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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A B S T R A C T

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.

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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. (reClassified 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...
combined antimicrobial treatments that exhibit synergistic bactericidal effects on pathogenic bacteria, which are minimally invasive compared with individual treatments (Davidson and Branan, 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 microbial activities against various bacteria (Desbois and Smith, 2010; Jang and Rhee, 2009; Sprong et al., 2001). CTA is approved as a 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 10^3 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 deoxycholate 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 Thermocounter TH200 (Novasina, Zurich, Switzerland). After 30 days, the sample was reconstituted in sterile distilled water, which yielded an initial bacterial concentration of approximately 10^9 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 10^8 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
prepared by dissolving them in dimethyl sulfoxide (Sigma Chemical Co.) and sterile distilled water, respectively, which were stored at −20 °C and 4 °C (concentration of SYTO9 and PI stock solution was 5 and 1.5 mM, respectively). Resuspended samples (1 ml) were dual stained with SYTO9 (5 μM of the working concentrations) and PI (30 μM) in a dark room. After staining, the samples were incubated for 15 min at 37 °C, washed twice, and placed on ice until the flow cytometric assay.

The flow cytometric analysis was performed using a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with 488 nm excitation by a 15 mW argon ion laser. All of the parameters were detected as logarithmic signals. The green fluorescence from SYTO9 stained cells was detected at 530 ± 150 nm (FL 1 channel, R2 region) and the red fluorescence emission from PI was detected at >670 nm (FL 3 channel, R1 region). Signals were detected from the forward light scatter (FSC) photodiode. For each sample, 50,000 events were recorded at a low rate setting (12 μl/min). BD CellQuest Pro (Becton Dickinson, San Jose, CA, USA) was used for the analysis and the results were expressed as density plots (FL1 versus FL3). In all experiments, the control samples comprised cells that were not stained with SYTO9 and a lethal culture (treated 30 min at 80 °C) stained with PI only, which were analyzed to check the settings of the instrument.

2.8. Transmission electron microscopy (TEM)

Cells were fixed overnight in a mixture of cold 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), and 2% paraformaldehyde in 0.1 M phosphate or cacodylate buffer (pH 7.2). These cells were post-fixed for 1.5 h in 2% osmium tetroxide in 0.1 M phosphate or cacodylate buffer for 1.5 h at room temperature. The samples were washed briefly

![Graphs showing bacterial reductions](image)

Fig. 1. Reductions of (A) Cronobacter sakazakii at 40 °C, (B) Salmonella Typhimurium at 40 °C, (C) Cronobacter sakazakii at 45 °C, and (D) Salmonella Typhimurium at 45 °C when treated with different concentrations of CA, CTA, and VNL in reconstituted infant formula. Treatments with 2% ethanol were served as the controls for the solvents. Data represent the average log reduction in bacterial population from three repeated experiments. CA, caprylic acid; CTA, citric acid; VNL, vanillin.
with deuterated H2O2 and dehydrated using the following graded ethanol series: 50, 60, 70, 80, 90, and 100% ethanol (×2). The samples were then treated with a mixture of propylene oxide and EPON epoxy resin mix (Embed 812, nadic methyl anhydride, poly Bed 812, dodecenylsuccinic anhydride, dimethylaminomethyl phenol; Electron Microscopy Polysciences), then finally embedded in epoxy resin only. The epoxy resin-treated samples were loaded into the capsules and polymerized at 38 °C for 12 h, followed by 60 °C for 48 h. The sections for light microscopy were cut at 1.0 nm and stained with 1% toluidine blue for 45 s on a hot plate at 80 °C. Thin sections were produced using an ultramicrotome (RMC MT-XL) and collected on a copper grid. Appropriate areas for thin sectioning were cut at 65 nm and stained with saturated 4% uranyl acetate and 4% lead citrate before examination with a transmission electron microscope (JEM-1400, Tokyo, Japan) at 80 kV.

2.9. Statistical analysis

For each treatment, the independent replicate trials were pooled and evaluated using the general linear model procedure in SAS (version 9.13; SAS Institute Inc., Cary, NC, USA). The model included the treatment concentration, time, and temperature as the major effects. If ANOVA detected a significant result ($P<0.05$), the mean values were tested using Tukey's multiple range test.

3. Results

3.1. Antimicrobial activity of individual compounds

Fig. 1 shows the effects of different concentrations of CA, CTA, and VNL on *C. sakazakii* or *S. Typhimurium* in reconstituted infant formula at 40 °C and 45 °C. The bacterial population was not inhibited in the control samples (sterile distilled water or 2% ethanol) throughout the entire treatment period (data not shown).

