Amaranthus tricolor crude extract inhibits *Cronobacter sakazakii* isolated from powdered infant formula

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ABSTRACT

The purpose of this study was to elucidate the antibacterial activity and possible mechanism of action of *Amaranthus tricolor* crude extract (ATCE) against *Cronobacter sakazakii* isolated from powdered infant formula (PIF). The antibacterial activity of ATCE was assessed by measuring the diameter of inhibition zone (DIZ), minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC). The possible mechanism of action of ATCE was revealed by analyzing the effects of ATCE on growth curves and changes in cell membrane potential, intracellular pH, content of bacterial protein and genomic DNA, and cell morphology. Finally, ATCE was applied to the disinfection of *C. sakazakii* in biofilm on stainless steel tube. The results showed that the DIZ, MIC, and MBC of ATCE against *C. sakazakii* strains were from 14.35 ± 0.67 to 14.84 ± 0.67 mm, 20 mg/mL, and 40 mg/mL, respectively. Treatment with ATCE ended the logarithmic growth phase of *C. sakazakii*, and led to depolarization of the cell membranes, reducing intracellular pH and bacterial protein and genomic DNA contents, and resulting in cytoplasmic leakage and deformation. In addition, ATCE effectively inactivated *C. sakazakii* in biofilm, reducing viable bacteria by approximately 6.5 log cfu/mL bacterial count after treatment with 1 MIC (1 MIC = 20 mg/mL) of ATCE for 20 min at 25°C. Our findings showed that ATCE inactivated *C. sakazakii* strains isolated from PIF and has potential as a natural disinfectant to reduce the contamination of PIF by *C. sakazakii*.

Key words: *Cronobacter sakazakii*, *Amaranthus tricolor* crude extract, powdered infant formula, antibacterial activity

INTRODUCTION

Cronobacter species, as emerging and opportunistic food-borne pathogens, are gram-negative, motile, non-spore-forming, peritrichous, rod-shaped facultatively anaerobic bacteria (Gurtler et al., 2005; Al-Nabulsi et al., 2009). The genus is diverse and currently divided into 7 species: *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, *C. dublinensis*, *C. universalis*, and *C. condimenti* (Joseph et al., 2012; Fei et al., 2018b). Among them, *C. sakazakii* is considered to be the dominant species isolated from powdered infant formula (PIF), and it can cause severe necrotizing enterocolitis, septicemia, and meningitis, with a mortality rate of 40 to 80% (Caubilla-Barron et al., 2007; Sonbol et al., 2013). Contamination of PIF and its production environments by *C. sakazakii* are ongoing; for example, Fei et al. (2015, 2017) found that the contamination rate of PIF with *C. sakazakii* in China was 3.5% in 2009–2012, and 2.8% from January 2015 to March 2017. Lu et al. (2019) isolated 37 *C. sakazakii* strains from 835 environmental samples collected from 4 PIF factories. Therefore, it is important to develop and enhance prevention and control measures to reduce the contamination of *C. sakazakii* strains in PIF.

Some studies have found that *C. sakazakii* is sensitive to heat and may not survive in PIF after the spray-drying process, although the main points of contamination of this pathogen are primary and secondary packaging (Brandl et al., 2014; Wu et al., 2018). Moreover, *C. sakazakii* has a strong ability to produce form biofilms that can allow pathogens to stick to the surfaces of stainless steel equipment and various packaging materials, and that play a role in protection of pathogens, resulting in increased resistance to disinfectants and environmental stresses and the risk of contamination.

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tion (Kim et al., 2006; Henry and Fouladkhah, 2019). *Cronobacter sakazakii* is widely distributed in water, air, and soil; in particular, air is considered the most likely vector for the spread of pathogenic bacteria in PIF factories (Brandl et al., 2014; Fei et al., 2015). Therefore, *C. sakazakii* strains in the environment need to be strictly controlled during the production process of PIF. Currently, sodium hypochlorite and hydrogen peroxide are the most commonly used disinfectants for environmental sterilization in PIF factories, and long-term use is likely to improve the tolerance of *C. sakazakii* strains to these 2 disinfectants (Park et al., 2016; Kim et al., 2019). In addition, sodium hypochlorite has been banned in some European countries because of its potential harm to the environment (Meireles et al., 2016). In view of this, research is focused on developing environmentally friendly strategies to eliminate *C. sakazakii* in the environment (Shi et al., 2016a,b; Guo et al., 2020a).

