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New decontamination method based on caprylic acid in combination with citric acid or vanillin for eliminating *Cronobacter sakazakii* and *Salmonella enterica* serovar Typhimurium in reconstituted infant formula

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ABSTRACT

The antimicrobial effects of natural compounds (caprylic acid, CA; citric acid, CTA; and vanillin, VNL) on the inactivation of *Cronobacter sakazakii* and *Salmonella enterica* serovar Typhimurium were examined in reconstituted infant formula. The samples were treated with: 1) CA, CTA, or VNL alone (0, 10, 20, 30, 40, 60, and 80 mM); 2) a combination of CA (10 and 20 mM) and CTA (15 and 30 mM); and 3) a combination of CA (10 and 20 mM) and VNL (15 and 30 mM), at mild feeding temperatures (40 °C and 45 °C), and the bacterial populations were assayed periodically (0, 5, 10, and 30 min). For both bacteria, the combined treatments had marked synergistic antimicrobial effects compared with the sum of the effects of each individual treatment. For example, there was no noticeable reduction ($P > 0.05$) in the population of *C. sakazakii* following an individual treatment with 20 mM CA or 30 mM CTA for 5 min at 40 °C, whereas the population was reduced to undetectable levels (reduction > 7.3 log CFU/ml) following treatment with a combination of CA and CTA (20 CA + 30 CTA for 5 min at 40 °C). As the temperature increased, the bactericidal effect was stronger at all time points with a synergistic effect. In a validation assay using a low level inoculum (approximately 10^3 CFU/ml) of desiccation-stressed bacteria in certain conditions, the combined treatments (e.g., CA 10 mM + CTA 30 mM for 5 min at 45 °C for *C. sakazakii*, and CA 10 mM + VNL 15 mM for 10 min at 45 °C for *S.* Typhimurium) completely destroyed the bacteria with no recovery of cell viability. Disintegration of the membrane and changes in the cell structure or morphology, such as plasmolysis and membrane disruption, were detected by flow cytometry and electron microscopy, respectively. These methods use antimicrobials that could be applied as food additives in infant formula, which may help to eliminate bacteria.

1. Introduction

Infant formula, which is used as a substitute for human breast milk, occasionally contains pathogenic bacteria capable of causing serious illness. In 2004, the Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) categorized *Cronobacter* spp. (reCAssified from *Enterobacter sakazakii*) and *Salmonella enterica* as "clear evidence of causality" (category A), both of which have been found in powdered infant formula (FAO/WHO, 2007). *Cronobacter* spp. are considered to be emerging opportunistic pathogens that cause life-threatening bacterial infections, including fatal neonatal meningitis, sepsis, necrotizing enterocolitis, and death in low birth weight (<2.5 kg) and premature (<37 weeks) infants (Bar-Oz et al., 2001; Muytjens et al., 1983; van Acker et al., 2001) where infant formula was identified as the main vehicle of transmission (Kim et al., 2011; van Acker et al., 2001; Weir, 2002). Many studies have addressed the prevalence of *Cronobacter* spp. in powdered infant formula where the contamination rate ranged from 2.4% to 22.7% (FAO/WHO, 2006, 2007; Muytjens et al., 1988; Nazarowec-White and Farber, 1997a). For *Salmonella enterica*, *S.* Typhimurium is the most common serotype that causes human infection and 287 infants acquired salmonellosis with diarrhea and meningitis from powdered infant formula during 1985–2005 (Cahill et al., 2008). Salmonellosis occurs in infants at an eight times higher frequency than other age groups (CDC, 2004).

