Contamination, persistence and dissemination of *Cronobacter* during the production of powdered infant formula in China in 2016

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Abstract

A total of 620 samples collected from two factories in China producing powdered infant formula (PIF) between July and November 2016 were analyzed for *Cronobacter*. Antimicrobial susceptibility, pulsed-field gel electrophoresis (PFGE), and biofilm formation of *Cronobacter* were carried out. The results showed that 2.26% samples were positive for *Cronobacter* among the 33 isolates that were identified. All *Cronobacter* isolates were susceptible to 12 antimicrobial agents tested except one isolate which showed intermediate resistance to Chloramphenicol. PFGE analysis showed that nine clusters comprising the 33 isolates were identified, among which C8, C4, and C5 were the predominant types. All 33 isolates were capable of forming biofilm, and particularly, *C. malonaticus* isolates showed a good biofilm-forming ability at both 28°C and 37°C. The results illustrated that it is necessary for PIF manufactures to develop control measures for reducing *Cronobacter* contamination and its associated foodborne illness among infants.

Keywords: biofilm; *Cronobacter*; PFGE; powdered infant formula

Introduction

Cronobacter spp. (formerly *Enterobacter sakazakii*) are Gram-negative opportunistic pathogens but fatal in neonates and immunocompromised infants (Ling *et al.*, 2018). The genus *Cronobacter* currently comprises seven species: *C. sakazakii, C. dublinensis, C. turicensis, C. malonaticus, C. muytjensii, C. condiment,* and *C. universalis.* It is well known that *Cronobacter* had been classified as pathogenic organisms to a restricted population, especially causing life-threatening infections in neonates and immunocompromised infants (Forsythe, 2018). These organisms are widely distributed in foods including powdered infant formulas (PIF), infant cereal foods, dairy products, fruits, and elements in the environment like soil, water, and dust (Tutar *et al.*, 2018; Ueda, 2017). *Cronobacter* infections in infants occur mostly through the consumption of contaminated PIF. As a non-sterile product, PIF is a major source of nutrition for infants under 6 months of age who could not be breastfed; however, the production of PIF involves stages where the product is susceptible to microbial, especially *Cronobacter* contamination, the most vulnerable phase occurring after pasteurization and prior to packaging (Drapala *et al.*, 2018; Kalyantanda *et al.*, 2015; Pires *et al.*, 2020). In several outbreaks caused by *Cronobacter*, the source of infection was traced to contaminated PIF or to spoons and blenders used in preparation of PIF (Chenu and Cox, 2009; Friedemann, 2008). Additionally, *Cronobacter* spp. display remarkable resistance to

desiccation compared with other *Enterobacteriaceae*, which may contribute to their long-term survival in PIF and on surfaces of the production environment (Gurtler and Beuchat, 2007). *Cronobacter* contamination can occur in any step during PIF production, especially in raw ingredients, during processing—originating from the surrounding environment, as a result of improper flushing, and in the prepared PIF for infant consumption. Besides, multiple studies illustrated that some strains of *Cronobacter* are able to form biofilms on glass, stainless steel, polyvinyl chloride, polycarbonate, silicone, and enteral feeding tubes through different media (Henry and Monica, 2019; Iversen *et al.*, 2004). Biofilm formation by *Cronobacter* species is a major concern to the PIF industry, as biofilms on raw ingredients or PIF contact surfaces represent possible sources of product contamination with *Cronobacter*.

Our previous study showed that four (3.4%, 4/119) out of 119 retailed powdered formula samples collected from 16 provinces between 2014 and 2015 were positive for *Cronobacter* (Gan *et al.*, 2018). Based on this result, we carried out a 5-month successive surveillance for *Cronobacter* along the entire PIF production chain in two PIF manufactures of which their PIF at retail level were contaminated by *Cronobacter.* The aim of this study was to determine the *Cronobacter* contamination pathways, to characterize the antimicrobial susceptibility as well as biofilm formation of the major species of *Cronobacter* recovered in these two factories. Meanwhile, pulsed-field gel electrophoresis (PFGE) of *Cronobacter* isolating from different origins was also conducted in order to track the source of contamination across the entire PIF production chain.