3.1.1. Caprylic acid (CA)

The antimicrobial activity of CA was significantly enhanced in a concentration- and temperature-dependent manner. At 40 °C, the logarithmic populations of *C. sakazakii* or *S. Typhimurium* were not reduced with 20 mM and 10 mM of CA, respectively, compared with the control samples. A 4.5 log reduction of *C. sakazakii* was observed with a concentration of 80 mM and a treatment time of 30 min while most of the *S. Typhimurium* population was inactivated when the samples were treated with 40 mM of CA for 10 min. The bactericidal effects on *C. sakazakii* and *S. Typhimurium* were enhanced significantly by increasing temperature. At 45 °C, the bacterial populations were reduced to an undetectable level with CA concentrations of 40 (treatment time $>$30 min), 60, and 80 mM for *C. sakazakii*, and 30 (treatment time $>$10 min), 40 ($>$10 min), 60, and 80 mM for *S. Typhimurium*.

![Graphs showing antimicrobial activity of CA and CTA on *C. sakazakii* and *S. Typhimurium*](image)

**Fig. 2.** Combined effects of CA and CTA on (A) *Cronobacter sakazakii* at 40 °C, (B) *Salmonella Typhimurium* at 40 °C, (C) *Cronobacter sakazakii* at 45 °C, and (D) *Salmonella Typhimurium* at 45 °C in reconstituted infant formula. Treatments with distilled water or 2% ethanol were served as the controls for the solvents. Data represent the average bacterial population from three repeated experiments. The bars represent the standard deviations. CA, caprylic acid; CTA, citric acid.
3.1.2. Citric acid (CTA)

At 40 °C, CTA concentrations of 10–40 mM and 10–60 mM reduced populations of C. sakazakii or S. Typhimurium, respectively, by ~0.8 logs with a treatment period of 30 min (not significantly different from the control). At 45 °C, there were no noticeable reductions in the pathogen populations when the CTA concentration was <30 mM, whereas a >40 mM resulted in a gradual and significant reduction. With 80 mM CTA at 45 °C, there were 5.7 and 5.3 log CFU/ml reductions in the populations of C. sakazakii and S. Typhimurium, respectively, after a treatment period of 30 min.

3.1.3. Vanillin (VNL)

The bacterial population was reduced only slightly (<1.0 log CFU/ml reduction) with VNL concentrations of <40 mM and <30 mM at 40 °C and 45 °C, respectively. However, the C. sakazakii and S. Typhimurium counts decreased significantly when the VNL concentrations were higher than these concentrations. The C. sakazakii or S. Typhimurium populations were reduced to undetectable levels by the following VNL treatments; 60 mM (treatment time >30 min) and 80 mM (>10 min) of VNL for C. sakazakii, and 60 mM (>30 min) and 80 mM (>30 min) for S. Typhimurium, respectively.

3.2. Combined effects of CA with CTA or VNL

The concentrations of each substance in the combined treatments were determined based on the results with the individual treatments. To test the synergistic effects of the combined treatments, concentrations that produced low bactericidal effects in the individual treatments were selected as follows: 10 mM and 20 mM of CA, and 15 mM and 30 mM of CTA and VNL.

3.2.1. Combination of CA and CTA

Fig. 2 shows the combined effects of CA with CTA on C. sakazakii or S. Typhimurium at 40 °C and 45 °C. At 40 °C, 10 mM of CA with 15 mM CTA (CA 10 + CTA 15) slightly reduced the bacterial population in reconstituted infant formula. However, all other treatments resulted in remarkably effective synergistic reductions of C. sakazakii and S. Typhimurium. Dramatic reductions of C. sakazakii or S. Typhimurium were observed with CA 10 + CTA 30 at 40 °C and undetectable levels of bacteria were observed after a treatment period of 30 min. When bacteria were treated with CA 20 + CTA 30, a ~6 log reduction of C. sakazakii was observed after 5 min and all of the cells were inactivated completely after 10 min, while S. Typhimurium was undetectable after 5 min.

When the temperature was increased to 45 °C, all of the combined treatments produced remarkable synergistic effects compared with the groups treated at 40 °C. When the samples were treated with CA 10 + CTA 30, the C. sakazakii count was reduced to an undetectable level after 10 min. With CA 20 + CTA 30, there were no viable C. sakazakii cells at all treatment time points. The antimicrobial effect against S. Typhimurium was very high with all treatment conditions at 45 °C. At 45 °C, all treatment combinations except CA 10 + CTA 15 eliminated S. Typhimurium completely, i.e., CA 10 + CTA 30 (>30 min), CA 20 + CTA 15 (>30 min), and CA 20 + CTA 30 (>5 min).

