Tea polyphenols inactivate *Cronobacter sakazakii* isolated from powdered infant formula

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ABSTRACT

This study evaluated the antimicrobial activity of tea polyphenols (TP) against 4 *Cronobacter sakazakii* strains with different sequence types (ST) isolated from powdered infant formula (PIF). The results showed that in normal saline, 5 mg/mL of TP (pH 3.44) could eliminate approximately 7.0 log cfu/mL of *C. sakazakii* within 1 h; in rehydrated PIF, after acidification with HCl (pH 3.55), TP showed a stronger antibacterial activity compared with the controls (malic acid, ascorbic acid, and citric acid). Further, some differences were obvious in tolerance to TP between *C. sakazakii* strains with different ST. The tolerance of *C. sakazakii* CE1 (ST4) to TP was found to be greater than that of the other 3 *C. sakazakii* strains (ST1, ST8, and ST64). The results of recovered test and transmission electron microscope analysis revealed that the action of TP against *C. sakazakii* was an irreversible bactericidal process caused by leakage of cytoplasm. Taken together, these results indicated that TP had an effective bactericidal effect against *C. sakazakii*, and provided a new idea for preventing and inactivating *C. sakazakii* in PIF.

Key words: tea polyphenols, *Cronobacter sakazakii*, inactivation, powdered infant formula

INTRODUCTION

Cronobacter spp. (formerly classified as *Enterobacter sakazakii*) is an emerging gram-negative, motile, non-spore-forming, rod-shaped, opportunistic foodborne pathogen (Healy et al., 2010; Yan et al., 2012; Sonbol et al., 2013). Although powdered infant formula (PIF) is considered the main source of infection, *Cronobacter* has been detected in a wide range of environmental

and food sources (Craven et al., 2010). As a matter of fact, it has been isolated from tea, which is used as a drink for infants >4 mo (Chap et al., 2009). Because of ingesting contaminated PIF by *Cronobacter* strains, the neonates, especially low birthweight individuals, can be infected to suffer from bacteremia, meningitis, and necrotizing enterocolitis, with a high fatality rate of 40 to 80% (Drudy et al., 2006; Lin and Beuchat, 2007; Holý and Forsythe, 2014). *Cronobacter sakazakii* had been shown to be the dominant species in *Cronobacter* spp. isolated from PIF (Forsythe et al., 2014). Similarly, this view was in agreement with the finding of our team by identifying and typing 75 strains of *Cronobacter* spp. isolated from a wet-mixing PIF manufacturing facility with different methods (Lu et al., 2014). Further, *C. sakazakii* sequence type (ST) 4 characterized by multi-locus sequence typing was proven to be the predominant sequence type associated with neonatal meningitis (Joseph and Forsythe, 2011). *Cronobacter sakazakii* ST1 and *C. sakazakii* ST8 were the main sequence types isolated from PIF and clinical sources, respectively (Joseph et al., 2012a). Meanwhile, *C. sakazakii* ST64 was found to be one of the dominant sequence types isolated from PIF in China by our team (Fei et al., 2015). Thus, for the *C. sakazakii* strains with different sequence types, this is an important issue needing further study if differences are present in tolerance to the same antibacterial material. To the best of our knowledge, this is the first report on the tolerance of *C. sakazakii* associated with its ST.

In recent years, compared with some sterilization techniques and synthetic antibiotics, various natural extracts, including polyphenols and organic acids, have received attention as potential antimicrobials because they are natural and acceptable to consumers. Cranberry pomace extract had been reported to control the growth of *Listeria monocytogenes* (Vattem et al., 2004). The methanol extract of *Lactuca sativa* was able to inhibit the growth of various gram-negative and gram-positive bacteria with the lowest minimal inhibitory concentration of 2.5 mg/mL (Edziri et al., 2011). The

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aqueous extract and crude alcohol extract of *Mangifera indica* seed kernel had significant antimicrobial activity against the isolated pathogen *Shigella dysenteriae* (Rajan et al., 2011). Furthermore, a variety of studies showed that organic acids could inhibit the growth of *Cronobacter* strains. Tartaric, citric and malic acid could delay the growth of *Cronobacter* strains with viable counts of 3.0 to 5.0 log after 24 h incubation at pH 5.0, whereas lactic, acetic, butyric, and propionic acid showed an absolute growth inhibition at the same pH (Zhu et al., 2013). To gain further insight into this phenomenon, malic acid, citric acid, and ascorbic acid (VC), as the most common organic acids with increasingly wide application, were selected in our study.

