Inactivation of Cronobacter spp. (Enterobacter sakazakii) in infant formula using lactic acid, copper sulfate and monolaurin

M.A. Al-Holy¹, L.F. Castro² and H.M. Al-Qadiri³

¹ Department of Clinical Nutrition and Dietetics, Faculty of Allied Health Sciences, Hashemite University, Zarqa, Jordan
² School of Food Science and Human Nutrition, Washington State University, Pullman, WA, USA
³ Department of Nutrition and Food Technology, Faculty of Agriculture, The University of Jordan, Amman, Jordan

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Correspondence
Murad A. Al-Holy, Department of Clinical Nutrition and Dietetics, Faculty of Allied Health Sciences, Hashemite University, Zarqa, Jordan. E-mail: murad@hu.edu.jo

Abstract
Aims: To investigate the effect of lactic acid (LA), copper (II), and monolaurin as natural antimicrobials against Cronobacter in infant formula.

Methods and Results: The effect of LA (0-1, 0-2 and 0-3% v/v), copper (II) (10, 50 and 100 µg ml⁻¹) and monolaurin (1000, 2000, and 3000 µg ml⁻¹) suspended into tween-80™ or dissolved in ethanol against Cronobacter in infant formula was investigated. Reconstituted infant formula and powdered infant formula were inoculated with five strains of Cronobacter spp. at the levels of c. 1 x 10⁶ CFU ml⁻¹ and 1 x 10³ CFU g⁻¹, respectively. LA at 0-2% v/v had a bacteriostatic effect on Cronobacter growth, whereas 0-3% v/v LA resulted in c. 3 log₁₀ reduction. Copper (II) at the levels of 50 µg ml⁻¹ and 100 µg ml⁻¹ elicited 1 and 2 log₁₀ reductions, respectively. The combination of 0-2% LA and 50 µg ml⁻¹ copper (II) resulted in a complete elimination of the organism. Monolaurin exhibited a slight inhibitory activity against Cronobacter (c. 1·5 log₁₀ difference) compared to the control when ethanol was used to deliver monolaurin.

Conclusions: A complete elimination of Cronobacter was obtained when a combination of sublethal concentrations of LA (0-2%) and copper (II) (50 µg ml⁻¹) was used.

Significance and Impact of the Study: The use of the synergistic interactive combination of LA and copper (II) could be beneficial to control Cronobacter in the infant formula industry.

Introduction
Cronobacter is an emerging human pathogen that contaminates powdered infant formula (PIF) (Iversen and Forsythe 2003) and has been associated with various cases of neonatal meningitis (CDC 2002). Although the incidence rate of the infection is low, the mortality rate ranges from 40 to 80% among infected infants, and those who survive the infection usually develop irreversible neurological sequelae (Bowen and Braden 2006). Recently, Cronobacter has been proposed as a new genus to replace Enterobacter sakazakii as a single species. Cronobacter currently encompasses six different species which are potentially pathogenic to neonates (Iversen et al. 2008).

Contaminated PIF is a recognized source of Cronobacter where the organism was detected in about 14% of 141 samples of PIF (Muytjens et al. 1988). The problem with infant formula is that it is not sterile, and its nutritional characteristics provide excellent conditions for bacterial growth following reconstitution (Mullane et al. 2006). Cronobacter does not survive the heat of pasteurization used in the production of powdered milk; therefore, the organism mostly originates from the processing environment or from heat-sensitive ingredients added after pas-
teurization despite rigorous hygienic practices (Kandhai et al. 2004). Therefore, an end-product control measure is necessary to prevent further the presence of the organism in the formula. Preservatives are commonly used in the production of food to ensure safety and stability. Nonetheless, there is a growing awareness among consumers about food-related diseases and a mounting demand on manufacturers to produce pathogen-free food with minimal use of artificial preservatives (Brul and Coote 1999). Many research articles have been published on survival conditions and inactivation of Cronobacter spp. (Gurtler and Beuchat 2007; Arku et al. 2008; Al-Holy et al. 2009), but little attention was given to the use of antimicrobials that are perceived as ‘more natural’ to inhibit Cronobacter in PIF and baby foods.

Lactic acid (LA) is a weak-organic acid that has been widely used to control the growth of spoilage and pathogenic bacteria in food (Jay 2000). LA also acts as a permeabilizer of the Gram-negative bacterial outer membrane, and this is of considerable consequence, because the weak acid could be used to potentiate the antimicrobial activity of other antimicrobials against Gram-negative bacteria (Alakomi et al. 2000).