Natural products have been widely developed as environmentally friendly potential bacteriostatic agents to inhibit food-borne pathogen contamination in the food industry (Guo et al., 2020a). For example, olive oil polyphenol extract (**OOPE**) has been shown to have good antibacterial effect on food-borne pathogens, including *C. sakazakii*, *Bacillus cereus*, *Listeria monocytogenes*, *Salmonella* Typhimurium, and *Staphylococcus aureus* (Fei et al., 2018a, 2019; Guo et al., 2019, 2020b). Analogously, *Amaranthus tricolor* and its extract contain alkaloids (betaine), polyphenols (flavonoids, steroids, catechuic acid, and tannins), terpenoids, and saponins, all of which have been reported to have varying degrees of antibacterial activity (Kueete et al., 2011; Peter and Gandhi, 2017). Other species of *Amaranthus*, including *A. viridis*, *A. hybridus*, *A. spinosus*, and *A. caudatus*, have been confirmed to have a broad spectrum of antibacterial effects (Maiyo et al., 2010; Bulbul et al., 2011). Therefore, there is sufficient evidence that the extract of *Amaranthus tricolor* should have good antibacterial activity.

Based on the factors described above, we analyzed the antibacterial effect and possible mechanism of *A. tricolor* crude extract (**ATCE**) against *C. sakazakii* isolated from PIF. The antibacterial activity of ATCE against *C. sakazakii* was assessed based on the values of diameter of inhibition zone (**DIZ**), MIC, and minimal bactericidal concentration (**MBC**). We demonstrated the effects of ATCE on the growth curves of *C. sakazakii* and clarified the mechanism of action of ATCE against *C. sakazakii* by revealing the changes in membrane potential, intracellular pH (**pH_{in}**), bacterial protein content, cellular DNA level, and cell morphology of tested strains.

MATERIALS AND METHODS

ATCE Material

The ATCE was prepared by the Key Laboratory of Dairy Science in Northeast Agricultural University (Harbin China). After cleaning, the *A. tricolor* leaves were dried at 25°C in a vacuum desiccator (Parkoo, Guangzhou, China) until there was no significant moisture in the samples, and then ground into powder using a mechanical grinder (Yongkang Boou Machinery Co. Ltd., Zhejiang, China). The powder was then added to 70% (vol/vol) ethanol at a ratio of 1:25 (g/mL) and soaked at 40°C for 2 h for extraction of active ingredients. The extracted solution was filtered, collected, and concentrated into the paste using a reduced pressure rotary evaporator (KNF, Freiburg, Germany). Finally, the paste was dried into powder using a vacuum freeze dryer (Beijing Biocool Co. Ltd., Beijing, China).

Bacteria and Culture Conditions

A total of 10 *C. sakazakii* strains were used in this study, including *C. sakazakii* ATCC 29544 purchased from American Type Culture Collection (ATCC, Manassas, VA) and 9 *C. sakazakii* strains isolated from PIF (Fei et al., 2017). In all isolates, we determined DIZ, MIC, and MBC values to evaluate the antibacterial activity of ATCE. *Cronobacter sakazakii* ATCC 29544 was used in experiments to reveal the possible mechanism of action. To culture the strains, 100 µL of culture was activated in 10 mL of tryptic soy broth (**TSB**) at 37°C for 18 h. A loopful of *C. sakazakii* was streaked onto tryptic soy agar (**TSA**; Qingdao Haibo Biotechnology Co. Ltd., Qingdao, China) plates and incubated at 37°C for 24 h. A typical colony was inoculated into TSB followed by incubation at 37°C for 18 h to obtain the pure cultures of isolates.

Disk Diffusion Assay

The DIZ of ATCE against 10 *C. sakazakii* strains were measured using the disk diffusion method reported by Barry (1976). A TSA plate was evenly coated with 100 µL of bacterial suspension of *C. sakazakii* (approximately 10⁶ cfu/mL). Filter paper disks (6 mm diameter) were soaked with 15 µL of ATCE (80 mg/mL); one disk soaked with 15 µL of water was used as control. These disks were laid evenly on the TSA plate containing *C. sakazakii* strains, and incubated at 37°C for 24 h. The DIZ of TSB was used as a negative control. In 3 repetitions, the DIZ values of ATCE against *C. sakazakii* were determined.

