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Antimicrobial activity of syringic acid against *Cronobacter sakazakii* and its effect on cell membrane

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ABSTRACT

Syringic acid (SA) has been reported to exhibit antibacterial ability against various microorganisms, but little work has been done on its effect on *Cronobacter sakazakii*. In this study, minimum inhibitory concentrations (MICs) of SA against various *C. sakazakii* strains were determined. Moreover, changes in intracellular ATP concentration, intracellular pH (pH_{in}), membrane potential and membrane integrity were measured to evaluate the influence of SA on cell membrane. Finally, field emission scanning electron microscope (FESEM) was used to assess the morphological changes of bacterial cells caused by SA. It was shown that the MICs of SA against all tested *C. sakazakii* strains were 5 mg/mL. SA retarded bacterial growth, and caused cell membrane dysfunction, which was evidenced by intracellular ATP concentration decrease, pH_{in} reduction, cell membrane hyperpolarization and changes in cellular morphology. These findings indicated that SA has potential to be developed as a natural preservative to control *C. sakazakii* in foods associated with this pathogen and prevent related infections.

1. Introduction

Cronobacter sakazakii is an opportunistic pathogen that has been implicated in bacteremia, necrotizing enterocolitis, and neonatal meningitis (Iversen, Waddington, On, & Forsythe, 2004). The bacterium has been isolated from a range of food sources including dairy-based foods, dried meats, milk and fruit powders, vegetables, and rice etc. (Beuchat et al., 2009). In particular, powdered infant formula (PIF) has been epidemiologically linked with many of *C. sakazakii* infections reported (Yan et al., 2012). Infants with a low birth weight (<2500 g) and premature (<37 weeks) infants are most susceptible to infections, and the reported fatality rates for *C. sakazakii* infections in these two groups are 50–80% (Healy et al., 2010). In addition to neonates, *C. sakazakii* can also cause infections in immuno-compromised adults and the elderly (Yan et al., 2012).

To control the level of *C. sakazakii* in PIF or other related foods, various intervention technologies have been developed or explored, such as simultaneous near-infrared radiant heating, UV radiation, microwave processing and electron beam irradiation (Ha & Kang, 2014). However, these methods may cause a loss of sensitive nutrients from the formula and have limitations because of high capital costs for installation (Ha & Kang, 2014; Harouna et al., 2015). Chemical preservatives have also been commonly used to prevent microbial contamination in various foods, but their application in food is not favored by consumers due to their synthetic nature and possible negative effect on human health (Kashani, Nikzad, Mobaseri, & Hoseini, 2012). There is currently an increased trend in food industry to use natural antimicrobials, which gain increased consumer acceptance (Joshi, Howell, & D'Souza, 2014). Several plant extracts or phytochemicals have been reported to exhibit inhibitory effect on *C. sakazakii*, including blueberry proanthocyanidins, commercial blueberry juice, trans-cinnamaldehyde, caprylic acid and citric acid (Amalaradjou & Venkitanarayanan, 2011; Frankova et al., 2014).

Syringic acid (4-hydroxy-3, 5-dimethoxybenzoic acid, $C_9H_{10}O_5$, SA) is a hydroxybenzoic acid of phenolic compound (Fig. 1) (Campos et al., 2009). It has been found in *Lentinula edodes* (shiitake), cereal grains, *Herba dendrobii* (Shihu in Chinese), *Radix Isatidis* (Banlangen in Chinese) (Kong, Zhao, Shan, Xiao, & Guo, 2008) and the leaves of *Alpinia calcarata Roscoe* (Muthukumaran, Srinivasan, Venkatesan, Ramachandran, & Muruganathan, 2013). Previous studies have demonstrated that SA could suppress hepatic fibrosis in chronic liver injury (Itoh et al., 2010) and exhibit antidiabetic activity in experimental diabetes mice models (Muthukumaran et al., 2013). SA has also been reported to show

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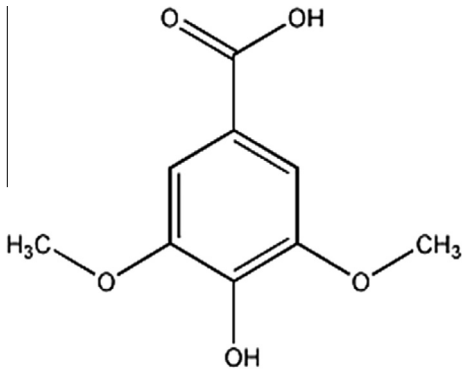

Fig. 1. Chemical structure of syringic acid (SA).

antibacterial activity against various microorganisms. Zaldivar and Ingram (1999) demonstrated that SA inhibited growth and ethanol production in *Escherichia coli* LY01 by collapsing ion gradients and increasing internal anion concentrations. In addition, SA could inactivate *Oenococcus oeni*, causing a noticeable decrease of viable cells after 90 min treatment (Campos, Couto, & Hogg, 2003).