Fig. 3. Combined effects of CA and VNL on (A) Cronobacter sakazakii at 40 °C, (B) Salmonella Typhimurium at 40 °C, (C) Cronobacter sakazakii at 45 °C, and (D) Salmonella Typhimurium at 45 °C in reconstituted infant formula. Treatments with distilled water or 2% ethanol were served as the controls for the solvents. Data represent the average bacterial population from three repeated experiments. The bars represent the standard deviations. CA, caprylic acid; VNL, vanillin.
3.2.2. Combination of CA and VNL

Fig. 3 shows the combined effects of CA with VNL on C. sakazakii or S. Typhimurium at 40 °C and 45 °C. All of the C. sakazakii populations were eliminated in samples treated with CA 20 + VNL 30 after a treatment period of 10 min. When S. Typhimurium samples were treated with CA + VNL at 40 °C, the combinations of CA 20 + VNL 15 for 30 min and CA 20 + VNL 30 for 5 min reduced the numbers of S. Typhimurium cells to undetectable levels.

An enhanced bacterial killing ability of CA + VNL was observed with increasing temperature. At 45 °C, a major reduction in C. sakazakii counts was observed after 5 min with all of the combined treatment conditions, except CA 10 + VNL 15. The magnitudes of the reductions at 5 min were as follows: CA 10 + VNL 30 (reduction of 2.3 log CFU/ml) < CA 20 + VNL 15 (reduction of 5.3 log CFU/ml) < CA 20 + VNL 30 (reduction of 5.6 log CFU/ml). After treatment for 10 min, no cells were detected with CA 10 + VNL 30 and CA 20 + VNL 30, while the counts decreased to 1.1 and 0.4 log CFU/ml within 30 min after treatment with combinations of CA 10 + VNL 15 and CA 20 + VNL 15, respectively. The highest antimicrobial effects among all treatments were achieved with S. Typhimurium. When the treatment period was 5 min at 45 °C, the populations were reduced to 5.6, 2.8, 0.2, and zero log CFU/ml for CA 10 + VNL 15, CA 10 + VNL 30, CA 20 + VNL 15, and CA 20 + VNL 30, respectively. After treatment at 45 °C for 30 min, no bacteria were detected with all of the combined treatments, except for CA 10 + VNL 15, which reduced the bacterial count to 1.4 log CFU/ml.

3.3. The pH of the solutions

The pH of infant formula containing individual antimicrobial agents ranged from 5.30 to 5.96 for CA, 3.32 to 5.08 for CTA, and 6.13 to 6.52 for VNL. CTA had the largest impact on pH followed by CA and VNL. In combined treatments, the pH of combined solutions was reduced with increasing CA and CTA concentrations (P < 0.05) whereas the concentration of VNL (15 mM and 30 mM) did not affect the pH (P > 0.05) (Table 1). The pHs of CA + CTA and CA + VNL were 4.05–4.78 and 5.62–5.84, respectively.

3.4. Validation of antimicrobial effects on desiccation-stressed cells

Table 2 shows the survival of desiccation-stressed bacteria cell in infant formula with low inoculum after the combined treatment at 40 °C and 45 °C. Both pathogens were inoculated into powdered infant formula and were exposed to desiccation stress at 22 °C for 30 days. During storage in this environment, there were no changes in the water activity levels of the inoculated samples (water activity of samples = 0.28). The initial populations of both pathogens were approximately 10^9 CFU/ml. C. sakazakii or S. Typhimurium cells were completely inactivated when treated under certain conditions (Table 2).