Materials and Methods

Sample collection

Two factories (referred to as A and B) located in Shan Xi province, China were chosen to carry out a 5-month surveillance from July to November 2016. Sampling plan was set up based on production flow of dry process. A total of 620 samples including 174 powder samples, 33 liquid samples, 135 air sedimentation samples, and 278 swab samples were obtained from 54 sampling areas and items including raw ingredients, surface of equipment, environment (soil and dust), swabs of the staff's hand, intermediate products, the end products etc. Among them, 195 samples including 28 powder samples, six liquid samples, 48 air sedimentation samples, and 113 swab samples were collected from Factory A. While, 425 samples including 146 powder samples, 27 liquid samples, 87 air sedimentation samples and 165 swab samples were obtained from Factory B. Brain Heart Agar (BHA) plates were used to collect air samples. Briefly, plates were exposed in the air in different areas of PIF production for 15–20 min. Additionally, 50 mL sterile centrifuge tubes were used to collect liquid samples including raw goat milk, concentrated milk, and water; they were collected from production pipeline. Powder samples including base powder, final PIF powder, soil around factory, floor powder, and raw ingredients were collected at amounts shown in Table 1 and stored in sampling bags. Swab samples were collected over a 25 cm^2 surface with sterile cotton swabs moistened in buffered peptone water (BPW). Details were given in Table 1. All samples were placed in insulation box with ice bag and tested on the same day.

Isolation and species identification of Cronobacter

Samples were detected for *Cronobacter* using a modified method based on China National Food Safety Standard (GB4789.40, 2016). For powder and liquid samples, an aliquot of 100 g (or) mL test portion was mixed with 900 mL BPW (unless otherwise indicated, all media from Beijing Land Bridge Technology Ltd., Beijing, China) and incubated at 37°C for 18 h ± 2 h. Swabs were inoculated into 10 mL BPW and incubated at 37°C for 18 h ± 2 h. The pre-enrichment samples were mixed well followed by transferring 1 mL into a tube containing 10 mL mLST/Vancomycin medium and incubated at 41.5°C for 24 h ± 2 h. A loop full of mLST/vancomycin culture was streaked on Brilliance Enterobacter sakazakii Agar (DFI) (Oxoid, England), and incubated at 41.5°C for 24 h ± 2 h. Five suspected colonies (all suspected colonies would be picked up when the number of colonies less than five) were selected and inoculated onto tryptone soya agar (TSA) plate and incubated at 37°C for 24 h. Colonies on TSA plate were further identified by both VITEK® 2 Compact (bioMérieux, France) and ITS (ITS-F 5'-GGGTTGTCTGCGAAAGCGAA-3', ITS-R 5'-GTCTTCGTGCTGCGAGTTTG-3') by polymerase chain reaction (PCR) based on the method published by Liu (Liu *et al.*, 2006). During determination of *Cronobacter* in the air of PIF production areas by disk sedimentation on TSA plate, all plates were incubated at 25°C for 48 h ± 4 h. Yellow pigment colonies were selected and streaked on TSA plate again and identified by both VITEK® 2 Compact and PCR, respectively.

For *Cronobacter* identification at species level, seven pairs of primers designed according to RNA polymerase β subunit (*rpoB*) gene were used to differentiate the seven species of *Cronobacter* according to the methods published previously (Lehner *et al.*, 2012; Stoop *et al.*, 2009). A total of 25 µl PCR reaction mixtures were prepared containing 12.5 µl of 2 × GoTaq Green Master Mix, each primer (10 µmol/L) for 1 µl, 9.5 µl of distilled deionized water and 1 µl of template. The primer sequences and PCR conditions are shown in Table 2.

Table 1. Distribution of samples collected from factories.

Sample categories	Sample description	No. of Factory A samples	No. of Factory B samples	Amount
Liquid	Raw goat milk	NT*	9	50 mL
	Concentrated milk	3	9	
	Internal cleaning water of production pipeline			
	A water sample before homogenization	1	3	
	B water sample after sterilization	1	3	
	C concentrated water sample after homogenization	1	3	
Powder	Goat milk powder (base powder 0)	3	15	300 g
	Base powder 1 (wet processing technology)	3	6	
	Final powder (dry processing technology)	3	12	
	Soil around factory	8	20	
	Floor powder of packaging room	3	6	100 g
	Whey powder	3	15	
	Lactose	NT	15	
	Concentrated whey protein	1	5	
	Galacto-oligosaccharide	1	5	
	Fructo oligosaccharides	NT	5	
	DHA powder	NT	5	
	ARA powder	NT	5	
	Casein calcium phosphate	NT	5	
	α-Lactoalbmin	NT	5	
	Lactorferrin	NT	4	
	Bifidobacterium	NT	12	
	Vegetable oil	NT	3	
	Floor powder of fluid-bed workshop	3	3	
Disk sedimentation	Release station	NT	12	15–20 min
	Bunker room	6	NT	
	Concentration room	6	9	
	Fluid-bed room	6	9	
	Inside packaging room	6	12	
	Excipients room	6	NT	
	Clean area	3	12	
	Non-clean area	6	12	
	Air purification room	6	9	
	Rear packaging room	3	12	
Swab	Door handles in clean and non-clean areas	10	12	25 cm^2
	Excipients conveyor belt (before and after UV disinfection)	6	12	
	Release platform	NT	9	
	Entrance of mixing tank	6	NT	
	Surface of mixing tank	6	12	
	Surface of precast powder bags	3	3	
	Fluid-bed	6	6	
	Ground of inside packaging room	6	12	
	Conveyor belt of inside packaging room	6	12	
	Air vent of inside packaging room	6	12	
	Staff's shoe soles of inside packaging room	8	12	
	Staff's hands of inside packaging room	6	12	

