster C1 consisted of four isolates cultured from Tang-yuan, which is made from rice flour, and all of these were identified as *C. sakazakii*. These four isolates were recovered from products from the same brand and manufacturer with the same address and were isolated on four separate occasions in September through October 2014. This may indicate that these strains had a common contamination source, and cross-contamination might be caused during food production, further indicating that the pathogen could persist in the food production environment for a long time. The pulsotype-designated cluster C2 consisted of six isolates identified as *C. sakazakii*, which were cultured from different food categories of rice kernels, Tang-yuan made from rice flour, and biscuits made from rice flour (ready to eat). Three of the isolates (CRNB2014012, CRNB2014013, CRNB2014014) were recovered from three different samples from the same brand, manufacturer with the same address, and the same production date in December 2013. The strain CRNB2014011 was also from rice kernels but from a different brand and production date. All the above four samples of rice kernels originated from Heilongjiang Province, China. The isolate CRNB2014111 was recovered from biscuits made from rice flour produced by a manufacturer in Hangzhou, Zhejiang Province, China. Another strain, CRNB2014164, was recovered from Tang-yuan made from rice flour produced by a manufacturer in Shanghai, China. The pulsotype of cluster C2 indicates that the pathogen could transmit via cereal processing.

Taken together, our results indicate that a high rate of the diverse cereals that we tested contained the bacteria and that cross-contamination might occur easily during processing procedures, especially during the production of cereal flour and cereal flour-based foods. Based on these findings, our former conclusion need to be updated that not only wheat flour but also other commonly used cereal flours might pose a risk of *Cronobacter* infection. On one hand, many leading baby food companies now incorporate an ingredient of a high caloric value called maltodextrin in infant formula products for premature and weaning babies (Rowan and Anderson, 1997). Maltodextrin is produced from starch by partial hydrolysis, while starch is produced from cereal flour. Thus, it might be a source of *Cronobacter* in powdered infant formula. On the other hand, people do not eat cereals and related food without heating; after appropriate treatment with heat, the related food is typically safe. However, flour made from wheat, rice, buckwheat, millet, corn, *etc.* is used widely as staple foods around the world — common household uses of cereal flour are making foods such as bread, noodles, dumplings, steamed buns, and so on. During the process of

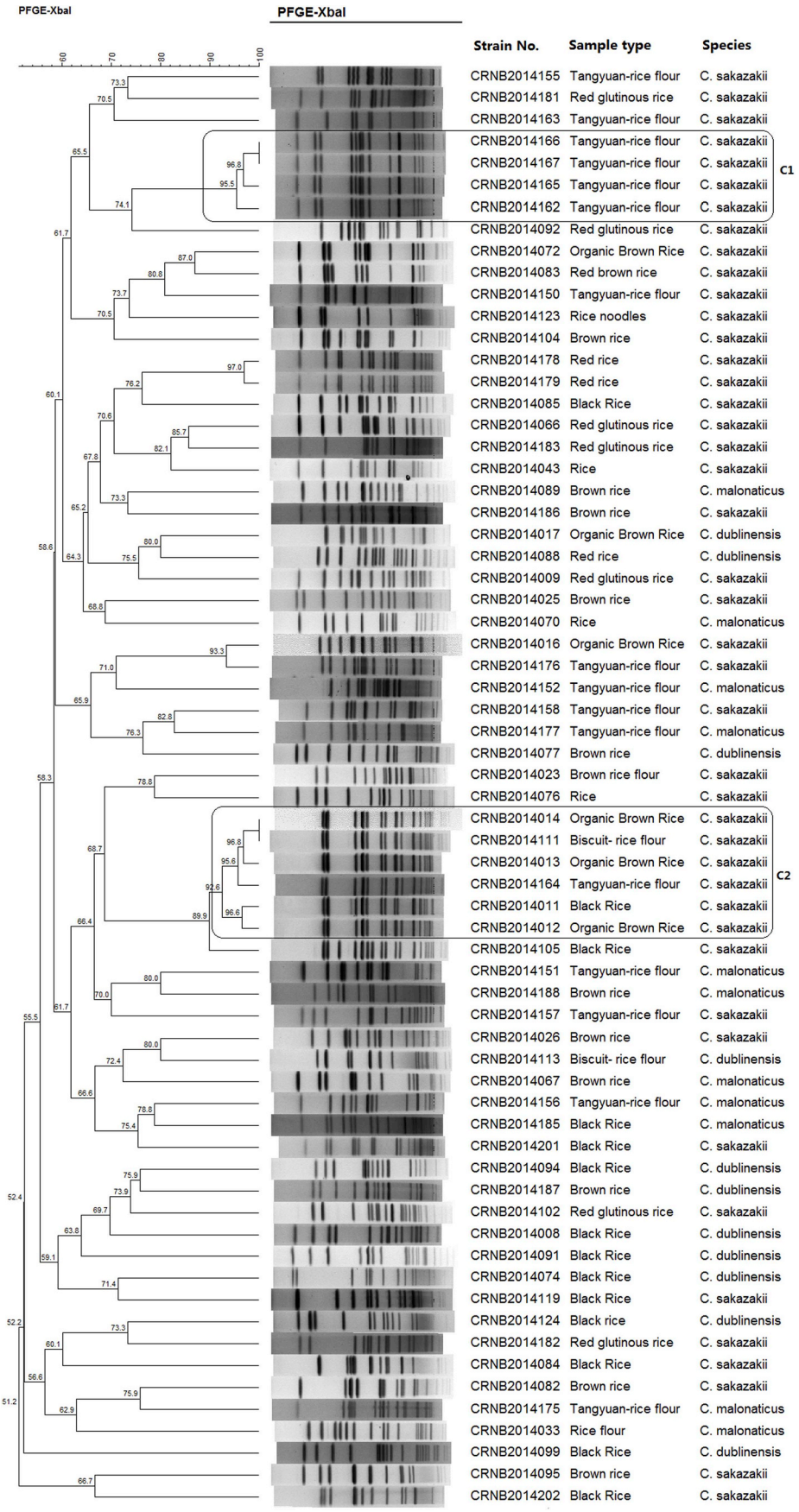

Fig. 1. Dendrogram showing *Xba*I-mediated plused-field gel electrophoresis(PFGE) profiles of Cronobacter strains in rice related food products. The dendrogram was obtained using the unweighted pair group method with arithmetic mean (UPGMA) and the DICE coefficient with 1.5% tolerance.

preparation, flour is easily diffused as it can move through the air and settle down as dust. This phenomenon may therefore lead to the contamination of indoor environments (Lou et al., 2019). Furthermore, when the flour is made into a dough with the addition of water during the dough mixing process, *Cronobacter* that are present might contaminate kitchen surfaces or equipment that they contact, and further cross-contamination might occur easily during the process of washing and storing equipment (Lou et al., 2019). This fact might explain why high levels of *Cronobacter* spp. have been detected in household vacuum dust or other aspects of indoor environments (Jaradat et al., 2009; Killer et al., 2015).

4. Conclusions

The main conclusion from this study is that the selected samples contained *Cronobacter* with a relatively high positive rate of 40.4%, and the majority of food categories (with the exception of infant nutrient rice flour) tested positive for *Cronobacter*. Our results also indicated that the processing of raw materials might be the critical source of these bacteria in different cereals. Moreover, cereal flour might pose a high risk for infection, and special care should be taken while preparing infant foods or formulas to avoid cross-contamination from this source. Reducing the use of cereal flour in residential homes with low birthweight or immunocompromised infants might further decrease the potential risk of *Cronobacter* spp. infection.

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