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Inhibitory effect of caprylic acid and mild heat on *Cronobacter* spp. (*Enterobacter sakazakii*) in reconstituted infant formula and determination of injury by flow cytometry

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ABSTRACT

The combined effects of caprylic acid and mild heat were investigated to ascertain their impact on *Cronobacter* spp. (*E. sakazakii*) in reconstituted infant formula. Samples containing a mixture of 3 strains of *Cronobacter* spp. (10^7 to 10^8 CFU/ml) were prepared with various concentrations of caprylic acid (5, 10, 20, and 30 mM) and were then heated to 45, 50, and 55 °C. The inhibitory effect of the combined treatment resulted in a synergistic effect, in which *Cronobacter* spp. numbers were reduced much more rapidly with increased temperatures and concentrations of caprylic acid. When samples were treated with 30 mM caprylic acid, the time required to reduce *Cronobacter* spp. cell numbers to an approximate reduction of 7.8 log CFU/ml was 60 min at 45 °C, 20 min at 50 °C, and 10 min at 55 °C. In the validation assay using a low population of *Cronobacter* spp. (approximately 10^3 log CFU/ml), no recovery of injured cells was observed after samples were treated with 10 mM caprylic acid for 20 min at 55 °C, 20 mM caprylic acid for 10 min at 50 °C and 55 °C, and 30 mM caprylic acid for 10 min at 45 °C to 55 °C. To determine the bactericidal mechanism of caprylic acid, membrane integrity was examined by fluorescent staining followed by flow cytometry and confocal microscopy. Increased cellular inactivation was associated with increased propidium iodide staining, indicating damage to the cell membrane of *Cronobacter* spp.. Overall, these data indicate that the addition of this natural antimicrobial agent to infant formula may have potential use for controlling microbes prior to consumption at lower heating temperatures. The study also provides a complementary understanding of the mode of action of caprylic acid on *Cronobacter* spp.

1. Introduction

Recently, the microbiological safety of infant formula has been of considerable concern in relation to emerging bacteria, particularly *Cronobacter* spp. (*Enterobacter sakazakii*). This pathogenic organism is primarily reported as being associated with powdered infant formula (Iversen and Forsythe, 2004; Leuschner et al., 2004; Muytjens et al., 1988; Nazarowec-White and Farber, 1997) and isolated from a wide range of environmental sources including hospital infant formula preparation areas (Masaki et al., 2001), food factories, and households (Kandhai et al., 2004). In addition, *Cronobacter* spp. is an opportunistic pathogen that has been implicated in infant infections including bacteremia (Muytjens et al., 1983), infant meningitis (Bar-Oz et al., 2001; Himelright et al., 2002), and enterocolitis (Stoll et al., 2004). Premature infants of low birthweight (<2500 g) and immunocompromised infants are at a greater risk from exposure than mature infants, children, or adults (FAO/WHO, 2004; Farber, 2004; Gurtler et al., 2005; Lai, 2001). Due to these significant epidemiological concerns, an FAO/WHO meeting (2004) addressed that *E. sakazakii* categorized into a Category A, considering it to have strong potential for infections based on clear evidence of causality.

Despite various attempts to reduce levels of *Cronobacter* spp. contamination in finished products of powdered infant formula (PIF), it has been hard to control effectively because PIF is not a commercially sterile product. In addition, improper handling during preparation and feeding allows for the amplification of the bacteria already presented. It has been generally recommended that PIF should be reconstituted with hot water at 70 °C (FAO/WHO, 2006), however, it is unavailable for feeding directly due to its high temperature and could potentially decrease the nutrient content of the formula.