3.5. Flow cytometric analysis

To analyze time-dependent integrity changes, the cells were treated with CA 10 + CTA 30 or CA 10 + VNL 30 at 40 °C, which reduced the bacterial cell population gradually. Fig. 4 shows the flow cytometric images of C. sakazakii or S. Typhimurium cells. Using dual staining with SYTO9/PI (FL1/FL3), the membrane integrity was assessed by flow cytometry where the viable cells had a strong green fluorescence (FL1, R2) and the permeabilized cells (membrane-disrupted cells) had a strong red fluorescence (FL3, R1). The magnitude of the R1 region density was used as a measure of the number of membrane-disrupted cells. Compared with the samples treated for 0 min (Fig. 4a), the dot plots of the exposed samples showed that the cells shifted from the R2 region to the R1 region as time progressed (Fig. 4b–d). There were large differences between the samples treated for 0 and 30 min. During treatment with CA 10 + CTA 30, the ratios in the R1 region for C. sakazakii or S. Typhimurium changed from 5.4% (Fig. 4Aa) to 73.5% (Fig. 4Ad) and from 9.8% (Fig. 4Ba) to 70.3% (Fig. 4Bd), respectively. With the CA + VNL treatments, the bacterial cell of C. sakazakii in the R1 region accounted for 9.0% (Fig. 4Ca) but this population density increased to 62.4% (Fig. 4Cd) after 30 min. For S. Typhimurium, the density of the R1 area was initially 4.7% (Fig. 4Da) but increased to 79.6% (Fig. 4Dd) after 30 min.

3.6. Transmission electron microscopy analysis

C. sakazakii or S. Typhimurium before and after treatment with CA 10 + CTA 30 and CA 10 + VNL 30 at 40 °C were examined by TEM (data not shown). Untreated C. sakazakii and S. Typhimurium cells had normal shapes and possessed intact membranes. After the combined treatment, the cells had a deformed structure and the outer and cytoplasmic membrane was totally destroyed. This cell destruction resulted in the release of cellular material outside the cell and subsequent cell death.

4. Discussion

The present study evaluated the antimicrobial effects of CA, CTA, and VNL on three strains of C. sakazakii or S. Typhimurium in reconstituted infant formula in various conditions at mild temperatures, i.e., individually and in combinations of the antimicrobials. In individual compound experiments, the bactericidal activities of CA and VNL increased with increasing temperature. The synergistic effects of CA and mild heat agree with previous studies showing that the bactericidal activity of fatty acids or monoglycerides is increased by heat. The authors suggested that this was due to the greater solubility of these compounds at higher temperatures, which increased their ability to disrupt cell membranes (Jang and Rhee, 2009; Nair et al., 2004). The enhanced bactericidal effects of phenolic compounds at higher temperatures were also

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH values of the bacterial suspensions (n = 3).</th>
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<tr>
<td>CA 10</td>
<td>6.73 ± 0.02*</td>
</tr>
<tr>
<td>CA 20</td>
<td>5.96 ± 0.03†</td>
</tr>
<tr>
<td>CA 30</td>
<td>5.54 ± 0.02‡</td>
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<tr>
<td>CA 40</td>
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<tr>
<td>CA 60</td>
<td>5.37 ± 0.01†</td>
</tr>
<tr>
<td>CA 80</td>
<td>5.30 ± 0.03°</td>
</tr>
<tr>
<td>CTA 10</td>
<td>5.08 ± 0.02‡</td>
</tr>
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<td>CTA 20</td>
<td>4.40 ± 0.03‡</td>
</tr>
<tr>
<td>CTA 30</td>
<td>4.05 ± 0.01‡</td>
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<td>CTA 40</td>
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<td>CTA 60</td>
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</tr>
<tr>
<td>CTA 80</td>
<td>3.32 ± 0.02‡</td>
</tr>
<tr>
<td>VNL 10</td>
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<td>VNL 80</td>
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<td>CA 10 + CTA 15*</td>
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<tr>
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<td>CA 20 + CTA 15</td>
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<td>CA 20 + VNL 30</td>
<td>5.63 ± 0.02β</td>
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</table>

The pH, value of caprylic acid, citric acid, and vanillin is 4.89, 3.13, and 7.40, respectively (Hsiao and Siebert, 1999; Wang et al., 2010).

*Mean ± standard deviation values denoted by different superscript letters are significantly different from the control values (P < 0.05).
†CA, caprylic acid; CTA, citric acid; VNL, vanillin.
‡Combined treatment with 10 mM caprylic acid and 15 mM citric acid.
suggested to be the result of the increased fluidity of the lipid bilayer in the presence of these compounds (Karatzas et al., 2000).

