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Short communication

# *Cronobacter sakazakii* reduction by blueberry proanthocyanidins

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ABSTRACT

Blueberry juice and blueberry polyphenols reportedly have antimicrobial properties against foodborne pathogens, without much currently known on their effects against *Cronobacter sakazakii*. This study evaluated the antimicrobial effects of blueberry proanthocyanidins (PAC) and commercial blueberry juice (BJ) against two strains of *C. sakazakii*, ATCC 29004 and 29544. BJ (pH 2.8), blueberry PAC (5 mg/ml) and controls (phosphate buffered saline (PBS), pH 7.2, and malic acid pH 3.0) were mixed with equal volumes of washed overnight cultures of *C. sakazakii* and incubated for 30 min, 1 h, 3 h and 6 h at 37°C. Reductions of ~1 and 1.50 log CFU/ml were obtained for strains 29004 and 29544, respectively after 30 min with BJ or blueberry PAC. Both *C. sakazakii* strains 29004 and 29544 were reduced to undetectable levels from 8.25 ± 0.12 log CFU/ml and 8.48 ± 0.03 log CFU/ml, respectively with BJ (pH 2.8) or blueberry PAC after 1 h, while malic acid (pH 3.0) showed ~1.3 log CFU/ml reduction for both strains. Scanning electron microscopy studies showed differences in cell membrane morphology with clumping and formation of blebs of the treated strains compared to untreated controls. These results warrant further *in vivo* studies with blueberry bioactives to determine potential for preventing and treating *C. sakazakii* infections.



## 1. Introduction

*Cronobacter sakazakii* is a gram-negative, rod-shaped, opportunistic bacterial pathogen and a major cause of invasive infections in neonates. The clinical symptoms of *Cronobacter* infection include necrotizing enterocolitis, bacteremia, and meningitis, with reported case fatality rates of 50–80% (Healy et al., 2010). However, in addition to neonates, immunocompromised adults and elderly are also susceptible to *Cronobacter* infections. *Cronobacter* outbreaks have been associated with contaminated powdered infant formula as well as raw and processed foods of animal and plant origin including fresh, frozen, and ready-to-eat products (Friedemann, 2007). Based on the wide-range of reported *Cronobacter*-contaminated foods, *Cronobacter* remains a potential risk for food safety and numerous studies are being carried out towards developing improved strategies for inactivation.

Among the seven *Cronobacter* species, *C. sakazakii* has been associated with milk and milk products. Thermal inactivation methods for *Cronobacter* include ultra-high temperature (UHT) processing for milk and fluids, manothermosonication (MTS), ultraviolet light, x-ray irradiation, and high pressure processing (Arroyo et al., 2011; Gonzalez et al., 2006; Ha and Ha, 2011; Lee et al., 2006).

In addition to processing technologies and chemical inactivation methods, there is currently an increased trend towards research on natural products as antimicrobials. The advantages of naturally occurring antimicrobials include increased consumer acceptance and availability. Trans-cinnamaldehyde (TC), a component of cinnamon oil, at 38 mM and 750 μM was shown to cause 4.0 and 3.0 log CFU/ml reduction of *C. sakazakii* after 96 h, respectively, inhibiting biofilm formation on various surfaces at temperatures of 12 °C and 24 °C, with downregulation of biofilm associated genes (Amalaradjou and Venkitanarayanan, 2011). Caprylic acid at 30 mM was found to cause 7.8 log CFU/ml reduction of *C. sakazakii* after 60 min at 45 °C (Jang and Rhee, 2009). The minimum inhibitory concentrations (MIC) against *C. sakazakii* were 2 mg/ml for ethyl vanillin, 3 mg/ml for vanillin, and 0.8 mg/ml for vanillic acid (Yemis et al., 2011), while carvacrol and thymol were shown to have MICs of 0.0625 mmol/l at 37 °C (Lee and Jin, 2008).

Various natural extracts including polyphenols from fruits and berries are also being studied as potential antimicrobials for bacteria, viruses, and parasites (Al-Habib et al., 2010; Su and D'Souza, 2011; Su et al., 2010; Takeshita et al., 2009). Muscadine seed extracts were found to be effective against *C. sakazakii* strains Fec39 and MSDH resulting in complete reduction (6 log CFU/ml) within 1 h of treatment at 37 °C (Kim et al., 2009).

Blueberries are known to exhibit strong antioxidant capacity associated with phenolic acids, catechins (flavanols), and proanthocyanidins (condensed tannins) (Huang et al., 2012). Blueberry

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PACs have antimicrobial activity against *Listeria monocytogenes*, *Helicobacter pylori*, *Salmonella* Typhimurium, *Escherichia coli* and *Candida albicans* (Chatterjee et al., 2004; Lacombe et al., 2012; Ofek et al., 1996). Water and ethanol extracts of blueberries were shown cause 5.9 log CFU/ml reduction of *L. monocytogenes* after 24 h (Park et al., 2011).

Therefore, the objectives of this research were to (1) determine the antimicrobial effects of commercial blueberry juice (BJ) and 5 mg/ml blueberry proanthocyanidins (PAC) against two isolates of *C. sakazakii* over 6 h at 37°C and (2) determine the mode of action of blueberry PACs against *C. sakazakii* using Scanning Electron Microscopy.