Determination of MIC and MBC

The MIC of ATCE for all *C. sakazakii* strains were determined using the agar dilution method according to Fei et al. (2018a). Briefly, ATCE was added to warm TSA that had been autoclaved and cooled to $\sim 50^{\circ}\text{C}$; the final concentrations of ATCE were adjusted to 0, 0.3125, 0.625, 1.25, 2.5, 5, 10, 20, and 40 mg/mL. The above system was uniformly mixed, and then 500 μL of the mixtures were quickly added into sterile 24-well plates. When the TSA medium with different concentrations of ATCE solidified, 2 μL (10^6 cfu/mL) of each *C. sakazakii* strain was inoculated to the agar block. The bacterial solution was allowed to dry and then the block was incubated at 37°C for 24 h. The MIC was defined as the lowest concentration of ATCE in which *C. sakazakii* strains could not grow visibly. One hundred microliters (10^6 cfu/mL) of suspensions after treatments with ≥ 1 MIC of ATCE for 30 min were spread on the TSA, and then cultured at 37°C for 24 h. The MBC was defined as the lowest concentration of ATCE that prevented growth of colonies of *C. sakazakii*.

Growth Curve of *C. sakazakii* ATCC 29544

The growth curve of *C. sakazakii* ATCC 29544 was plotted with reference to a previous report (Shi et al., 2016a). *Cronobacter sakazakii* was cultured in TSB medium until the optical density at 600 nm (OD_{600}) value of the bacterial solution reached 0.2. Two milliliters of this bacterial solution was added to 96-well microtiter plates. The ATCE was mixed in the bacterial solution, and adjusted to 0, 0.5, 1, 1.5, and 2 MIC. For the *C. sakazakii* to grow, all plates were incubated at 37°C for 24 h. During this period, cell growth was monitored by measuring OD_{600} at 2-h intervals using a multimode plate reader (Tecan, Infinite M200 PRO, Männedorf, Switzerland).

Determination of Membrane Potential

Based on a previous study, the membrane potential of *C. sakazakii* ATCC 29544 was measured (Fei et al., 2019). Briefly, a suspension of *C. sakazakii* at 10^7 cfu/mL was prepared and seeded in black cell enzyme label plates followed by incubation at 37°C for 30 min. Then, 1 $\mu\text{mol/L}$ bis-(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC4 (3); Beijing Solarbio Sciences and Technology Co. Ltd., Beijing, China] was added to the plates as a membrane potential-sensitive fluorescent probe, and ATCE was added to reach final concentrations of 0, 1, and 2 MIC. The system was incubated at 37°C for 30 min and then fluorescence was measured at excitation and emission wavelengths of 492 and 515

nm, respectively, using a fluorescence microplate reader (Beijing Potent Technology Co. Ltd., Beijing, China).

Determination of Intracellular pH

The changes in pH_{in} of *C. sakazakii* ATCC 29544 after treatment with ATCE were analyzed based on our previous study (Guo et al., 2020c). Briefly, a suspension of 10^8 cfu/mL of *C. sakazakii* was prepared and incubated at 37°C for 30 min; then, 1 μM carboxyfluorescein diacetate succinimidyl ester (cFDA-SE; Shanghai Yaoyun Biological Technology Co. Ltd., Shanghai, China) was added as a fluorescent probe. The cultures were centrifuged at $11,200 \times g$ for 5 min, the pellet was resuspended in sterilized normal saline, and 10 mM glucose mixed into the suspension. The mixed samples were incubated at 37°C for 30 min and centrifuged at $11,200 \times g$ for 5 min followed by washing twice. The pellets were resuspended in normal saline (125 μL) containing ATCE (1 MIC and 2 MIC), incubated at 37°C for 20 min, and added into black opaque 96-well plates. Under excitation wavelengths of 440 nm and emission wavelengths of 490 nm, the fluorescence intensities were measured at 25°C using a microplate reader (Tecan, Infinite M200 PRO). The actual fluorescence value of the sample was equal to the measured value minus the value of cell-free control. The pH_{in} was defined as the ratio of the actual fluorescence signal at 490 nm/440 nm.