All living organisms including bacteria require copper at low concentrations. Copper is used as a cofactor for metalloproteins and enzymes, but at high concentrations, the inhibition of growth in bacteria can be accrued (Faundez et al. 2004). In addition, fatty acids and their esters may pose antimicrobial activity against certain pathogens. The monoglyceride of lauric acid (monolaurin) demonstrated a considerable antibacterial activity against Listeria monocytogenes in beef emulsion and hotdog and against Escherichia coli O157:H7 in UHT (ultra high temperature) milk (Bränen and Davidson 2004; Mbandi et al. 2004).

The objective of this study was to investigate the effect of both LA and copper (II) alone and in combination on the inactivation of Cronobacter spp. in infant formula. In addition, the effect of monolaurin against Cronobacter was also explored.

Materials and methods

Bacterial strains and preparation of the inoculum

Five strains of Cronobacter [Cronobacter sakazakii (ATCC 29004, ATCC 12868, ATCC 29544), Cronobacter muytjensii ATCC 51329 and a Cronobacter isolate FSM 287 (PIF isolate)] were used in this study. Strains were kept refrigerated in tryptic soy agar (TSA) (Difco, Becton Dickinson, Sparks, MD, USA) slants. Prior to the experiment, the strains were individually inoculated into 10-ml tubes containing sterile brain heart infusion (BHI) broth (Difco) and incubated at 37°C for 24 h. After incubation, the five strains were added to a 50-ml sterilized centrifuge tube, creating a bacterial cocktail. The tube was then centrifuged at room temperature for 15 min at 4000 g (Fisher AccuSpin™ Model 400 Benchtop Centrifuge; Fisher Scientific, Pittsburgh, PA, USA) to harvest bacterial cells. The pellet was resuspended into 9 ml of sterile 0.85% saline solution and centrifuged as described earlier. The supernatant was discarded, and the pellet was resuspended into 9 ml of sterile 0.1% peptone water. Tenfold serial dilutions using 0.1% peptone water were carried out to obtain the required concentration of inoculum to be used for the inoculation of the reconstituted infant formula (RIF).

Inoculation of PIF milk with Cronobacter

Milk-based PIF was bought from a local grocery store. Cronobacter cells were desiccation-stressed by inoculating 24-h old cultures of a cocktail of five strains of Cronobacter (listed earlier) as per the method described by Al-Holy et al. (2008). The inoculated formula was kept in a sterile beaker for 3 months at room temperature in a desiccator. The water activity of the PIF was measured before and after the inoculation at day zero and after 3 months of storage using an Aqua lab water activity meter (Model 3TE; Aqualab, Pullman, WA, USA). The initial level of Cronobacter was determined by the overlay method (Al-Holy et al. 2008). The initial level of Cronobacter was c. 1 × 10^6 CFU g⁻¹. The desiccated cells in PIF were further diluted with PIF to obtain a final concentration of c. 1000 CFU g⁻¹.

Inoculation of RIF with Cronobacter

Commercial PIF was reconstituted with sterile distilled water according to the manufacturer’s instructions. Ten millilitres of the RIF was placed into a sterile test tube and inoculated with 100 μl of the inoculum to give approximately 1 × 10^6 CFU ml⁻¹.

Effect of copper (II) and LA on the growth of Cronobacter in RIF

The effect of copper (II) [added as copper sulfate pentahydrate (Fisher)] and LA on the growth of Cronobacter inoculated into RIF was studied. Concentrations of 10, 50 and 100 μg ml⁻¹ of copper (II) were used. The effect of 0·1, 0·2 and 0·3% v/v of LA (Acros Organics, NJ, USA) on the growth of Cronobacter in RIF was also investigated. In a separate experiment, the effect of LA (0·2% v/v) or copper (II) (50 μg ml⁻¹) alone or in combination on the growth of Cronobacter in 10-ml samples of RIF (pH = 5·01) was studied. RIF inoculated with Cronobacter but without treatment was used as a control. In a separate
trial, the pH of RIF treated with 0-2% LA and 50 μg ml⁻¹ copper (II) was controlled to 6.54 by adding 1 mol l⁻¹ NaOH (Sigma-Aldrich, St Louis, MO, USA). The pH of the RIF was monitored by means of pH meter (Model 744; Metrohm, Herisau, Switzerland).

Effect of LA and copper (II) on the growth of Cronobacter in PIF
An aliquot of 800 μl of the 10% v/v solution of LA was added to 15.5 g of PIF milk; the powder was reconstituted according to manufacturer’s instruction and mixed using a sterile spatula to make a final concentration of 0-2% v/v of LA. For the copper treatment, copper sulfate powder was added to inoculated PIF and rehydrated according to manufacturer’s instruction to make a final concentration of copper (II) equivalent to 50 μg ml⁻¹. The combined effect of 0-2% v/v of LA and 50 μg ml⁻¹ of copper (II) was also studied. Untreated PIF that had been inoculated with Cronobacter was used as a control.