Although antimicrobial activity of SA has been reported for certain microorganisms, its antimicrobial activity against *C. sakazakii* has been rarely reported and its effects on the pathogen's cell membrane remains unclear. In this study, minimum inhibitory concentrations (MICs) of SA against several *C. sakazakii* strains from clinical and food source were determined. Moreover, changes in cell membrane potential, intracellular ATP concentration, membrane integrity and intracellular pH (pH_{in}) were analyzed to explore possible mechanism of action. Finally, morphological changes of cell membrane were assessed by using field emission scanning electron microscope (FESEM).

2. Materials and methods

2.1. Reagents

SA (CAS: 530-57-4, HPLC purity $\geqslant$98%) was purchased from the Chengdu Must Bio-technology Co., Ltd (Chengdu, Sichuan, China). SA was dissolved in water containing 2% anhydrous ethanol (v/v) and filter-sterilized before use to minimize oxidation of the compound. All other chemicals were of analytical grade.

2.2. Bacterial strains and culture conditions

C. sakazakii strains ATCC 29544, ATCC 29004, ATCC 12868 and ATCC BAA-894 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Five other *C. sakazakii* strains were taken from our laboratory collection, which were isolated from various infant formula or powdered infant rice cereal in China. A loopful of each strain was inoculated into 30 mL of tryptone soya broth (TSB) and incubated for 18 h at 37 °C.

2.3. MIC determinations

MIC of SA was determined by agar dilution method as described by the European Committee with modification (European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical & Infectious, 2000). Ampicillin was used as reference antibiotic. Melted tryptic soy agar (TSA) was aseptically transferred into sterile 24-well plates containing either SA or ampicillin. The content (a final volume 500 μL) of each well was gently mixed. The final concentrations of SA were 0, 0.3125, 0.625, 1.25, 2.5, 5, and 10 mg/mL, whereas that of ampicillin was 0.1 mg/mL. After hardening, the agar in each well was spotted with 2 μL (approximately 10^4 CFU) of the tested bacterium. The spots were left to dry and then plates were incubated at 37 °C for 24 h. The lowest concentration of SA that results no visible growth of test organisms was determined as MIC.

2.4. Growth curves

The growth curves were constructed by the method described by Qiu et al. (2010) with some modifications. Briefly, *C. sakazakii* strain ATCC 29544 was grown to an OD_{600} value of 0.2 (approximately 1.5×10^8 CFU/mL) in TSB. Then 125 μL of the culture was transferred into each well on 96-well microtiter plates. SA was dissolved in 2% anhydrous ethanol (v/v) and added to the cultures to obtain final concentrations of 1/64MIC, 1/32MIC, 1/16MIC, 1/8MIC, 1/4MIC, 1/2MIC, MIC and 2MIC, and TSB containing 2% anhydrous ethanol (v/v) was used as a negative control. Bacteria were further cultured at 37 °C, and cell growth was monitored by enzyme micro-plate reader at 600 nm at 1 h intervals, using a multimode plate reader (Tecan, Infinite™ M200 PRO, Männedorf, Switzerland).

2.5. Measurement of intracellular ATP concentrations

The intracellular ATP concentration of *C. sakazakii* strain ATCC 29544 was analyzed according to the method described by Sanchez, Garcia, and Heredia (2010), with some modifications. The overnight culture of *C. sakazakii* strain ATCC 29544 was harvested by centrifugation (5000*g*, 5 min). Then, the supernatant was removed and cells were washed three times with 0.1 mol/L of phosphate-buffered saline (PBS, pH 7.0). Cells were re-suspended in PBS to achieve an OD_{600} of 0.5 (approximately 4×10^8 CFU/mL), and 2 mL of cell solution was taken into Eppendorf tubes. SA was then added to each tube to achieve final concentrations of 0 (control), MIC, and 2MIC, respectively. And the samples were incubated at 37 °C for 30 min. ATP were extracted by ultrasound on ice. Then the samples were centrifuged for 5 min at 5000*g*, the supernatant was taken and stored on ice to prevent ATP loss. ATP was measured by using an ATP assay kit according to manual instructions (Beyotime Bioengineering Institute, Shanghai, China).

2.6. Membrane potential determinations

The method described by Sanchez et al. (2010) was followed with minor modifications. Cells were grown in 30 mL of TSB at 37 °C to an OD_{600} of 0.5. Cells were then harvested by centrifugation (5000*g*, 5 min) and washed twice with PBS (pH 7.0). Next, 125 μL of cell suspensions were placed in black, opaque 96-well microtiter plates (Nunc, Copenhagen, Denmark) for 30 min at 37 °C. Then, 1 μM of the membrane potential-sensitive fluorescent probe bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4$ (3); Molecular Probes, Sigma, Louis, USA) was added, which is an anionic, fluorescent membrane potential dye that has been used as an indicator of changes in membrane potential. The mixture was then incubated for 30 min at 37 °C. SA was added to achieved final concentrations of 0, MIC, and 2MIC. After 5 min, fluorescence was measured at the excitation and emission wavelengths of 492 and 515 nm, using a fluorescence microplate reader (Tecan, Infinite™ M200 PRO, Männedorf, Switzerland). The excitation and emission slit widths were 3 and 5 nm, respectively. Background fluorescence resulting from the medium was determined and the results corrected.