(Continues)

Table 1 Continued

Sample categories	Sample description	No. of Factory A samples	No. of Factory B samples	Amount
	Staff's clothes of inside packaging room	6	12	
	Big bag packaging machine	3	3	
	Receiving sample window of rear packaging room	3	12	
	Final product conveyor belt	3	12	
	Purification system and clean room	NT	3	
	Air inlet of purification system	6	3	
	Air outlet	6	6	
	Drain	7	NT	
	Delivery window	4	NT	
	Entrance of preparing tank	3	NT	
	Outlet of preparing tank	3	NT	

*Not tested.

Table 2 Primer sequences and PCR conditions of Cronobacter identification at species level.

Species	Primer	Sequence(5′→3′)	Predenaturation	denaturation	annealing	extension	Fragment size
C. condimenti	Con-F	AAC GCC AAG CCA ATC TCG	95 ± 2 min	95 ± 1 min	58 ± 30s	72 ± 1 min	689 bp
	Con-R	GTA CCG CCA CGT TTT GCT					
C. sakazakii	Sak-F	ACG CCA AGC CTA TCT CCG CG	94 ± 3 min	94 ± 1 min	69 ± 30s	72 ± 1 min	514 bp
	Sak-R	ACG GTT GGC GTC ATC GTG					
C. malonaticus	Mal-F	CGT ATC TCT GCT CTC	94 ± 3 min	94 ± 1 min	64 ± 30s	72 ± 30s	251 bp
	Mal-R	AGG TTG GTG TTC GCC TGA					
C. turicensis	Tur-F	CGG TAA AAG AGT TCT TCG GC	94 ± 3 min	94 ± 1 min	61 ± 30s	72 ± 1 min	628 bp
	Tur-R	GTA CCG CCA CGT TTC GCC					
C. dublinensis	Dub-F	GCA CAA GCG TCG TAT CTC C	94 ± 3 min	94 ± 1 min	62 ± 30s	72 ± 30s	418 bp
	Dub-R	TTG GCG TCA TCG TGT TCC					
C. muytjensii	Muy-F	TGT CCG TGT ATG CGC AGA CC	94 ± 3 min	94 ± 1 min	67 ± 30s	72 ± 30s	289 bp
	Muy-R	TGT TCG CAC CCA TCA ATG CG					
C. universalis	Uni-F	ACA AAC GTC GTA TCT CTG CG	94 ± 3 min	94 ± 1 min	61 ± 30s	72 ± 30s	506 bp
	Uni-R	AGC ACG TTC CAT ACC GGT C					

Antimicrobial susceptibility testing

Minimal inhibitory concentrations (MIC) of 12 antimicrobials including Tetracycline (TET), Nalidixic acid (NAL), Ciprofloxacin (CIP), Chloramphenicol (CHL), Sulfamethoxazole-Trimethoprim (SXT), Amoxicillin (AMX), Ampicillin (AMP), Ampicillin-Sulbactam (SAM), Gentamicin (GEN), Cefotaxime (CTX), Ceftazidime (CAZ), and Imipenem (IMP) were determined via broth microdilution method for all confirmed *Cronobacter* isolates. *Escherichia coli* ATCC 25922 was used as a quality control in antimicrobial susceptibility testing (AST) experiment. All susceptibility results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) interpretive standards (CLSI M100S, 2016).