To ensure the microbiological safety of infant formula, FAO/WHO recommended that powdered infant formula should be reconstituted with water, heated to >70 °C, and cooled quickly to the appropriate temperature before feeding (FAO/WHO, 2006). However, it is difficult to follow these steps on a daily basis because they are time-consuming and demand energy inputs, while it is not feasible to consume the infant formula immediately and there may be a loss of nutrients from the formula with high temperature treatments. At present, consumer demands are focused on fresh, good quality food so minimally invasive and safe treatments are required to meet these demands (Abee et al., 1995; Gao et al., 2006). Thus, there is considerable interest in developing

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combined antimicrobial treatments that exhibit synergistic bactericidal effects on pathogenic bacteria, which are minimally invasive compared with individual treatments (Davidson and Branen, 2005). In addition, many consumers avoid chemical additives and they prefer the use of natural antimicrobial compounds or preservatives.

Caprylic acid (CA), citric acid (CTA), and vanillin (VNL) are food additives derived from natural sources, which are acceptable food additives in infant formula according to Codex Alimentarius and the regulations of several countries (CAC, 1981, 2006; KFDA, 2004). CA and CTA have a generally recognized as safe (GRAS) status (Code of Federal Regulations Title 21 of CA: §184.1025 and CTA: §184.1386) (FDA, 2006). CA, a medium-chain fatty acid found in breast milk, has a broad spectrum of microbicidal activities against various bacteria (Desbois and Smith, 2010; Jang and Rhee, 2009; Sprong et al., 2001). CTA is used widely as a preservative, flavoring agent, and acidulant in food and beverages (Brul and Coote, 1999; Dppres, 2005) while VNL, which has a desirable flavor recognized by most young children, also exhibits antimicrobial properties (Burt, 2004; Fitzgerald et al., 2004). These antimicrobial agents have been applied to various foods to eliminate bacterial contamination but few studies have investigated their combined antimicrobial effects.

The objective of this study was to determine the effects of combined treatments using minimal amounts of CA with CTA or VNL at mild feeding temperatures on the inactivation of *C. sakazakii* and *S.* Typhimurium in reconstituted powdered infant formula. To confirm the bactericidal effects of natural compounds in infant formula, the membrane integrity and any changes in cell morphology were determined by flow cytometry and electron microscopy, respectively.

2. Materials and methods

2.1. Bacterial strains

Three strains of *C. sakazakii* (ATCC 12868, 29004, and 29544) and three strains of *S.* Typhimurium (ATCC 19585, 43174, and DT104 killer cow) were used in this study. All of the strains were obtained from the Food Microbiology Culture Collection at Korea University (Seoul, Korea). They were maintained at −20 °C in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) containing 20% glycerol. Each strain was resuscitated in 10 ml of sterile TSB in screw-cap tubes at 37 °C for 24 h. Following incubation, equal quantities of each *C. sakazakii* culture or each *S.* Typhimurium culture were mixed in a sterile 50 ml centrifuge tube and centrifuged (Centra-CL2, IEC, Needham Heights, MA, USA) at 3000 ×*g* for 15 min to harvest the bacterial cells. The supernatant was discarded and the pellet was washed twice with 0.85% sterile saline, and then resuspended in the same buffer.

2.2. Sample preparation and inoculation

Commercial brand powdered infant formula was purchased from a retail store. The powdered infant formula (25.5 g) was rehydrated in 180 ml of sterile distilled water, according to a published method (Jang and Rhee, 2009). Prior to inoculation with the bacteria, the reconstituted infant formula was pasteurized at 63 °C for 30 min. The mixed suspension of *C. sakazakii* or *S.* Typhimurium was added to the reconstituted infant formula to yield an initial inoculation level of approximately 10^7 CFU/ml.