Tea polyphenol (TP) is a significant natural compound extracted from tea, mainly composed of catechins, flavonoids, phenolic acids, and anthocyanins, among them, the catechins account for 60 to 80% (Mukhtar and Ahmad, 2000). It has many beneficial functions to human health, such as antioxidant effect, bacteriostasis, and anticarcinogenic and antiinflammatory activities (Koňariková et al., 2015; Yang and Jiang, 2015). Meanwhile, TP had effective inhibitory effects on mixed biofilm formation of *Staphylococcus aureus* and *Salmonella* Enteritidis through inhibiting the bacterial communication signal autoinducer 2 synthesis (Zhang et al., 2014). In addition, it had been reported that TP could effectively inhibit the growth of *Serratia marcescens* through cell membrane damage, and the inhibition halos increased as the concentration of TP increased within certain limits (Yi et al., 2014). Despite various studies that reported the antimicrobial activity of TP against a variety type of bacteria, the antibacterial mechanism of TP against different bacteria might be different. So far, the literature available on TP inhibiting *C. sakazakii* was limited, especially in reconstituted PIF.

In our study, we investigated the effect of TP on the 4 *C. sakazakii* strains with different sequence types isolated from PIF, analyzed the influence of low pH condition on the bactericidal action of TP, and compared the differences in antimicrobial activity of TP against the *C. sakazakii* strains with different sequence types.

MATERIALS AND METHODS

Bacterial Strains

All the 4 *C. sakazakii* strains in this study were isolated from PIF in China and identified by multi-locus sequence typing; details of isolates are shown in Table 1. These strains were stored in cryogenic vials at -20°C in the presence of 40% glycerol as cryoprotectant. To

reactivate the *C. sakazakii* strains, 0.1-mL portions of cultures were inoculated into 10 mL of Luria-Bertani (LB) broth and cultivated at 37°C for 20 h.

Preparation of Unstressed *C. sakazakii* Cell Suspensions

After incubation, 2 mL of each culture was transferred to a sterile Eppendorf tube. Working stationary-phase cell suspensions were collected after centrifugation at $8,000 \times g$ for 10 min, then washed twice with normal saline (NS) at room temperature. The supernatants were discarded and the pellets were re-suspended in 1 mL of sterile NS. For enumeration, 0.1 mL of each cell suspension was spread-plated in duplicate on tryptic soy agar (TSA) plates after serial dilution in NS and incubated at 37°C for 24 h to obtain the required concentration of inoculum.

Treatment with TP and Organic Acids in PIF

A commercial PIF (Wondersun, Harbin, Hei Longjiang Province, China) was purchased and reconstituted according to the manufacturer's instruction. Briefly, 15 g of the PIF was reconstituted in 100 mL of sterile distilled water. The rehydrated PIF was pasteurized at 63°C for 30 min before use, and no *C. sakazakii* strains were detected. Based on related studies, an appropriate concentration of 5 mg/mL was selected as the optimum dosage (Amalaradjou et al., 2009; Joshi et al., 2014). Thus, 5 mg/mL of TP was added to the rehydrated PIF and shaken gently to make sure it was fully dissolved. Meanwhile, as controls, malic acid, citric acid, and VC were processed in the same way. An Accumet pH meter (Corning Inc., Corning, NY) was used to determine the pH of all samples. The initial pH of rehydrated PIF was 6.8, 2 and the value of pH decreased to pH 3.62, 3.55, 4.55, and 6.46 after 5 mg/mL of malic acid, cit-

Table 1. *Cronobacter sakazakii* strains isolated from powdered infant formula in China

<i>C. sakazakii</i> strain	ID ¹	ST ²	CC ³
CE21	858	1	1
CE1	872	4	4
CE13	890	8	8
CE25	905	64	64

¹ID = strain identification code in the pubMLST.org/cronobacter database (<http://www.pubmlst.org/cronobacter>).