PFGE subtyping of Cronobacter

PFGE analysis was performed on all *Cronobacter* isolates. Cell suspensions were adjusted to 4–4.5 malt concentration. After solidification, plugs [0.5% (w/v)] were lysed in 5 mL cell lysis buffer (50 mmol/L Tris-HCl, 50 nmol/L EDTA, 1% sodium lauroyl-sarcosine) and 25 μl proteinase K (20 mg/mL) at 55°C with 170 r/min shaking for 2 h. Plugs were washed twice with 15 mL deionized water for 10 min followed by washing with 15 mL TE buffer at 50°C

shaking at a speed of 170 r/min for 3 times, 15 min for each. After cutting into 3 mm slices, the plug was digest with XbaI at 37°C for 2 h. Treated plugs were cast into a 1% (w/v) SKG gel and separated by electrophoresis in 0.5 × TBE buffer at 14°C for 18 h. The PFGE gel was then dyed with 0.01% (w/v) GelRed for 30 min and then soaked in deionized water for 30 min. DNA fingerprints were analyzed by BioNumerics V7.6 (Applied Math, Belgium) using DICE coefficient and unweighted pair group method with arithmetic mean (UPGMA). Patterns indistinguishable by computer and visual inspection were assigned the same pattern designation.

Biofilm formation assay

Quantification of biofilms grown in microtiter dishes was performed by following the procedure as described by Yan (Yan *et al.*, 2015). M9 minimal medium (6 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 0.5 g/l NaCl, 1 g/l NH_4Cl, 2 mM $MgSO_4$, 0.1% glucose and 0.1 mM $CaCl_2$) adjusted to 0.1 Malt concentration was used as the growth medium. *Cronobacter* isolates were inoculated into tryptic soy broth (TSB) medium and incubated at 37°C overnight. Briefly, a triplicate 200 µl cultures was transferred into 96-well microplates and incubated at 28°C and 37°C for 72 h, respectively. Each well was washed with 200 µl of phosphate-buffered saline (PBS) for 3 times, fixated with 200 µl of methanol for 20 min and stained with 200 µl 0.4% (w/v) crystal violet (CV) for 15 min after air-drying for 15 min. The plate was washed with 200 µl PBS for 3 times, dissolved biofilm with 200 µl of 33% (v/v) acetic acid for 30 min, and measured the optical density (OD) at 570 nm. *Salmonella* Typhimurium ATCC 14028 with a strong biofilm-forming capacity was selected as the positive control (Martins *et al.*, 2013). All tests were carried out in triplicate.

Results

Prevalence of Cronobacter contamination in samples

Table 3 shows that among 620 samples, 14 (2.26%, 14/620) were positive for *Cronobacter* and 33 isolates were obtained. Among the positive samples, seven (50%, 7/14) samples were soil origin, four were swabs of drain, air inlet and soles of staff, two were PIF raw ingredients of lactose, and one was air sedimentation, respectively. In terms of 33 *Cronobacter* isolates, 21 (63.64%, 21/33), seven (21.21%, 7/33), four (12.12%, 4/33), and one (0.30%, 1/33) were cultured from soil, lactose, environmental swabs, and air sedimentation samples, respectively. Regarding PIF factories, 13 isolates cultured from eight samples (57.14%, 8/14) including four soil samples, four swabs of air inlet of purification room, drain, and soles from Factory A and 20 isolates from six samples (42.86%, 6/14) including three soil samples, two lactose samples and one rear packaging room air sedimentation samples from Factory B were contaminated by *Cronobacter.* No significant difference on the incidence of *Cronobacter* contamination between these two PIF factories were found (Chi-square test: $X^2 = 3.251$, $P = 0.071$); however, contamination of raw ingredients was found in Factory B. In 14 *Cronobacter* positive samples, 12 samples including soil, environmental swabs, and lactose were contaminated by *C. sakazakii* and two (including soil and air sedimentation) by *C. malonaticus*, respectively. Among 33 *Cronobacter* isolates, *C. sakazakii* was the predominant species detected and accounted for 87.88% (29/33)

Table 3 Contamination of *Cronobacter* monitored during PIF production.

Factory	Sampling date	Source categories	Species	No of strains
A	July	Soil-3	*C. sakazakii*	1
		Soil-4(2)	*C. sakazakii*	2
		Air inlet of purification system-1	*C. sakazakii*	1
		Drain-4	*C. sakazakii*	1
		Staff's sole-1	*C. sakazakii*	1
		Staff's sole-2	*C. sakazakii*	1
B	August	Lactose-1	*C. sakazakii*	4
		Lactose-2	*C. sakazakii*	3
		Soil-2	*C. malonaticus*	3
A	September	Soil-2	*C. sakazakii*	3
		Soil-3	*C. sakazakii*	3
B	September	Soil-2	*C. sakazakii*	3
B	October	Disk sedimentation of rear packaging room-3	*C. malonaticus*	1
		Soil-3(6)	*C. sakazakii*	6

followed by *C. malonaticus* (12.12%, 4/33). All 13 isolates from Factory A were *C. sakazakii*. Whereas, 20 *Cronobacter* isolates from Factory B were diverse at the species level: 16 (80%, 16/20) for *C. sakazakii* from soil and lactose, and four for *C. malonaticus* from soil and air sedimentation, respectively. Precisely, two different species were detected from soil samples collected in Factory B (Table 3).