## 2. Materials and methods

### 2.1. Bacteria and culture conditions

Two strains of *C. sakazakii* (ATCC 29004 and ATCC 29544) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The stock cultures were grown in Tryptic Soy Broth (TSB) and transferred twice at 37°C overnight, streaked on Tryptic Soy Agar (TSA) plates and incubated overnight at 37°C. Single colonies of each strain were inoculated into TSB and incubated at 37°C and transferred twice. Eighteen-h old cultures of both strains were used to test the effect of blueberry PAC and commercial blueberry juice (BJ).

### 2.2. Treatments with blueberry PAC and BJ

Blueberry PAC was obtained from Dr. Amy Howell, Rutgers University (Chatsworth, NJ) and commercial blueberry juice (BJ) was obtained from a local grocery store. Blueberry PAC was isolated following the procedure of Howell et al. (2005). Blueberry PAC was dissolved in 10% ethanol and filter-sterilized through 0.2-micron filters to obtain a 10 mg/ml stock. Overnight cultures of *C. sakazakii* were washed twice and resuspended in phosphate buffered saline (PBS, pH 7.2). Each washed culture (100 μL) was individually mixed with equal volumes of blueberry PAC at 5 mg/ml, BJ (pH 2.8), neutralized BJ (pH 7 using 4 M sodium hydroxide (NaOH)), malic acid (pH 3.0), 10% ethanol (final concentration 5%) or PBS and incubated at 37°C for 30 min, 1 h, 3 h, or 6 h. The initial pH of broth containing pure culture was 6.8 for both strains that dropped to pH 5 after mixing with either malic acid or BJ. After each time-point, the treatments were neutralized/stopped using TSB (Tryptic Soy broth) with 3% beef extract, serially diluted in PBS, 0.1 ml was spread-plated on TSA plates and incubated at 37°C for 24 h. Colonies were enumerated and recovered bacterial counts were expressed in log Colony Forming Units (CFU)/ml.

### 2.3. Determination of recovered treated cells after 6 and 12 h of incubation

*C. sakazakii* 29004 and *C. sakazakii* 29544 following treatment with blueberry PAC (5 mg/ml) and BJ at 37 °C for 1 h were added to fresh TSB and incubated at 37°C for 6 and 12 h to determine bacteriostatic or bactericidal effects. Their ten-fold serial dilutions after 6 and 12 h were plated on TSA plates and incubated at 37°C to determine counts.

### 2.4. Scanning electron microscopy (SEM) sample preparation and observation

Overnight (18 h) washed cultures of *C. sakazakii* were mixed with equal volumes (100 μL each) of 5 mg/ml blueberry PAC, BJ or PBS and incubated for 3 h at 37°C. Samples were fixed using primary fixation with 3% glutaraldehyde in 0.1 M cacodylate and secondary fixation with 2% osmium tetroxide in 0.1 M cacodylate. Samples were dehydrated through graded ethanol series (25% ethanol to 100% dry ethanol) followed by critical point drying. One sample per treatment was used for the SEM study. Six to eight fields of observation were included for analysis. Samples were visualized using SEM at the Advanced Microscopy and Imaging Center at the University of Tennessee-Knoxville.

### 2.5. Statistical analysis

Each treatment was assayed in duplicate and replicated thrice. Statistical analysis was carried out using Analysis of Variance (ANOVA) with SAS 9.3 (SAS Institute, Cary, NC) and Tukey's test for mean separation on a completely randomized design with six sets of data under each treatment condition.

## 3. Results

### 3.1. Reduction of C. sakazakii by blueberry juice and blueberry proanthocyanidins

*C. sakazakii* strain ATCC 29004 was found to be reduced to undetectable levels from initial counts of 8.25 ± 0.12 log CFU/ml after 1 h with 5 mg/ml blueberry PAC and BJ (Fig. 1B). After 30 min with

**Table 1**
Recovery of *C. sakazakii* 29004 and *C. sakazakii* 29544 treated with 5 mg/ml blueberry PAC and BJ at 37 °C for 1 h after incubation for 6 h and 12 h at 37 °C in tryptic soy broth (TSB) and plating on tryptic soy agar (TSA).

| Incubation time | 10-fold Serial dilutions | *C. sakazakii* 29004 | | | | *C. sakazakii* 29544 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Treatment with BB PAC (5 mg/ml) | | Treatment with BJ | | Treatment with BB PAC (5 mg/ml) | | Treatment with BJ | |
| | | Visible growth in TSB broth | Growth on TSA plates | Visible growth in TSB broth | Growth on TSA plates | Visible growth in TSB broth | Growth on TSA plates | Visible growth in TSB broth | Growth on TSA plates |
| 6 h | −2 | + | ++ | − | ++ | + | ++ | − | ++ |
| | −3 | − | + | − | + | − | + | − | + |
| | −4 | − | + | − | + | − | + | − | + |
| | −5 | − | + | − | + | − | + | − | + |
| | −6 | − | + | − | + | − | + | − | + |
| 12 h | −2 | + | ++ | + | ++ | + | ++ | + | ++ |
| | −3 | + | ++ | + | ++ | + | ++ | + | ++ |
| | −4 | − | ++ | − | ++ | − | ++ | − | ++ |
| | −5 | − | + | − | + | − | + | − | + |
| | −6 | − | + | − | + | − | + | − | + |

(−) Indicates no growth.
(+) Indicates countable growth.
(++) Indicates uncountable growth (too numerous to count).