²ST = sequence types.

³CC = clonal complex defined as clusters of sequence types with single locus variants, as given by Joseph et al. (2012b).

Table 2. Results of the final pH after 7 h treatment and turbidity measurements¹

Item	Before acidification					After acidification			
	PIF	VC	Malic acid	Citric acid	TP	HCl	VC	Malic acid	TP
Initial pH	6.82	4.55	3.62	3.55	6.46	3.55	3.55	3.55	3.55
Final pH after 7 h treatment	6.80	4.57	3.64	3.58	6.49	3.53	3.54	3.54	3.53
Turbidity (NTU ²)	447.6 ^a	448.0 ^a	446.6 ^a	447.3 ^a	446.5 ^a	447.8 ^a	446.9 ^a	447.6 ^a	447.7 ^a

^aTurbidity measurements did not differ between treatments.

¹PIF = powdered infant formula; VC = ascorbic acid; TP = tea polyphenols.

²NTU = nephelometric turbidity units.

ric acid, VC, and TP, respectively, were added (Table 2). One hundred microliters of cell suspension of the 4 *C. sakazakii* strains was separately added to 10 mL of rehydrated PIF with different treatments to obtain a final desired concentration of approximately 7.0 log cfu/mL. The PIF inoculated with *C. sakazakii* strains but without treatment was used as a control. The cell suspensions with different treatments were incubated at 37°C for 1, 3, 5, and 7 h. Then, at each time point, 0.1-mL portions of appropriately diluted cultures were spread-plated on TSA in duplicate and incubated at 37°C for 24 h to enumerate the surviving populations of the pathogen. The final pH values of differently treated media were measured after 7 h of treatment.

Treatment with Inorganic Acids in PIF

To investigate the role played by the pH of different treatments in *C. sakazakii* reduction, the pH of all samples was adjusted to 3.55 (the lowest pH value of the aforementioned samples) with 4 M HCl. The HCl-treated PIF of pH 3.55 was used for comparison purposes. The following inoculation, cultivation, and enumeration processes were performed in accordance with the above description.

Turbidity Measurements

The addition of organic acids and acidification with HCl in PIF could reduce the pH of treated media, and the acidic environment might affect the colloidal stability of casein micelles (Post et al., 2012). To approximate changes in casein micelles, solution turbidity as a measure of protein-protein aggregation was monitored by the transmittance at 450 nm using a Shimadzu 1601 PC UV-visible spectrometer as described by Madadlou et al. (2009). In brief, a 3-mL portion of the treated medium was poured into a 1-cm-pathlength cuvette and placed into the cuvette holder. All the aforementioned treatments in nonacidified and acidified PIF containing

5 mg/mL of malic acid, citric acid, VC, and TP were measured.

Treatments with TP in NS

To avoid the influence of proteins in PIF on antibacterial effect of TP (Staszewski et al., 2011), the study was conducted in sterile NS. Accordingly, 5 mg/mL of TP was added to the sterile NS, the initial pH of the samples were all 6.93, which decreased to pH 3.44 after mixing with 5 mg/mL of TP. The following inoculation, cultivation, and enumeration processes were performed in accordance with the above description.

Recovery of the Stressed *C. sakazakii* Cells

According to the previous report, after treatment with 5 mg/mL of acidified TP for 7 h in nutritious rehydrated PIF, the 4 *C. sakazakii* strains were transferred to fresh LB broth and PIF immediately, and then incubated at 37°C for 6 h and 12 h to estimate bactericidal or bacteriostatic effects (Joshi et al., 2014). Moreover, the 4 *C. sakazakii* strains treated with 5 mg/mL of TP in NS for 1 h were also observed in the same way. The counts of *C. sakazakii* strains were determined by plating 0.1 mL of appropriate diluents on TSA plates and incubating the plates at 37°C for 24 h. Besides, considering the unfavorable environmental conditions, microorganisms might exist in a state known as viable but nonculturable. Cox et al. (2015) reported that these nonculturable cells could not be recovered by various cultivation methods. So this form of *C. sakazakii* cells did not affect the results because it could not be determined under the current experimental conditions.