Effect of monolaurin on the growth of Cronobacter in RIF
The effect of monolaurin (Tokyo Chemical Industry, Tokyo, Japan) at different concentrations (1000, 2000, and 3000 μg ml⁻¹) on the growth of Cronobacter was investigated. Stock solutions of monolaurin were prepared either as a suspension in Tween-80™ (TW) (Fisher) or as a solution in 96% ethanol (Fisher); thereafter, 100 μl of monolaurin solutions was added to RIF, and the number of Cronobacter was determined at 0, 2, 4 and 6 h. Untreated RIF (control) or samples with 100 μl of TW or ethanol without monolaurin were used for comparison purposes.

Enumeration of Cronobacter
Tenfold serial dilutions in 0-1% peptone water were prepared at 0, 2, 4 and 6 h for the RIF and the PIF. The enumeration of Cronobacter was performed using a minor modification of the overlay method reported by Al-Holy et al. (2008). All samples were spread plated on TSA, instead of TSA supplemented with 0-1% (w/v) of sodium pyruvate. The plates were incubated for 2 h at 37°C, and then a thin layer of Violet Red Bile Lactose Agar (Difco) (8 ml) was overlaid onto TSA. The plates were incubated for an additional 22 h at 37°C. This method was specifically used to enumerate intact and injured Cronobacter cells.

Statistical analysis
At least three independent replicate trials were conducted, and standard deviations were determined. Cronobacter counts were log transformed (log10), and data were analysed with a computer software package (SAS Institute, Cary, NC, USA) using analysis of variance and Fisher’s least significant difference test for mean separations (P ≤ 0.05).

Results
The effect of different concentrations of LA on the growth of Cronobacter in RIF is shown in Fig. 1. LA (0-1% v/v) apparently does not have an inhibitory effect on the growth of the micro-organism. Nonetheless, the number of Cronobacter (6.70 log10) was significantly (P ≤ 0.05) lower compared to the control (7.56 log10) at the end of the storage period. LA at 0-2% v/v imparted a bacteriostatic effect on the growth of Cronobacter. In contrast, LA at 0-3% v/v elicited the most pronounced bactericidal effect against Cronobacter. In this treatment, the numbers of Cronobacter were significantly (P ≤ 0.05) lower (c. 3 log10 difference after 6 h of storage) compared to the other LA concentrations (0-1 and 0-2% v/v).

The antibacterial activity of copper (II) against Cronobacter in RIF is shown in Fig. 2. A dose-dependent response was shown. At a concentration of 10 μg ml⁻¹, a slight but a significant (P ≤ 0.05) decrease in the number of Cronobacter cells was observed after 2 h, but a re-growth occurred, and the number of Cronobacter reached about 6.4 log10 after 6 h. At 50 μg ml⁻¹ of copper (II), a slight but steady decrease in the number of Cronobacter cells was observed. Copper (II) at 100 μg ml⁻¹ exerted the highest antimicrobial activity against Cronobacter, where about 1.8 log10 reduction at the end of the storage period was noticed. Figure 3 shows the effect of either 0-2% LA or copper (II) (50 μg ml⁻¹) alone or in combination against Cronobacter in RIF. A synergistic effect of LA and copper (II) against Cronobacter was observed.
More than 5-log₁₀ reduction in the organism was obtained after 2 h, and a total elimination was rendered at the end of the storage period. In comparison, either LA or copper (II) treatments alone only resulted in <1 log₁₀ reduction in Cronobacter. A similar inactivation pattern was observed for the combined treatment of 0.2% LA and 50 μg ml⁻¹ of copper (II) when the pH of the RIF was controlled at 6.54 (Fig. 4). The practical validity of the combined treatment [LA and copper (II)] against Cronobacter was further explored in PIF. The water activity of the inoculated PIF milk after 3 months of storage was 0.21. As shown, in Fig. 5, after applying the combined treatment [(0.2% LA and 50 μg ml⁻¹ copper (II)], a synergistic effect was obtained, where a complete elimination of the organism was obtained after 2 h and until the end of the storage period. The impact of different concentrations of monolaurin (1000, 2000 and 3000 μg ml⁻¹) as a suspension in TW (Fig. 6a), or dissolved in absolute ethanol (Fig. 6b), on the growth of Cronobacter in RIF was investigated. Surprisingly, TW containing no monolaurin exhibited a significantly (P ≤ 0.05) lower count (≤ 1 log₁₀) of Cronobacter compared to the control after 2 h and until the end of the storage period. The effect of monolaurin in TW at all of the tested levels was comparable to TW alone, where a slight inhibition of Cronobacter growth took place during the first 2 h of storage followed by an increase in Cronobacter count till the end of storage period. Nonetheless, there was a 1-log₁₀ difference between the control vs the TW with or without monolaurin throughout the 6-h storage period, revealing the inhibitory activity of TW on Cronobacter growth. Increasing monolaurin concentration from 1000 to 3000 μg ml⁻¹ did not result in further inhibition. However, it is worthwhile to mention that monolaurin in
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Figure 6 Effect of monolaurin (µg ml⁻¹) suspended in tween-80 (TW) (a) or dissolved in absolute ethanol (Et-OH) (b) on the growth of Cronobacter spp. (Log₁₀ CFU ml⁻¹) in reconstituted infant formula stored at room temperature (21°C). Control (untreated) (●), TW without monolaurin (□), Et-OH without monolaurin (○), 1000 µg ml⁻¹ (▲), 2000 µg ml⁻¹ (◦) and 3000 µg ml⁻¹ (○).