To meet consumer criteria, an effective method for reducing *Cronobacter* spp. that utilizes mild heat treatment would be useful. Food manufacturers have increasingly relied on mild intervention techniques using natural antimicrobial substances, which maintain the natural appearance and nutritive quality of the product (Abee et al., 1995). There have been a number of reports on the antimicrobial properties of free fatty acids and their monoglycerides against a wide spectrum of pathogens including *Campylobacter jejuni*, *Salmonella* spp. (Thormar et al., 2006), *L. monocytogenes* (Tokarskyy and Marshall, 2008), *E. coli* O157:H7 (Nair et al., 2004b), *S. aureus*

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(Bergsson et al., 2001; Kamdem et al., 2008), *E. sakazakii* (Nair et al., 2004a), *Chlamydia trachomatis* (Bergsson et al., 1998), and *Helicobacter pylori* (Bergsson et al., 2002). In addition, we have recently reported an inhibitory effect of caprylic acid on *E. sakazakii* and *B. cereus* in reconstituted infant formula (Jang and Rhee, 2008). Caprylic acid (ocatnoic acid) is generally recognized as safe (GRAS; 184.1025), based on an extensive history of use that reveals no associated health problems. This compound is an eight-carbon fatty acid that is naturally found in breast milk, bovine milk, and goat and ewe milk (Jensen et al., 1990; Jensen, 2002; Park et al., 2007; Sprong et al., 2001). The milk-derived components have been shown to prevent infections, because such components have antimicrobial or immunomodulatory activities, or interfere with the expression of virulence factors (Baranyi et al., 2003; Cravioto et al., 1991; Jia et al., 2001; Sun et al., 2002).

The underlying mode of action for caprylic acid against *Cronobacter* spp. is not yet fully understood. It is probable that the lipid exerts a bactericidal effect by disrupting membranes (Bergsson et al., 1998; Bergsson et al., 2001); however, complementary approaches to assess cell viability and membrane damage are necessary to provide clear evidence of the mechanism. Flow cytometry and microscopy based on fluorescent staining are the most widely used methods for cell determination, including analyzing the physiological states of damaged cells (Ueckert et al., 1997). Flow cytometry analysis is based on the unique properties of dyes. SYTO9 (green fluorescence) and propidium iodide (PI), both of which stain nucleic acids, are used to analyze membrane integrity.

The present study was designed to investigate the combined effects of caprylic acid and mild heat for the inactivation of *Cronobacter* spp. in reconstituted infant formula. Furthermore, a clearer review of the mode of action and activity of caprylic acid was performed using flow cytometry and confocal laser scanning microscopy with respect to membrane damage.

2. Materials and methods

2.1. Bacterial strains

Three strains of *Cronobacter* spp. (ATCC 29004, ATCC 29544, and ATCC 51329) were obtained from the Food Microbiology Culture Collection at Korea University (Seoul, Korea). According to the U.S. Food and Drug Administration isolation and enumeration method (U.S. FDA, 2002), each strain was cultured separately in 10 ml of sterile Enterobacteriaceae enrichment (EE) broth in screw-cap tubes at 37 °C for 18 h. A loopful of each strain was streaked with a flamed loop onto violet red bile glucose agar (VRBGA; Oxoid, Hampshire, England) and incubated at 37 °C for 24 h, and then confirmed typical colonies (purple colonies surrounded by purple halo) were streaked onto a trypticase soy agar (TSA; Difco, Becton Dickinson, Sparks, MD, USA). Following incubation at 37 °C for 24 h, the strain identification was biochemically performed with API 20E (Biomerieux, Marcy l'Etoile, France). The culture suspensions were centrifuged (EF-1300, Tomy Kogyo Co., Tokyo, Japan) twice at 10,000 ×*g*, and 4 °C for 3 min, and then the pellets were resuspended in 10 ml of tryptic soy broth (TSB; Difco). The suspended TSB solutions were dispensed into 2-ml vials with glycerol (20%). The cultures were immediately stored at −70 °C. All three culture transfers were performed to maintain vigorous condition.