AST of Cronobacter

Results of AST show that all 33 *Cronobacter* isolates cultured from different matrix samples were susceptible to all the 12 antimicrobials tested except one isolate with an intermediate resistance to Chloramphenicol.

PFGE subtyping analysis

The 33 *Cronobacter* isolates comprising 29 *C. sakazakii* and four *C. malonaticus* were analyzed using PFGE with XbaI and differentiated into nine PFGE pulsotypes with similarities above 95% (Figure 1). The predominant pulsotype of *C. sakazakii* was C8, which included seven isolates (7/33, 21.2%) cultured from lactose collected from Factory B, followed by C4 (6/33, 18.2%) and C5 (6/33, 18.2%) cultured from soil collected from Factory A and Factory B, respectively. Pulsotypes of C3 and C6 consisted of four *C. malonaticus* isolates from both soil and air sedimentation and three *C. sakazakii* isolates from Factory B, respectively. Three pulsotypes (C1, C2, and C7) of *C. sakazakii* cultured from soil, air inlet, drain, and workers' sole collected from Factory A, respectively were found to contain two isolates for each. And the remaining C9 pulsotype contained one isolate obtained from soil in Factory A. The PFGE results revealed that the drain and air inlet in the workshop of Factory A had cross contamination of *Cronobacter* with soil around the factory, respectively. Factory B also existed cross contamination between soil and rear packaging room environment. And the isolates collected form staff's sole of Factory A were different from other strains isolated from this factory. The contamination of *Cronobacter* during the production of PIF may come from both raw ingredients like lactose and the environment including soil, air, workers movement, and so forth.

Biofilm formation

All 33 *Cronobacter* isolates were studied for biofilm formation using the microplate method with M9 minimal medium as the growth medium at two different incubation temperatures: 28°C and 37°C. A temperature of 28°C was chosen to simulate that of PIF processing environment, while 37°C was the one similar to human body temperature. Biofilm formation by *Cronobacter* isolates is shown in Table 4 and Figure 2. In all the 33 *Cronobacter* isolates tested, 17 isolates (51.52%, 17/33) of *C. sakazakii* produced biofilm which were deemed "weak" on their rating scale with an optical density (OD) at 570 nm (OD_{570}) <0.2 at both 28°C and 37°C on the basis of criteria described by Lee (Lee *et al.*, 2012). Eight (8/33, 24.24%) isolates including three *C. malonaticus* and five *C. sakazakii* showed intermediate capacity of biofilm formation with an OD_{570} ranged from 0.5 to 1.0 at 28°C and two of *C. sakazakii* isolates exhibited strong biofilm formation with an OD_{570} >1 at 28°C. Whereas, two (2/33, 6.06%) *C. sakazakii* and three *C. malonaticus* (3/33, 9.09%) isolates showed intermediate and strong biofilm formation ability at 37°C, respectively. No significant difference of isolates in OD_{570} >0.5 biofilm-forming ability between 28°C and 37°C was found (Fisher test: P = 0.240). Additionally, intermediate and strong biofilm formation ability of four *C. malonaticus* isolates at 28°C and 37°C, respectively, was observed. No significant difference in the biofilm-forming capacity at 28°C was found between *C. malonaticus* and *C. sakazakii* isolates (Fisher test: P = 0.073). Whereas, the capacity of 3 *C. malonaticus* isolates in forming biofilm at 37°C was significantly stronger than those of *C. sakazakii* (Fisher test: P = 0.007). Regarding the effect of temperature on biofilm formation, Figure 2 revealed that there is no significant difference in biofilm formation by *Cronobacter* between 28°C and 37°C (T tested: P = 0.176). Compared with *S. Typhimurium* ATCC 14028, 11 *Cronobacter* isolates were of stronger capacity in biofilm formation at 37°C.

Discussion

The results of this study indicated that *Cronobacter* can enter processing plants through raw ingredients, soil, and PIF production environment and be disseminated throughout, especially in the situation of areas of high foot traffic were contaminated by staff's soles with *Cronobacter*, and consequently can cross-contaminate processing equipment and persist for long periods in these facilities. This persistence is also attributable to biofilms—a well-known factor for protecting bacteria from harsh environmental stresses such as desiccation, antimicrobials, and disinfectants—which is of concern to public health and food safety. The result of PFGE in this study further illustrated that carry-over of *Cronobacter* from raw ingredient as well as PIF processing environment contamination by this bacterium are the major sources of *Cronobacter* contamination in PIF. Therefore, PIF manufactures should strengthen the management of raw ingredients and production environments, especially they should pay more attention to external and internal environment contamination of PIF processing workshop.