Discussion

LA is a weak-organic acid, which presents an antimicrobial activity and has been used as antimicrobial agent in foods. LA in the undissociated form can penetrate the cytoplasmic membrane, which results in reduced intracellular pH and disruption of the transmembrane proton motive force, which accounts for a significant part of its antibacterial action (Alakomi et al. 2000). LA is also a strong outer membrane disintegrating agent. LA permeabilizes the outer membrane of Gram-negative bacteria, a property that could help other antimicrobials penetrate bacterial cells and produce a toxic effect (Alakomi et al. 2000).

The toxic effect of copper on bacteria is possibly attributed to the displacement of essential ions, hence inactivating enzymes and obstructing functional groups of proteins, producing free radicals from hydroperoxide compounds and thus affecting membrane integrity (Nies 1999).

Usually, under the condition of relatively high pH, it is expected that mostly LA is present in the dissociated form of the acid, which is incapable of crossing the cytoplasmic membrane of the bacteria. Nonetheless, a complete elimination of Cronobacter cells was noticed by the end of the storage period (Figs 3, 4 and 5). It is possible that LA in the dissociated form may chelate copper (II) ions and consequently allowing them to penetrate through the cytoplasmic membrane and pose toxic effect against Cronobacter. Metallic copper surfaces inhibited the growth of Salmonella enterica and Campylobacter jejuni; two of the most common foodborne pathogens, (Faundez et al. 2004). But the ions have to enter the cell to have a physiological or toxic effect (Faundez et al. 2004). It is possible that at concentrations as low as 10 and 50 µg ml⁻¹, not enough ions are penetrating the cell membrane to produce a toxic effect. The permeabilizer properties of LA could have facilitated the entry of copper ions into the Cronobacter cells and consequently imparting the toxic effect, as well as producing a toxic effect itself. The combination of inhibitory activity and disruption of the outer membrane of Cronobacter by LA may have lead to potentiation of the antimicrobial activity of copper (II) and consequently resulted in the inhibition of Cronobacter growth. Furthermore, the toxic effect of copper (II) on micro-organisms can occur because of displacement of essential metals from their native binding sites or by ligand interactions. In addition, copper (II) may also change the conformational structure of nucleic acids and proteins and interfere with oxidative phosphorylation and osmotic balance (Borkow and Gabbay 2005). Ibrahim et al. (2008) reported that the combination of copper (II) and LA was effective in inhibiting the growth of Salmonella spp. and E. coli O157:H7 in laboratory medium and apple juice. Monolaurin exhibited a slight inhibitory activity against Cronobacter spp. (Fig 6a,b), Branen and Davidson (2004) reported that monolaurin posed some inhibitory activity against E. coli O157:H7 in UHT milk. In another study, monolaurin and lauric acid at the level of 500 µg g⁻¹ exhibited a remarkable antilisterial activity in hotdog emulsion stored at 5°C (Mbandi et al. 2004). The mechanism of inhibition of bacteria by monolaurin is not known, but the cell membrane is thought to be the target (Kabara 1993).

LA and copper (II) showed a noticeable inhibitory activity against the growth of Cronobacter in RIF. Using a combination of sublethal concentrations of LA (0.2%) and copper (II) (50 µg ml⁻¹) resulted in a synergistic inhibitory activity against Cronobacter in RIF stored at room temperature; where a complete elimination of inoculated strains of Cronobacter was obtained. However, the effect of these compounds on the organoleptic and nutritional properties of infant formula needs to be explored.
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References