2.3. Antimicrobial activity assays

The CA (Sigma Chemical Co., St Louis, MO, USA) and VNL (Acros Organics, NJ, USA) stock solutions were prepared in 98% ethanol while CTA (Sigma Chemical Co.) was dissolved in sterile distilled water. The bactericidal activities of CA, CTA, and VNL at various concentrations were tested using *C. sakazakii* or *S.* Typhimurium. For individual treatments, 100 μl aliquots of 1, 2, 3, and 4 M or 200 μl aliquots of 3 and 4 M solutions of the three antimicrobials were added to 9.9 or 9.8 ml of inoculated samples so the final concentration was adjusted to 10, 20, 30, 40, 60, and 80 mM. For the combined treatments (CA + CTA or CA + VNL), a 100 μl aliquot of each stock solution was added to the dispensed samples (9.8 ml) to obtain the appropriate final concentrations of CA (10 and 20 mM) with CTA (15 and 30 mM) or VNL (15 and 30 mM), respectively. The prepared samples were treated at 40 °C or 45 °C in a shaking water bath (Vision Scientific co., Ltd., Daejeon, Korea) at 100 rpm for 0, 5, 10, and 30 min. Distilled water and 2% ethanol were also added, which served as the controls for the solvents. Each experiment was performed in triplicate.

2.4. Enumeration of survivors

The samples were 10-fold serially diluted with 0.85% sterile saline. One hundred microliters of the diluents was spread-plated onto chromogenic *Enterobacter sakazakii* agar (DFI Formulation, Oxoid, Hampshire, England) for *C. sakazakii* or xylose lysine desoxycholate agar (XLD; Difco) for *S.* Typhimurium, respectively. To facilitate a lower detection limit, 0.2 ml aliquots of the undiluted samples were spread-plated onto five plates i.e., in total, 1 ml of each undiluted sample was spread-plated. Thus, the detection limit was 1 CFU/ml. Colonies were enumerated (log CFU/ml) following incubation for 24 h at 37 °C.

2.5. Validation of combined treatments using desiccation-stressed cells

Stationary phase cells of *C. sakazakii* or *S.* Typhimurium were prepared as described above. The inoculation of bacteria into powdered infant formula was performed as described in a previous study (Kim et al., 2010). Powdered infant formula was distributed equally on a sterile stainless-steel tray in a laminar flow biosafety cabinet. The bacterial culture suspension was placed in a sterile sprayer and sprayed vertically onto the samples from a distance of 30 cm above. After the moisture was removed, the sample was blended thoroughly using a sterile spatula and dried at ambient temperature for 24 h on a clean bench. The sample was then transferred to a sterile beaker, covered, and stored at a constant temperature of 22 °C up to 30 days. The water activity was measured before and after inoculation at day 0 and after 15 and 30 days using a Thermoconstanter TH200 (Novasina, Zurich, Switzerland). After 30 days, the sample was reconstituted in sterile distilled water, which yielded an initial bacterial concentration of approximately 10^3 CFU/ml. Following the combined treatment, a 0.2 ml aliquot of undiluted sample was spread onto the surface of five plates containing chromogenic *Enterobacter sakazakii* agar or XLD (direct plating). To examine the recovery of injured cells, 1 ml of treated sample was enriched in 10 ml TSB and incubated for 24 h at 37 °C. The resulting culture was streaked onto chromogenic *Enterobacter sakazakii* agar or XLD agar using a sterile flamed loop in duplicate (plating after enrichment). The results were recorded as positive or negative following incubation.

2.6. pH measurement

The pH of the reconstituted infant formula containing individual or combined antimicrobial agents was measured using a combination electrode (MP220 basic; Mettler–Toledo, Greifensee, Switzerland).

2.7. Flow cytometric analysis

The samples containing bacteria (*C. sakazakii* or *S.* Typhimurium) were treated with combinations of the antimicrobial agents (10 mM CA + 30 mM CTA and 10 mM CA + 30 mM VNL at 40 °C) and then diluted to approximately 10^6 CFU/ml in 0.85% saline. One milliliter aliquots of the samples were transferred to 1.5 ml Eppendorf tubes, centrifuged (13,000 ×*g*, 4 °C for 3 min), and washed twice with sterile PBS (pH 7.4). Stock solutions of SYTO9 (Molecular Probes, Invitrogen, Eugene, OR, USA) and propidium iodide (PI; Molecular Probes) were