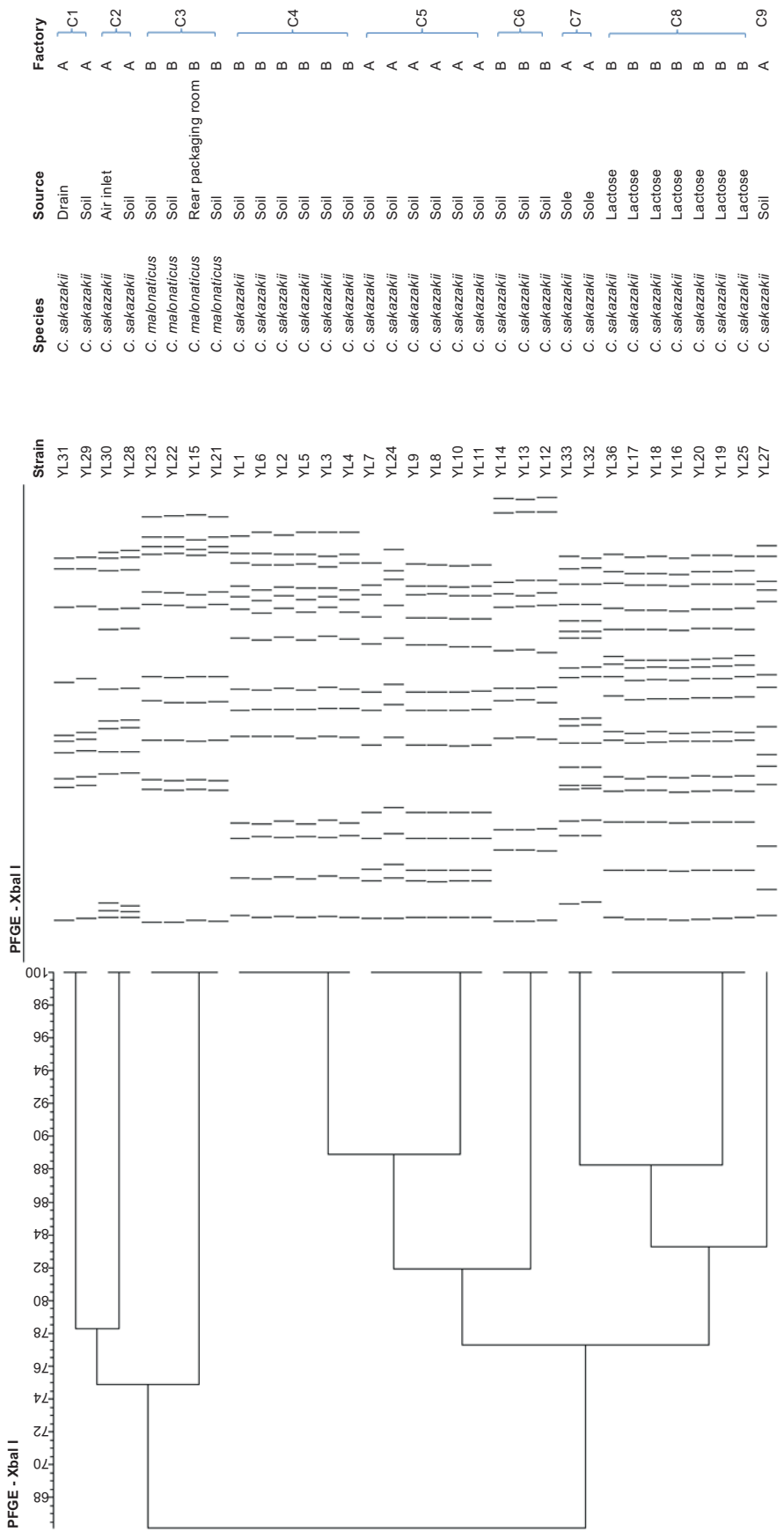

Figure 1 PFGE typing of 33 *Cronobacter* isolates.

Table 4 Biofilm formation by *Cronobacter* expressed as OD_{570nm} at different temperatures.