SDS-PAGE

We carried out SDS-PAGE based on a previous report (Fei et al., 2019). Briefly, *C. sakazakii* ATCC 29544 culture (10^7 cfu/mL) was prepared and treated with 1 MIC and 2 MIC of ATCE at 37°C for 3, 6, 9, and 12 h. Untreated *C. sakazakii* was used as the control. All samples were centrifuged at $8,000 \times g$ for 10 min at 4°C to obtain the supernatant. The supernatant (15 μL) was mixed with SDS-PAGE loading buffer (5 μL) followed by heat treatment at 95°C for 10 min. Twenty microliters of sample was analyzed by SDS-PAGE using a 5% stacking gel and a 15% separating gel. The gels were dyed with Coomassie Brilliant Blue R-250 (Shanghai Regal Biology Technology Co. Ltd., Shanghai, China), and scanned for imaging using an E-Gel imager (Thermo Fisher, Waltham, MA).

Agarose Gel Electrophoresis for DNA Fragmentation

The effect of ATCE on the genomic DNA of *C. sakazakii* ATCC 29544 was analyzed as previously described (Guo et al., 2019). Briefly, after treatments with 0 (control), 1, and 2 MIC of ATCE at 37°C for 2, 4, and 10

h, the genomic DNA of *C. sakazakii* (10^8 cfu/mL) was extracted using a bacterial genomic DNA extraction kit (Tiangen Biotech Co. Ltd., Beijing, China), and electrophoresed using 1.5% agarose gel at 100 V for 30 min. Finally, the gel was stained with ethidium bromide (10 mg/mL) for 15 min, and imaged using a gel imaging system (Bio-Rad Laboratories, Hercules, CA).

Transmission Electron Microscopy Analysis

The cell morphology of *C. sakazakii* ATCC 29544 was observed using transmission electron microscope (TEM) as previously reported (Fei et al., 2018a). Briefly, *C. sakazakii* cultures were treated with 1 and 2 MIC at 37°C for 4 h, and untreated culture was used as control. The above mixtures were centrifuged at $8,000 \times g$ for 5 min at 4°C to obtain cells from the working culture, which were fixed in 0.1 M sodium phosphate buffer containing 2.5% glutaraldehyde for 12 h followed by washing twice with sterilized normal saline. To further fix the cells, the pellets were treated with 1% osmium tetroxide at 25°C for 6 h and washed 3 times with normal saline. The cells were dehydrated in a graded series of ethanol (50, 70, 90, and 100%) for 10 min, and embedded in Epon Lx-112 (Ladd Research, Williston, VT). Finally, the cells were double-stained with uranyl acetate and lead citrate and observed using TEM (Hitachi, Tokyo, Japan).

Activity of ATCE Against *C. Sakazakii* in Biofilm on Stainless Steel

The activity of ATCE against *C. sakazakii* in biofilm on stainless steel was analyzed as previously described (Guo et al., 2020a), with minor modifications. Briefly, sterile American Iron and Steel Institute (AISI)-304 stainless steel coupons (50 × 20 mm) were added to a 50-mL tube followed by the addition of 30 mL of bacterial suspension (10^7 cfu/mL). The coupons were cultured at 4°C for 24 h and washed twice with 300 mL of sterile distilled water (SDW) for 10 s. To further simulate the presence of *C. sakazakii* on equipment surfaces in a PIF plant, the coupons were incubated in 30 mL of TSB 25°C for 24 h to form more biofilms, followed by washing twice with 300 mL of SDW for 10 s. These prepared coupons were incubated in 30 mL of ATCE solution at concentrations of 0, 1, and 2 MIC for 0, 10, 20, and 30 min at 25°C, respectively. At each sampling point, the coupons were washed twice with 300 mL of SDW for 10 s and transferred to 50-mL tubes containing 30 mL of sterile PBS and 3 g of glass beads (300–600 μm, Yongqing Huayang Glass Products Co Ltd., Yongqing, China). After 5 min of vortexing, the

number of bacteria in PBS was determined by the plate count method.