Transmission Electron Microscopy Analysis

To eliminate the influence of proteins in PIF on the observation of cell morphology (Karlsson et al., 2007), the treatments with TP in NS for 1 h were prepared

and the treatments in LB broth for 1 h served as controls. All the 4 *C. sakazakii* strains treated with 5 mg/mL of TP in NS or without TP in LB broth were drawn after 1 h of contact time and centrifuged at $3,000 \times g$ for 5 min at room temperature. To fix the cells, the pellets were resuspended in 0.1 M sodium phosphate buffer (pH 7.1) containing 3% glutaraldehyde solution. *Cronobacter sakazakii* cells were washed twice with the same buffer after fixation for 12 h, and then 1% osmium tetroxide was used to fix the cells for 6 h at 25°C, and washed 3 times with the same buffer. The cells were dehydrated with a graded series of ethanol (50, 70, 90, and 100%) for 10 min, and embedded in Epon Lx-112 (Ladd Research, Williston, VT) after washing with acetone. Thin sections were cut with a knife on a Power Tome XL microtome (Boeckeler Instruments, Tucson, AZ) and double-stained with uranyl acetate and lead citrate. Samples were examined in a transmission electron microscope (Hitachi, Tokyo, Japan). Micrographs were digitally recorded.

Statistical Analysis

Each treatment was carried out with 3 replications. Statistical analysis was calculated by ANOVA with the SPSS 20.0 software (SPSS Inc., Chicago, IL). Tukey's multiple range test was used to determine the significant differences ($P < 0.05$) between treatments.

RESULTS

Reduction of *C. sakazakii* in PIF Treated with TP

After 7 h treatment, the final pH of samples containing 5 mg/mL malic acid, citric acid, VC and TP were 3.64, 3.58, 4.57, and 6.49, respectively (Table 2). As a result, the pH values of different treatments were almost stable after 7 h, which had less than 0.1 unit alteration to the initial pH. The growth of *C. sakazakii* strains treated with 5 mg/mL of TP in PIF (pH 6.46) was significantly inhibited ($P < 0.05$) compared with *C. sakazakii* strains growing in PIF (pH 6.82; Figure 1). As controls, 5 mg/mL of malic acid (pH 3.62), citric acid (pH 3.55), and VC (pH 4.55) were also found to significantly reduce ($P < 0.05$) *C. sakazakii* strains in PIF. The order of inhibition of different treatments was citric acid (pH 3.55) > malic acid (pH 3.62) > VC (pH 4.55) > TP (pH 6.46) > PIF (pH 6.82).

Reduction of *C. sakazakii* in PIF Treated with Acidified TP

Table 2 shows that the final pH of all samples were almost stable after 7 h of treatment. The inhibition

effect of TP acidified with HCl against *C. sakazakii* was obviously stronger ($P < 0.05$) than the effect of control groups (Figure 2). Further, none of the 4 *C. sakazakii* strains treated with 5 mg/mL of TP acidified with HCl in PIF (pH 3.55) could be detected on TSA after 7 h of treatment. The inhibition effect of HCl as an inorganic acid against *C. sakazakii* was greater ($P < 0.05$) than the effect of citric acid and malic acid.

Turbidity Analysis

The hydration level of the casein micelles could be influenced by pH, temperature, and pressure, which might bring about the changes of casein in particle size, aggregation, and gelation (Orlien et al., 2010). The results of solution turbidity were illustrated in Table 2. As shown in Table 2, the turbidity remained relatively stable ($P > 0.05$) after the addition of organic acids and acidification with HCl in PIF, which indicated that acid-treated PIF did not lead to casein aggregation and precipitation.

Reduction of *C. sakazakii* in NS Treated with TP

In NS, TP showed strong antimicrobial properties against *C. sakazakii* and resulted in a complete elimination of the organism. As a consequence, no *C. sakazakii* strains could be detected on TSA after 1 h treatment with 5 mg/mL of TP in NS (data not shown).