Strain ID	Species	OD_{570nm} at different temperatures	
		28°C	37°C
YL1	*C. sakazakii*	0.097	0.072
YL2	*C. sakazakii*	0.108	0.091
YL3	*C. sakazakii*	0.092	0.114
YL4	*C. sakazakii*	0.095	0.109
YL5	*C. sakazakii*	0.121	0.117
YL6	*C. sakazakii*	0.113	0.112
YL7	*C. sakazakii*	0.090	0.128
YL8	*C. sakazakii*	0.178	0.108
YL9	*C. sakazakii*	0.182	0.178
YL10	*C. sakazakii*	0.092	0.115
YL11	*C. sakazakii*	0.226	0.146
YL12	*C. sakazakii*	0.100	0.076
YL13	*C. sakazakii*	0.114	0.078
YL14	*C. sakazakii*	0.166	0.080
YL15	*C. malonaticus*	0.517	0.436
YL16	*C. sakazakii*	0.749	0.392
YL17	*C. sakazakii*	0.430	0.631
YL18	*C. sakazakii*	0.161	0.199
YL19	*C. sakazakii*	0.740	0.531
YL20	*C. sakazakii*	0.745	0.436
YL21	*C. malonaticus*	0.633	1.097
YL22	*C. malonaticus*	0.486	1.025
YL23	*C. malonaticus*	0.694	1.288
YL24	*C. sakazakii*	0.386	0.134
YL25	*C. sakazakii*	0.596	0.289
YL26	*C. sakazakii*	0.552	0.419
YL27	*C. sakazakii*	0.338	0.204
YL28	*C. sakazakii*	0.095	0.113
YL29	*C. sakazakii*	0.138	0.049
YL30	*C. sakazakii*	0.103	0.118
YL31	*C. sakazakii*	0.467	0.204
YL32	*C. sakazakii*	1.043	0.352
YL33	*C. sakazakii*	1.203	0.459
14028	*Salmonella Typhimurium*	1.891	0.298

Among the 33 *Cronobacter* isolates, 29 (87.88%, 29/33) were *C. sakazakii* and four (12.12%, 4/33) were *C. malonaticus*. *C. sakazakii* is the dominant species isolated, which was the same as previous studies (Fei *et al.*, 2015, 2017). Since all seven species of *Cronobacter* have pathogenicity and the clinical reports of neonatal *Cronobacter* infection are primarily because of *C. sakazakii*, *C. malonaticus,* and *C. turicensis*. According to Stephen J. Forsythe, grouped *Cronobacter* species were divided into two groups: Group 1 comprises *C. sakazakii* and *C. malonaticus* as the majority of clinical isolates in all age groups. *C. sakazakii* is mainly related to neonatal infections while *C. malonaticus* is related to adult infections (Forsythe, 2018; Jaradat *et al.*, 2014). Hence, we can speculate that infants who consumed PIF produced by these two factories that is contaminated with one or more *Cronobacters* like *C. sakazakii* and *C. malonaticus* would be at a high risk of *Cronobacter* infection on the basis of our study.

Antibiotic resistant bacteria has been the focus of global attention; the spread of antibiotic resistant bacteria directly leads to the reduction or ineffectiveness of clinical antibiotic treatments, extend patient's course, and could cause complications. All the 33 *Cronobacter* isolates obtained in the present study were susceptible to all 12 antimicrobials tested except one isolate that was of intermediate resistance to Chloramphenicol. Our previous studies revealed that eight out of 417 (1.92%) *Cronobacter* isolates cultured from four kinds of infant foods from 27 provinces in China during 2012–2014 were resistant to antimicrobials, including AMX (3), AMP (2), CHL (2), CTX (1), NAL (1), SXT (1), and TET (1) (Gan *et al.*, 2015). These data imply that *Cronobacter* isolates were generally sensitive to antimicrobials, similar to the results reported by Gu, Chen and Pei (Chen *et al.*, 2011; Gu *et al.*, 2008; Pei *et al.*, 2007).