Statistical Analysis

Each experiment involving data analysis was carried out in triplicate. All experimental data are presented as mean values ± standard deviations (SD). Differences between treatments were analyzed by ANOVA using the SPSS 20.0 software (IBM Corp., Armonk, NY). $P < 0.05$ was considered to indicate a significant difference.

RESULTS

DIZ, MIC, and MBC of ATCE on *C. sakazakii* Strains

The DIZ, MIC, and MBC of ATCE against the 10 *C. sakazakii* strains were determined and results are shown in Table 1. The negative control did not produce an inhibition zone, whereas the mean DIZ of ATCE against *C. sakazakii* strains ranged from 14.35 ± 0.67 to 14.84 ± 0.67 mm. We observed no difference ($P > 0.05$) in DIZ of ATCE against all tested strains. The MIC of ATCE against all *C. sakazakii* strains was 20 mg/mL, and the MBC of ATCE against all strains was 40 mg/mL.

Growth Curve

As shown in Figure 1, the effects of ATCE at 0.5 to 2 MIC on the growth of *C. sakazakii* ATCC 29544 were analyzed. After treatments with different concentrations of ATCE, growth of the *C. sakazakii* strain was significantly inhibited. As the concentration of ATCE

Table 1. Antibacterial activity¹ of *Amaranthus tricolor* crude extract against 10 *Cronobacter sakazakii* strains isolated from powdered infant formula (PIF)

Strain ²	DIZ (mm; mean ± SD)	MIC (mg/mL)	MBC (mg/mL)
ATCC 29544	14.50 ± 0.80^a	20	40
FP1	14.35 ± 0.67^a	20	40
FP5	14.47 ± 0.38^a	20	40
FP7	14.27 ± 0.49^a	20	40
CBM5	14.49 ± 0.65^a	20	40
CBM9	14.69 ± 0.59^a	20	40
ML10	14.84 ± 0.67^a	20	40
ML12	14.56 ± 0.62^a	20	40
AD6	14.59 ± 0.70^a	20	40
AD9	14.73 ± 0.58^a	20	40

^aValues within a column with the same letters do not differ significantly ($P < 0.05$).

¹DIZ = diameter of inhibition zone; MBC = minimum bactericidal concentration.

²Strains were isolated from PIF except for ATCC 29544, which was from the American Type Culture Collection (Manassas, VA).

Figure 1. Effects of different concentrations of *Amaranthus tricolor* crude extract on the growth curve (measured as optical density at 600 nm, OD₆₀₀) of *Cronobacter sakazakii* ATCC 29544. Bars represent the SD (n = 3).

increased, the growth of *C. sakazakii* slowed, but did not stop completely. When the concentration of ATCE was ≥ 1 MIC, the logarithmic phase of *C. sakazakii* ended and was replaced by slow proliferation.

Changes in Membrane Potential

The changes in membrane potential of *C. sakazakii* ATCC 29544 after treatments with ATCE are shown in Figure 2. Compared with untreated cells, *C. sakazakii* treated with ATCE at 1 MIC and 2 MIC had a significant increase in the amount of fluorescence ($P < 0.05$), indicating that ATCE treatment led to the depolarization of *C. sakazakii* cells. In addition, no significant change in amount of fluorescence was found between 1 MIC and 2 MIC, which may be because the depolarization of membrane potential of the tested strains reached a maximal concentration between 1 MIC and 2 MIC.

Changes in pH_{in}

We observed good linearity between fluorescence intensity ratio (490 nm/440 nm) and pH_{in} ($y = -1.0689x + 12.847$; $R^2 = 0.9973$). The pH_{in} value of *C. sakazakii* ATCC 29544 after treatment with 1 and 2 MIC of ATCE decreased significantly ($P < 0.05$; Figure 3). Further, the pH_{in} of untreated *C. sakazakii* was 7.49 ± 0.71 , which decreased to 4.56 ± 0.98 and 3.31 ± 0.81 , respectively, after treatment with 1 MIC and 2 MIC of ATCE at 37°C for 20 min. No difference ($P > 0.05$) was

found in pH_{in} value between *C. sakazakii* cells treated with 1 MIC and 2 MIC of ATCE.