Antimicrobial Ability of TP and Acidified TP Against *C. sakazakii* with Different ST

The differences in antimicrobial ability of TP and acidified TP with HCl against *C. sakazakii* strains with different ST, including ST1, ST4, ST8, and ST64, are shown in Figure 3. The results revealed that the survival rates of the 4 *C. sakazakii* strains with different ST were different in rehydrated PIF with 5 mg/mL of nonacidified and acidified TP. In treatments with nonacidified TP in PIF, from 1 to 3 h, the surviving bacterial counts of *C. sakazakii* CE1 (ST4) were significantly higher ($P < 0.05$) than those of the other 3 *C. sakazakii* strains with ST1, ST8, and ST64. On the contrary, the surviving bacterial counts of *C. sakazakii* CE25 (ST64) were the lowest among the 4 *C. sakazakii* strains. From 5 to 7 h, significant differences ($P < 0.05$) were found in survival rates among the 4 *C. sakazakii* strains with different ST, and the order of survival rates was *C. sakazakii* CE1 (ST4) > *C. sakazakii* CE21 (ST1) > *C. sakazakii* CE13 (ST8) > *C. sakazakii* CE25 (ST64). In treatments with TP acidified with HCl in PIF, after

Figure 1. Effect of 5 mg/mL of tea polyphenols (TP) against *Cronobacter sakazakii* CE1 (A), CE25 (B), CE13 (C), and CE21 (D) in rehydrated powdered infant formula (PIF) at 37°C; the rehydrated PIF and 5 mg/mL of malic acid, citric acid, and ascorbic acid (VC) serve as controls. Error bars represent standard deviation. Different letters (a–e) represent significant differences between treatments within the same time points ($P < 0.05$).

1 h, the antimicrobial effects of acidified TP against *C. sakazakii* CE1 (ST4) and *C. sakazakii* CE13 (ST8) were significantly stronger ($P < 0.05$) than the effects against *C. sakazakii* CE21 (ST1) and *C. sakazakii* CE25 (ST64). From 3 to 5 h, the antimicrobial ability of acidified TP against *C. sakazakii* strains showed significant differences ($P < 0.05$), and the order of survival rates was *C. sakazakii* CE1 (ST4) > *C. sakazakii* CE13

(ST8) > *C. sakazakii* CE21 (ST1) > *C. sakazakii* CE25 (ST64). After 7 h, all 4 *C. sakazakii* strains were reduced to undetectable levels.

Recovery of the Stressed *C. sakazakii* Cells

The 4 *C. sakazakii* strains with different ST following treatment with 5 mg/mL of TP acidified with HCl in

Figure 2. Effect of 5 mg/mL of tea polyphenols (TP), malic acid, and ascorbic acid (VC) acidified with 4 M HCl in rehydrated powdered infant formula (PIF) against *Cronobacter sakazakii* CE1 (A), CE25 (B), CE13 (C), and CE21 (D) at 37°C. Error bars represent standard deviation. Different letters (a–f) represent significant differences between treatments within the same time points ($P < 0.05$).

PIF for 7 h showed no growth after 6 and 12 h incubation in fresh recovery (LB broth and PIF) medium. Consistently, no *C. sakazakii* cells were able to restore growth in LB broth or in PIF after treatment with 5 mg/mL of TP in NS for 1 h. It should be apparent from these results that the action of TP against *C. sakazakii* was a bactericidal rather than bacteriostatic process.

Transmission Electron Microscope Analysis Results

The 4 *C. sakazakii* strains with different ST treated with 5 mg/mL of TP in NS or without TP in LB broth for 1 h were examined by transmission electron microscopy. The transmission electron microscopy images (Figure 4) showed that, compared with controls, the

Figure 3. (A) Effect of 5 mg/mL of tea polyphenols (TP) in rehydrated powdered infant formula (PIF) against *Cronobacter sakazakii* CE1 (ST4), CE25 (ST64), CE13 (ST8), and CE21 (ST1) at 37°C. (B) Effect of 5 mg/mL of TP acidified with 4 M HCl in rehydrated PIF against *C. sakazakii* strains CE1 (ST4), CE25 (ST64), CE13 (ST8), CE21 (ST1) at 37°C. Error bars represent standard deviation. Different letters (a–d) represent significant differences between the 4 *C. sakazakii* strains with different sequence type (ST) within the same time points ($P < 0.05$).

cell morphology of all *C. sakazakii* strains with different ST treated with TP was seriously damaged, and the cytoplasm leaked out.