2.2. Sample preparation and inoculation

A commercially manufactured powdered infant formula was purchased from a local retail store. The formula (25.5 g) was reconstituted in 180 ml of sterile distilled water according to the methods described by Nair et al. (2004a) with agitation. The mixture was then pasteurized at 63 °C for 30 min before inoculation with a cocktail suspension of *Cronobacter* spp. A mixture of the three strains of *Cronobacter* spp., obtained as described below, was used as an inoculum. The bacterial cultures of each *Cronobacter* spp. strain were combined in a plastic 50-ml centrifuge tube (Becton Dickinson, Franklin Lakes, NJ, USA). The cells were harvested by centrifugation at 2600 ×*g* for 15 min (Centra-CL2, IEC, Needham Heights, MA, USA). After the supernatant fluid was decanted, the pellet was washed twice in 30 ml of 0.85% sterile saline, and the final pellet was resuspended in 2 ml of sterile distilled water. A 2 ml volume of the appropriately diluted three-strain mixture of *Cronobacter* spp. was added to the reconstituted infant formula sample under constant stirring in order to obtain a uniform distribution and achieve a yield of 10^7 to 10^8 CFU/ml.

2.3. Caprylic acid

The stock solutions of caprylic acid (purest grade; Sigma Chemical Co., St. Louis, MO, USA) were prepared by dissolving in dimethyl sulfoxide (DMSO; Sigma) to obtain concentrations of 0.5, 1.0, 2.0, and 3.0 M. The concentrations of caprylic acid were chosen based on a previous study (Jang and Rhee, 2008). The stock solutions were vortexed at high speed for 1 min to create emulsions (Thormar et al., 2006), which appeared to be clear at room temperature.

2.4. Combined treatment with caprylic acid and mild heat

The inoculated samples of reconstituted infant formula were transferred to sterile glass-tubes, each with a volume of 9.9 ml, and caprylic acid was added to achieve final concentrations of 5, 10, 20, and 30 mM (1%) of caprylic acid, respectively. Samples without caprylic acid served as controls (reconstituted formula alone or with 1% DMSO). A concentration of 2% DMSO was previously found not to affect bacterial viability in milk (Isaacs et al., 1995). To achieve the various treatment conditions, the caprylic acid-treated samples were subjected to mild heat using a shaking water bath (Vision Sci. Co., Incheon, Korea; 100 rpm) to activate the antimicrobial effect on *Cronobacter* spp.. The samples were treated at 45, 50, and 55 °C for 0, 10, 20, 30, 60 min and 2 h.

2.5. Determination of antimicrobial activity

The treated samples were serially diluted (10^1 to 10^5) with 9 ml of 0.85% sterile saline. Following 10-fold serial dilutions, 0.1 ml of each of the dilutions was spread-plated onto Chromogenic *Enterobacter sakazakii* agar (DFI Formulation, Oxoid, Hampshire, England) in duplicate, and 0.2 ml of the nondiluted samples were spread-plated on five plates, which was test for healthy cells (Gurtler and Beuchat, 2005). The plates were incubated at 37 °C for 24 h. The experiments were repeated three times. Each microbial count was expressed as a mean logarithmic value (log CFU/ml).

2.6. Validation of the combined treatments

To examine the extent of recovery of injured cells, the effectiveness of the different treatments was validated in a low inoculum level of *Cronobacter* spp. in reconstituted infant formula. The mixed strain cultures were inoculated into the reconstituted formula by the same method described above, yielding initial populations of approximately 10^3 to 10^4 CFU/ml. Following the treatments, 0.2 ml of each nondiluted sample (10^0) was immediately spread-plated onto Chromogenic *Enterobacter sakazakii* agar. One milliliter from each of the treated samples was inoculated into 10 ml of TSB and incubated at 37 °C for 24 h. The enriched samples were streaked with a flamed loop onto the Chromogenic *Enterobacter sakazakii* agar. Following incubation, the results were recorded as positive or negative. The experiments were performed in triplicate.