SDS-PAGE Analysis

The SDS-PAGE images (Figure 4) showed significant changes in bacterial protein levels of *C. sakazakii* ATCC 29544 within 12 h of ATCE treatment at 1 MIC and 2 MIC. As shown in Figure 4A, the protein bands of *C. sakazakii* treated by 1 MIC of ATCE became fainter as treatment time increased. Figure 4B shows that the protein bands of *C. sakazakii* treated at 2 MIC were weaker compared with those of control group. Furthermore, protein bands of *C. sakazakii* disappeared after treatment with 2 MIC of ATCE for 12 h.

DNA Cleavage Analysis

The effects of ATCE on genomic DNA levels of *C. sakazakii* ATCC 29544 are shown in Figure 5. Compared with untreated *C. sakazakii*, the DNA bands of strains after exposure to ATCE became fainter with increasing treatment time and concentration. The bands disappeared completely after 10 h of treatment with 2 MIC ATCE.

TEM Observation

Differences in cell morphology of *C. sakazakii* ATCC 29544 with and without ATCE treatment were ob-

Figure 2. Effects of *Amaranthus tricolor* crude extract (ATCE) concentration on the membrane potential (in relative fluorescence units) of *Cronobacter sakazakii* ATCC 29544. The mean values of independent triplicate experiments were analyzed. Bars represent the SD ($n = 3$). Different letters (a, b) denote significant differences ($P < 0.05$).

served, as shown in Figure 6. Compared with untreated cells (Figure 6A), *C. sakazakii* cells after treatment with ATCE at 1 MIC (Figure 6B) and 2 MIC (Figure 6C) showed severe surface depression, cytoplasmic leakage, and deformation; the damage to cell morphology became more severe with increasing ATCE concentration.

Effect of ATCE Against *C. sakazakii* in Biofilm on Stainless Steel

Figure 7 shows the number of viable bacteria after treatment with 0, 1, and 2 MIC of ATCE for 0, 10, 20, and 30 min at 25°C. Compared with the control group (0 MIC), counts of *C. sakazakii* strains in biofilm on stainless steel were significantly reduced after treatments with ATCE ($P < 0.05$). When the concentration of ATCE was 1 MIC, the time to inactivate ~ 6.5 log cfu/mL of *C. sakazakii* was 30 min; when the concentration was 2 MIC, the inactivation time was 20 min.

DISCUSSION

Natural extracts have been extensively studied as potential preservatives and disinfectants based on their antioxidant activity (Fei et al., 2018a; Wu et al., 2019; Xiang et al., 2019). In previous studies, the DIZ values of standard vincristine sulfate, chloroform, *n*-hexane, and ethyl acetate extracts of *A. spinosus* against 11 pathogens excluding *C. sakazakii* were reported to be 8 to 15 mm (Bulbul et al., 2011), whereas the MIC of *A. spinosus* extracts against *Salmonella typhi* was 129 mg/mL, and those of *A. hybridus* and *A. caudatus* extracts against 8 tested organisms were 200 to 755 mg/mL and 162.2 to 665 mg/mL, respectively (Maiyo et al., 2010). In this study, ATCE was prepared and used to inhibit *C. sakazakii* isolated from PIF. The MIC of ATCE against *C. sakazakii* strains was 20 mg/mL, a value higher than that of some other pure natural products, such as eugenol (MIC = 1.0 mg/mL), lipoic acid (MIC = 2.5–5 mg/mL), and syringic acid (MIC = 5.0 mg/mL) (Bajpai et al., 2013; Shi et al., 2016b,c),

Figure 3. Changes in intracellular pH, which was calculated by the ratio of fluorescence emission intensities at 490 and 440 nm ($-\square-$, pH_{in}) of *Cronobacter sakazakii* ATCC 29544 after treatment with *Amaranthus tricolor* crude extract (ATCE). Values represent the means of independent triplicate measurements. Bars represent the SD ($n = 3$). Different letters (a, b) denote significant differences ($P < 0.05$).

which indicated that further concentrations of ATCE should be evaluated to achieve a better bacteriostatic effect. Similarly, the average MIC of polyphenol-rich pomegranate peel extract (pH = 6.0) against *C. sakazakii* was 20 mg/mL, according to the report of Yemis et al. (2019), who deemed that a MIC of 20 mg/mL was acceptable for the crude extract, and that a concentration process be performed before using the extract as a disinfectant.