DISCUSSION

Natural extracts with antioxidant capacity such as organic acids and phenolics could effectively inhibit the growth of foodborne pathogens (Kim et al., 2009, 2012; Zhu et al., 2013). Kim et al. (2009) showed that hot water-soluble muscadine seed extracts in total phenolics (2.78 mg/mL), malic acid (1.87 mg/mL), and tannic acid (7.1 mg/mL) were able to inactivate 6.0 log cfu/mL *C. sakazakii* strains within 1 h at 37°C, and the various organic acids as the main active ingredients played an important role in *C. sakazakii* reduction. Besides, 0.5% *trans*-cinnamaldehyde could reduce *C. sakazakii* strains to undetectable levels from 7.0 log cfu/mL within 4 h at 37°C or 23°C (Amalaradjou et al., 2009). In our study, all the experiments were carried out at 37°C because it was near the optimum growth temperature of *C. sakazakii* (Iversen and Forsythe, 2003; Chenu and Cox, 2009). At this temperature, *C. sakazakii* strains had higher metabolic, growth, and death rates, which could help to better investigate the antimicrobial ef-

fect of TP. Indeed, a population of 7.0 log cfu/mL *C. sakazakii* was completely killed after treatment with 5 mg/mL of TP in NS (pH 3.44) for 1 h at 37°C. Further, 7.0 log cfu/mL *C. sakazakii* strains could be reduced to undetectable levels after 7 h treatment in rehydrated PIF supplemented with 5 mg/mL of TP acidified with HCl (pH 3.55) at 37°C. These results suggested that the antimicrobial effect of TP against *C. sakazakii* strains was satisfactory and acceptable.

The pH played an important role in the action of natural extracts against *C. sakazakii*. The antimicrobial effect of citric acid, malic acid, and VC was enhanced after acidification. This phenomenon was due to the synergistic counteractions, H⁺ dissociated from HCl could increase the accumulation in the undissociated organic acids, thus its effective concentration was altered (Zhu et al., 2013). Some studies found that organic acids due to undissociated form and higher pKa could easily penetrate the bacterial cell membrane to reduce intracellular pH of bacteria, further causing disturbance and damage of enzymatic reaction and the nutrient transport system; ultimately, the irreversible denaturation of acid-labile protein and DNA led to the death of cells (Alvarez-Ordóñez et al., 2014). However, TP at pH 3.55 led to a reduction of about 7.0 log cfu/

Figure 4. (A) Transmission electron microscopy images of the 4 *Cronobacter sakazakii* strains treated with Luria-Bertani broth at 37°C for 1 h. (B) Transmission electron microscopy images of the 4 *C. sakazakii* strains treated with 5 mg/mL of tea polyphenols (TP) in normal saline at 37°C for 1 h. ST = sequence type.

mL after 7 h treatment in PIF, which was greater than that caused by citric acid, malic acid, and VC. According to the previous reports, polyphenols and proteins were able to combine to form protein-polyphenol complexes, which could significantly influence their biological activities (Richard et al., 2006; Staszewski et al., 2011), but catechins, as the most abundant and biologically active compounds in TP, were very stable in acidic solutions (pH <4; Chen et al., 2001). So a low pH condition could inhibit the formation of protein-polyphenol complexes and improve the biological activity of TP to have a more efficient bactericidal action.