In the present study, combined treatments had marked synergistic effects against C. sakazakii and S. Typhimurium. For example, 20 mM CA and 30 mM CTA for 5 min at 40 °C reduced C. sakazakii to negligible levels (Fig. 1) while the same combined treatment (20 CA + 30 CTA for 5 min at 40 °C) reduced the C. sakazakii counts to undetectable levels (reduction >7.3 log CFU/ml) (Fig. 2). Highly enhanced antimicrobial activities were also determined with combined treatments of CA + VNL. Bacterial populations were not reduced significantly in the following individual conditions: CA 10 and VNL 30 for 30 min at 40 °C against S. Typhimurium (Fig. 1), whereas the combined treatment produced a reduction of 6.6 log CFU/ml with S. Typhimurium (Fig. 3). The anti-microbial activities also increased as a function of heat. As the temperature increased, the bactericidal effects were greater at all time points. A reduction of 1.1 log CFU/ml for C. sakazakii was obtained with CA 10 + VNL 30 for 10 min at 40 °C while a temperature increase to only 45 °C led to complete inactivation (>6.8 log CFU/ml). The effects of combined treatments containing all three compounds were also examined, i.e., CA, CTA, and VNL, but no major effects were observed at all time points (data not shown). Therefore, dual combinations were sufficiently effective for disinfecting reconstituted infant formula.

Under certain conditions (see Figs. 2 and 3), the combined treatments completely destroyed the colony-forming capabilities of C. sakazakii and S. Typhimurium in reconstituted infant formula. However, sublethal injury of bacteria cells might not have been detected by the selective media because the selective agents added to the media inhibited the growth of non-target organisms (Kim and Rhee, 2011b). If any surviving cells were present in the infant formula, it is possible that they could recover from injury via improper handling during preparation, holding, and feeding. In addition, Cronobacter spp. is usually present in powdered infant formula at low concentrations of 0.4–0.7 CFU per 100 g (Muytjens et al., 1988; Nazarowec-White and Farber, 1997b). To confirm that the combined effects would be effective in practical conditions, validation experiments were conducted using formula inoculated at a low concentration with desiccation-stressed cells, which were analyzed by direct plating and plating following enrichment. Some of the treatment conditions described in the results (see conditions in shaded areas in Table 2) failed to recover injured cells even after enrichment, which indicated the complete destruction of the pathogens. These results will be helpful for optimizing the treatment conditions before application in the dairy food industry.

The bacterial inactivation observed with the combination treatment suggested that the antimicrobial compounds used in these experiments negatively affected the cell structure and/or function. To analyze the mode of cell destruction with the combined treatment, especially membrane disruption, the cell membrane integrity was measured by flow cytometry and visualized by TEM. The results showed that the proportion of cells in the PI fluorescent region increased as a function of the treatment time (Fig. 4), which indicated that there was an increase in the number of membrane-damaged cells. The TEM images showed that major alterations and disruption of the cell membranes with cellular material leakage were caused by the combined treatments. These

<table>
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<th>Temp. (°C)</th>
<th>Agents and their concentrations (mM)</th>
<th>Treatment time (min)</th>
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*+, growth on chromogenic Enterobacter sakazakii agar or xylene lysine desoxycholate agar; –, no growth on chromogenic Enterobacter sakazakii agar or xylene lysine desoxycholate agar.
CA, caprylic acid; CTA, citric acid; VNL, vanillin.

a. Combined treatment with 10 mM caprylic acid and 15 mM citric acid.

b. These values were determined by direct plating using three replicate experiments.

c. The values in parentheses were determined by streaking enriched samples in three replicate experiments.
images verified the destruction of *C. sakazakii* and *S. Typhimurium* cells and provided clear evidence of the antimicrobial activity of the combined treatments.

The mechanism of microbial inactivation by CA is still poorly understood but the main target is the cell membrane. Many studies have suggested that fatty acids can diffuse into the bacterial cell membrane where they disrupt the electron transport chain and oxidative phosphorylation (Altieri et al., 2009; Bergsson et al., 2001; Desbois and Smith, 2010). Fatty acids create transient or permanent pores that may penetrate the cell membrane so membrane proteins or larger portions of the lipid bilayer are released, which ultimately disrupts the membrane permeability. This process depends on the amphipathic structure of fatty acids and it can affect the following cellular processes: 1) interference with cellular energy production, 2) disruption of oxidative phosphorylation, 3) bacterial growth inhibition or cell lysis, 4) inhibition of enzyme activity, 5) impairment of nutrient uptake, 6) generation of toxic peroxidation, and 7) generation of auto-oxidation products (Altieri et al., 2009; Bergsson et al., 2001; Desbois and Smith, 2010).

The primary antimicrobial action of CTA is its ability to inhibit the growth of many bacteria. The undissociated form of CTA penetrates the cell membrane and enters the cytoplasm where it reduces the intracellular pH and disrupts the transmembrane proton-motive force (Brul and Coote, 1999; Dppres, 2005). In addition, CTA is not a classical weak acid because it acts as a chelating agent and inhibits the growth of bacteria by chelating divalent metal ions (Brul and Coote, 1999).