Membrane potential is closely related to the normal physiological activities of bacteria and can help bacteria resist the absorption of antibiotics and bactericidal action (Guo et al., 2019). Without external interference, the membrane potential of bacteria is in a resting state; however, once the membrane potential is changed, normal growth of bacteria is inhibited (Bot and Prodan, 2009). Previous studies have shown that the most common changes in membrane potential are cell membrane depolarization and hyperpolarization. Cell membrane depolarization is associated with opening of the Na^+ channels, which allows Na^+ to migrate into the cell, reducing the intracellular negative charge. Cell membrane hyperpolarization is related to the increase in intracellular negative charge caused by leakage of K^+ ions out of the cell (Gries et al., 2013; Guo et al., 2020a). In this study, ATCE caused cell membrane depolarization of *C. sakazakii*; similarly, the antibacterial activity of OOPE and lipoic acid was associated with cell membrane depolarization (Shi et al., 2016b;

Fei et al., 2018a). Treatment of *C. sakazakii* cells with citral, syringic acid, and coenzyme Q_0 resulted in cell membrane hyperpolarization (Shi et al., 2016a,c; Guo et al., 2020a).

Homeostasis of pH_{in} is an important environmental condition for enzymatic biochemical reactions, DNA translation, and protein synthesis of bacterial cells (Bracey, et al., 1998). When cell membrane integrity is compromised, the cell cannot maintain pH_{in} through ion channels; therefore, changes in pH_{in} are a useful indicator of whether bacterial cells are damaged (Fitzgerald, et al., 2004). In the current study, the pH_{in} of *C. sakazakii* decreased significantly after treatment with ATCE, indicating that cellular integrity of the tested bacteria was severely damaged, leading to inhibition of bacterial growth. Similarly, a decrease in pH_{in} was an important bacteriostatic factor during the antibacterial process of oregano essential oil, thymol, and carvacrol against *Staphylococcus aureus*, and of lipoic acid against *C. sakazakii* strains (Lambert et al., 2001; Shi et al., 2016b).

Previous studies have shown that natural products can inhibit bacterial protein synthesis, accelerate protein breakdown, and improve membrane permeability, resulting in a significant reduction in bacterial protein content (Di Pasqua et al., 2010; Guo et al., 2020b). Our findings showed that *C. sakazakii* exposed to ATCE had lower levels of bacterial proteins than untreated cells. Similarly, Chen et al. (2017) found that after treat-

Figure 4. Sodium dodecyl sulfate-PAGE analysis of *Cronobacter sakazakii* ATCC 29544 proteins treated with concentrations (A) 1 MIC and (B) 2 MIC of *Amaranthus tricolor* crude extract (ATCE). Lane M = marker; lane 1: control; lanes 2, 3, 4, 5: treatment with 1 MIC or 2 MIC of ATCE for 3, 6, 9, 12 h, respectively.

Figure 5. Agarose gel electrophoresis analysis of the DNA of *Cronobacter sakazakii* ATCC 29544 treated by 1 MIC and 2 MIC of *Amaranthus tricolor* crude extract (ATCE). Lane 1 = control; lanes 2, 3, 4 = treatment with 1 MIC of ATCE for 2, 4, 10 h, respectively; lanes 5, 6, 7: treatment with 2 MIC of ATCE for 2, 4, 10 h, respectively.

ment with sugar beet molasses polyphenols, the protein content of food-borne pathogens was significantly reduced, including *Staph. aureus*, *Listeria monocytogenes*, *Escherichia coli*, and *Salmonella* Typhimurium. Fei et al. (2018a) reported that the inhibitory effect of OOPE on *C. sakazakii* was related to the decrease of bacterial protein level.