Moreover, in contrast with the complexation and degradation reactions of polyphenols in the nutrient-rich media of rehydrated PIF, TP in NS showed a noticeable inhibitory activity against the *C. sakazakii* strains and caused the same reduction of the tested strains within 1 h. Additionally, compared the pH value between the different TP treatments, in NS (pH 3.44) and in rehydrated PIF (pH 6.46), PIF could improve the initial pH of media from pH 3.44 to 6.46. Similarly,

the buffered effect of PIF also appeared in the study by Zhu et al. (2013). Therefore, the appropriate acidification of PIF was necessary to play a satisfactory antimicrobial effect of TP against *C. sakazakii* strains in PIF. As indicated earlier, the addition of organic acids and appropriate acidification with HCl did not result in the quality deterioration of PIF. The acidified TP could be used as a disinfectant to sterilize the air and the surface of equipment and floor that coated with milk powder particles in the dairy factory. As a preliminary study, this article lays the foundation for further research. Further in-depth studies about practical applicability of TP need to be pursued, and data in this study will be very useful.

The antibacterial mechanism of TP had been preliminary revealed, but the exact mechanism of action was not clear. However, in this study, the permeability of the outer and inner membrane of *C. sakazakii* dramatically increased after TP treatment, which caused severe disruption of cell membrane, followed by the release of cytoplasm. In contrast, *C. sakazakii* strains treated with

blueberry proanthocyanidins and blueberry juice were reduced because of cell clustered together along with the formation of blebs and viewing pores, and blueberry phenolics was considered to disturb cell membrane fluidity, change fatty acid profile, and destroy cellular metabolism (Joshi et al., 2014). Therefore, bactericidal effect of TP on *C. sakazakii* was an irreversible process compared with the bacteriostatic action of blueberry proanthocyanidins and blueberry juice. Furthermore, the antibacterial mechanism of TP was a complicated process, and a wide variety of reasonable explanations was reported. Navarro-Martínez et al. (2006) found that TP pigallocatechin-3-gallate inhibited ergosterol synthesis by disturbing folic acid metabolism in *Candida albicans*. Cho et al. (2008) demonstrated that TP could differentially stimulate the expression of various proteins in the bacteria and synergize the bactericidal activity of oxacillin for methicillin-resistant *Staphylococcus aureus*. Therefore, a more comprehensive study on the mechanism of TP against *C. sakazakii* should be performed as soon as possible. However, undoubtedly, as a potential natural antibiotic, TP was a good choice because of its efficiency and irreversible bactericidal action.

Cronobacter spp. is a diverse genus in the *Enterobacteriaceae* family. In this study, we researched the antibacterial effect of TP against the 4 *C. sakazakii* strains with different sequence types isolated from PIF, including ST4, ST1, ST8, and ST64, and found 5 mg/mL of TP could effectively kill 7.0 log cfu/mL the above 4 *C. sakazakii* strains in NS (pH 3.44) and in acidified PIF (pH 3.55) after 1 and 7 h of treatment, respectively. However, in the middle phase of treatment, namely from 3 to 5 h, the antibacterial effect of TP on the 4 *C. sakazakii* strains with different ST showed significant differences ($P < 0.05$). The reduction of *C. sakazakii* CE1 (ST4) was the lowest among the 4 *C. sakazakii* strains CE1 (ST4), CE21 (ST1), CE13 (ST8), and CE25 (ST64). Interestingly, Joseph et al. (2012b) applied the 7-loci method to *Cronobacter* spp. isolates obtained between 1950 and 2009 and showed that *C. sakazakii* sequence type 4 was the predominant ST associated with severe cases of neonatal meningitis. This result suggested that the dominant position of *C. sakazakii* CE1 (ST4) in *Cronobacter* genus might be due to its stronger tolerance ability. But this inference needs to be further confirmed, because *C. sakazakii* CE1 (ST4) cannot represent all the *C. sakazakii* ST4 strains.

CONCLUSIONS

The effective bactericidal ability of TP against *C. sakazakii* is a meaningful finding and provides a new option for preventing and killing *C. sakazakii* stains

using natural extracts. Irreversible cytochylema leakage improves the chance that TP becomes an efficient natural antibiotic. However, to use TP as a food additive to prevent *Cronobacter* contamination in PIF, it is critical that the TP dosage is optimized and that safety experiments in infants are conducted.

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