Lee defined $OD_{600nm} > 1$ as a strong biofilm-forming strain, $0.5 < OD_{600nm} < 1$ as a moderate biofilm-forming strain, and $OD_{600nm} < 0.5$ as a weak biofilm strain (Lee *et al.*, 2012). According to his definition, the biofilm formation ability of all 33 isolates were weaker than *S. Typhymurium* ATCC 14028 at 28°C. There were two *C. sakazakii* isolates, codex YL32 and YL33, which had strong biofilm formation ability. PFGE showed that both isolates cultured from the same source (swabs of staff's sole in Factory A); once these isolates are found to persist in the workshop, they will cause a potential long term threat such as PIF contamination. There were eight moderate biofilm-forming strains, including YL15, YL21, and YL23 which belonged to pulsotype C3 with 100% similarity and isolated from disk sedimentation of rear packaging room and soil sample. And YL16, YL19, YL20, YL25, and YL26 with moderate biofilm formation ability belonged to C8 and came from lactose powders. These isolates could cause persistent contamination on the surface of productive facilities and affect the safety of end products. Two *C. malonaticus* isolates were strong biofilm-forming strains at 37°C and moderate at 28°C. The National Institutes of Health (NIH) of America announced that over 80% of microbial infections were found involved in biofilm formation (Römling and Balsalobre, 2012). *Cronobacter* could form biofilm on different materials such as silicon, latex, polycarbonate, stainless steel, glass, and polyvinyl chloride (Choi *et al.*, 2014). Cells of *Cronobacter* in biofilm could survive for several weeks under dry conditions and resistant to

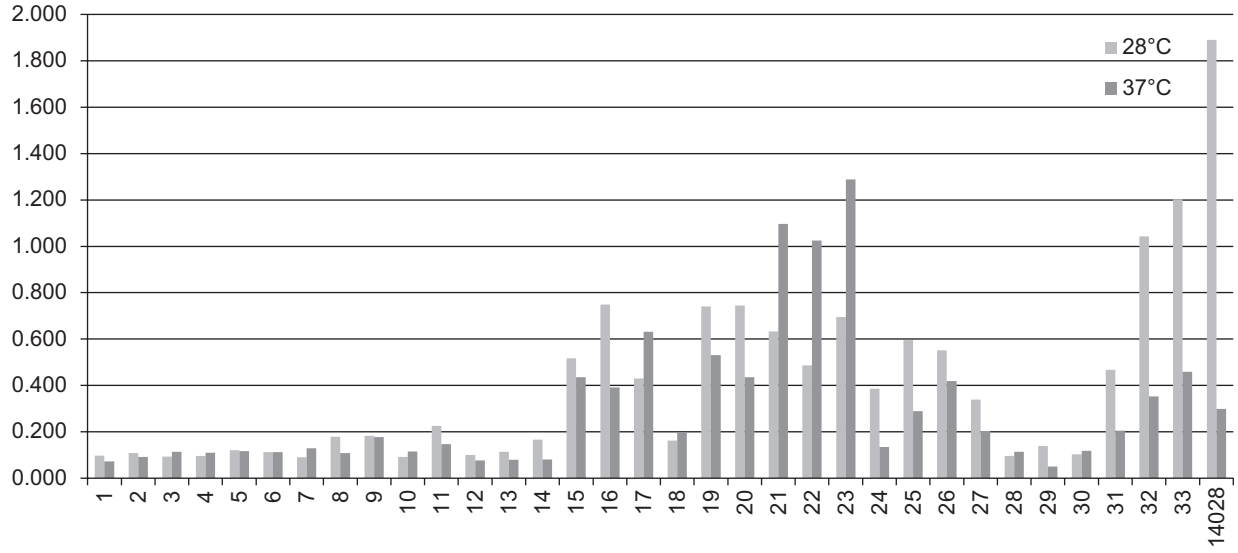

Figure 2 Comparison of biofilm formation by 33 *Cronobacter* isolates at 28°C and 37°C.

osmotic pressure (Dancer *et al.*, 2009). Biofilm has a great impact on PIF industry, persistent strains with biofilm formation ability could store on the surface of packaging materials and processing equipment for a long time, and resistant to common cleaning agents, disinfection and sterilization methods, and cause a long term and persistent contamination in production environment. A strict regulatory strategy for PIF manufactures including successive monitoring for environment, raw ingredients, semi and end products, particular disinfection procedures are needed. Hazard analysis and critical point (HACCP) systems could be the effective control measures and decrease the risk of *Cronobacter* contamination along the entire production chain.

Conclusion

Totally, 33 isolates of *Cronobacter* were isolated form raw ingredients, PIF processing environment, and workers' sole from two PIF processing factories in Shanxi province, China between July and November 2016. *C. sakazakii* was found to be the predominant species in different sources of our surveillance. The results suggest that two factories exited multi-source contamination of *Cronobacter*, especially in the PIF processing environment. *C. sakazakii* and *C. malonaticus* could form biofilm at either 28°C or 37°C, with strong biofilm formation ability at 37°C for *C. malonaticus*. The data obtained in the present study would provide the scientific basis for prevention and control measures of PIF contamination by *Cronobacter* during manufacturing. It is necessary to minimize the contamination of this bacterium from different sources and reduce the risk of infants being exposed to Cronobacter via PIF.

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