The DNA content of bacterial cells is closely related to their growth and proliferation; once synthesis of DNA is blocked or lost, growth of bacteria can be inhibited (Cui et al., 2018). In our study, DNA bands of *C. sakazakii* on agarose gel were fainter and eventually disappeared after treatment with ATCE, similar to the

results of Guo et al. (2020c), who revealed a change in bacterial DNA in the antimicrobial action of ATCE against *Staph. aureus*. Wang et al. (2017) reported that the antibacterial action of clove oil-containing phenols against *L. monocytogenes* was associated with DNA fragmentation and reduction. A previous report (Guo et al., 2019) listed 2 ways in which polyphenols reduced the DNA content of the pathogen: (1) cell morphology of tested bacteria was destroyed, leading to increased cell membrane permeability and DNA leakage; and (2) the secondary structure and morphology of DNA were altered by the association of polyphenols with the minor grooves in DNA.

Figure 6. Cell morphology of *Cronobacter sakazakii* ATCC 29544 (40,000×) under the transmission electron microscopy. (A) Untreated for 4 h, (B) treated with 1 MIC of *Amaranthus tricolor* crude extract (ATCE) for 4 h, (C) treated with 2 MIC of ATCE for 4 h.

Figure 7. Inactivation effect of *Amaranthus tricolor* crude extract (ATCE) against *Cronobacter sakazakii* in biofilm on a stainless steel tube at 25°C. Bars represent the SD (n = 3). Different letters (a–c) denote significant differences ($P < 0.05$) for different MIC within a timepoint.

The TEM results showed that ATCE caused cytoplasmic leakage and cell morphological damage of *C. sakazakii*; similar results were found for the action of OPE and tea polyphenols against *C. sakazakii* (Li et al., 2016; Fei et al., 2018a), which indicated that the effect of these natural products on pathogens is irreversible. Natural products with different components have different effects on bacterial cell morphology. Compared with the leakage of cell fluid, pores and bubbles appeared on the cell surface, indicating that the corresponding natural products had bacteriostatic rather than bactericidal effects on the tested bacteria. The bacteriostatic effect is reversible, and the treated bacteria can be resurrected (Joshi et al., 2014). Xu et al. (2017) found that *Staph. aureus* cells became larger and rougher after treatment with punicalagin. Shi et al. (2016c) reported that compared with untreated cells, the cell surface of *C. sakazakii* exposed to syringic acid collapsed and shrank. In addition, coenzyme Q₀ was shown to deform the rod shape of *C. sakazakii* but not cause cell disintegration or appearance of visible pores (Guo et al., 2020a).

In our study, ATCE was used to inactivate *C. sakazakii* on stainless steel. In the production of PIF, once production equipment becomes contaminated, *C. sakazakii* is very difficult to eliminate because of its adhesion to surfaces and strong resistance to desiccation; some encapsulated *C. sakazakii* strains have been revived after 2.5 years in a dry environment (Barron and Forsythe, 2007;

Henry and Fouladkhah, 2019). Therefore, we simulated the adhesion of *C. sakazakii* to the surface of stainless steel, resulting in approximately 6.5 log cfu/mL of *C. sakazakii* in biofilms on stainless steel. After treatment with 1 MIC or 2 MIC of ATCE at 25°C, *C. sakazakii* on the stainless steel was completely inactivated. In contrast, under the same conditions, the inhibitory effect of coenzyme Q₀ on *C. sakazakii* was not significant (Guo et al., 2020a), which may be because coenzyme Q₀ does not result in serious leakage of cell fluid of *C. sakazakii*. In addition, to reduce experimental error, the tested strains were immersed in ATCE solution rather than sprayed with ATCE. However, considering that *C. sakazakii* would not reach 6.5 log cfu/mL in the real-world environment and we could increase the concentration of ATCE or develop a compound disinfectant, ATCE should be used as a spray-type disinfectant.

CONCLUSIONS

We showed that ATCE could inhibit *C. sakazakii* isolated from powdered infant formula, and as a crude extract, ATCE showed moderate antibacterial activity against *C. sakazakii*. The possible mechanism of action of ATCE involved depolarization of the cell membrane, a decrease in pH_{in}, significant reductions in levels of bacterial proteins and genomic DNA, cytoplasmic leakage, and deformation. Our findings indicate that ATCE could be used as a spray-type disinfectant to inhibit

C. sakazakii strains in PIF production environments or on packaging materials. However, further study on the dosage of ATCE as the disinfectant is still necessary.

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