The antimicrobial mechanism of phenolic compounds in essential oil is still unclear but they function via similar bactericidal mechanisms to CA. Many studies have suggested that phenolic compounds, such as VNL, primarily target the cytoplasmic membrane via the accumulation of hydrophobic phenolic groups (hydroxyl group) in the lipid bilayer, which disrupt lipid protein interactions, alter the membrane structure, function, and permeability, accelerate the leakage of cell contents, disrupt the proton-motive force and electron influx, meaning that finally, compounds that destroy cell integrity can enter the cell (Burt, 2004; Char et al., 2010; Ramos-Nino et al., 1997).

The synergistic antimicrobial activity of combined CA and CTA or VNL may be associated with changes in bacterial permeability and the loss of cell membrane integrity. The synergistic bactericidal effects of CA with CTA or VNL were assumed to occur as follows: the CTA or CA disturbed the cell membrane, which may have created an environment that facilitated the entry of other antimicrobial compounds (CA, CTA, VNL, and hydrogen ion), thereby destabilizing the cell membrane and contributing to cell destruction. The study presented here provides definite proof of bacterial death by membrane damage using flow cytometry and TEM images. The results supported our hypothesis that the combination of antimicrobial substances disrupted the cell membrane or destabilized the outer membrane, which eventually led to permeabilization of the cells.

In particular, hydrogen ions cannot enter bacterial cells through a membrane (only undissociated molecule can enter cells and play a role in killing bacteria) thus individual treatment with CTA results in weak bactericidal activity despite the low pH of the sample. With CA + CTA treatment, antimicrobial compounds could attack the cell membrane, allowing the hydrogen ions in an aqueous environment to penetrate the cells through the damaged membrane, resulting in stronger bactericidal effects than those observed with individual treatment. Therefore, although the pH of the sample containing CTA 60 (pH 3.47) alone was lower than that of the sample containing CA 20 + CTA 15

![Fig. 4. Flow cytometric plots of SYTO9-PI stained (A) *Cronobacter sakazakii* and (B) *Salmonella Typhimurium*, when treated with a combination 1 (10 mM CA with 30 mM CTA), and (C) *Cronobacter sakazakii* and (D) *Salmonella Typhimurium* when treated with a combination 2 (10 mM CA with 30 mM VNL) for: (a) 0 min, (b) 5 min, (c) 10 min, and (d) 30 min at 40 °C. The quadrants show the division between damaged or dead cells in gate 2 (red fluoresence channel, R1) and live cells in gate 1 (green fluoresence channel, R2). CA, caprylic acid; CTA, citric acid; VNL, vanillin.](image-url)
(pH 4.73), the log reduction in the CFU of S. Typhimurium treated with the latter combination at 45 °C for 10 min (7.04 log reduction) was higher than that of the formal under the same conditions of temperature and time (0.89 log reduction).

The use of additional chemicals may cause consumers to avoid treated products. To address consumer demands, it is necessary to develop natural antioxidants that can be applied to foods. All of the antimicrobials used in this study (CA, CTA, and VNL) are natural compounds (Brul and Coote, 1999; Desbois and Smith, 2010; Dppres, 2005). CA and CTA are considered as GRAS (CFSR section of CA: §184.1025 and CTA: §184.1386) (FDA, 2006), and the Korean government has approved the use of CA, CTA, and VNL as food additives (KFDA, 2004). CA can be used as a nutritional supplement (KFDA, 2004) and CTA and VNL traditionally have been used to preserve foods and to enhance food flavor (Brul and Coote, 1999; Burt, 2004; Dppres, 2005; Fitzgerald et al., 2004). Important benefits of the new decontamination method based on combined treatments are its industrial feasibility, cost effectiveness, and the absence of negative consumer reaction to food additives because chemical compounds are not used. In addition, only mild treatment conditions (low concentrations of agents and mild temperatures) are required to obtain adequate bactericidal effects with the combined treatments compared with the individual treatments so as any loss of nutrients or undesirable changes would be minimal.

In conclusion, combinations of CA and CTA or VNL were shown to have remarkable antimicrobial effects. Clear evidence of bacterial destruction was obtained by using flow cytometry and TEM to analyze the cell viability and cell membrane damage, which confirmed the distinctive changes in cells. The results of this study may contribute to the fundamental understanding of antimicrobial mechanisms. This approach is a highly feasible and practical technique, which could be applied in the food industry.


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