Detergent and Sanitizer Stresses Decrease the Thermal Resistance of Enterobacter sakazakii in Infant Milk Formula


ABSTRACT: This study determined the effect of acid, alkaline, chlorine, and ethanol stresses on the thermal inactivation of Enterobacter sakazakii in infant milk formula. Unstressed or stressed cells were mixed with reconstituted powdered infant milk formula (PIMF) at temperatures between 52 and 58 °C for various time periods or mixed with PIMF prior to reconstitution with hot water between 50 and 100 °C. D- and z-values were determined using liner regression analysis. In general, detergent and sanitizer stresses decreased the thermal resistance of E. sakazakii in infant milk formula. The results of this study may be of use to regulatory agencies, manufacturers, and infant caregivers to design heating processes to eliminate E. sakazakii.

Keywords: detergent stresses, Enterobacter sakazakii, sanitizer stresses, infant milk formula, thermal inactivation

Introduction

Enterobacter sakazakii is a ubiquitous, Gram-negative, facultatively anaerobic rod that belongs to the Enterobacteriaceae family. E. sakazakii has been isolated from a wide range of foods, including powdered infant milk formula (PIMF), and food factory environments, including the milk powder production environment (Kandhai and others 2004). The occurrence of E. sakazakii in PIMF may be due to its survival during the pasteurization treatment or, most likely, due to postradiation contamination during mixing with other ingredients, filling, and packaging (FAO/WHO 2006). E. sakazakii can survive for at least 2.5 y in PIMF (Caubilla-Barron and Forsythe 2007). The presence of E. sakazakii in PIMF has been associated with outbreaks of severe forms of neonatal meningitis and necrotizing enterocolitis (Simmons and others 1989; Nazarowec-White and Farber 1997a; Himelright and others 2002). The ability of E. sakazakii to form biofilms and survive desiccation conditions may contribute to its survival in infant formula factory environments and subsequent desiccated products (Iversen and others 2004a).

Recently, WHO/FAO (2007) recommended the use of water at 70 °C to reconstitute the infant formula to eliminate possible contamination of E. sakazakii in the formula; however, water at high temperatures may cause some nutrient loss associated with infant formulas, particularly loss of vitamin C (FAO/WHO 2004). It was reported that E. sakazakii is more thermotolerant than most other members of Enterobacteriaceae (Nazarowec-White and Farber 1997b). Nonetheless, there is a great disparity in the heat resistance of different strains of E. sakazakii. Edelson-Mammel and Buchanan (2004) indicated that there was about 20-fold divergence in thermal resistance between 12 strains of E. sakazakii in reconstituted PIMF at 56 to 70 °C.

Although the thermotolerance of microorganisms is affected by their physiological states (Lou and Yousef 1996; Doyle and others 2001; Wesche and others 2005), all published thermal inactivation studies of E. sakazakii in infant milk formula have used unstressed cells grown under optimal laboratory conditions (Nazarowec-White and Farber 1997b; Breeuwer and others 2003; Edelson-Mammel and Buchanan 2004; Iversen and others 2004b). However, in infant formula processing environments, E. sakazakii may be exposed to chemical stresses from the use of detergents and sanitizers in cleaning and sanitizing equipment, pipes, and floors. Therefore, it is appropriate to study the thermotolerance properties of the prestressed E. sakazakii cells, as could occur prior to contamination of infant formula.

Osaili and others (2008) have already shown that desiccation and heat stresses caused significant reduction in D-values of the same strains of E. sakazakii as used in the present study.

To our knowledge, no information is available in the literature on the effect of detergent and sanitizer stresses on the thermal resistance of E. sakazakii in infant milk formula. Therefore, the objectives of the current study were to i) assess the effect of acid, alkaline, chlorine, and ethanol stresses on the D- and z-values of E. sakazakii in reconstituted PIMF; ii) evaluate the effect of reconstituting PIMF with hot water at different temperatures on the survival of stressed microorganism. Such information will be of interest to regulatory agencies, infant formula producers, and infant caregivers to design heating processes that are sufficient to kill E. sakazakii that may be present in infant milk formula.

Materials and Methods

E. sakazakii strains

One ATCC (S1329) strain and 4 food isolates originally isolated by Shaker and others (2007) from infant milk formulas (IMF1 and IMF2), infant food formula (IF1), and crushed wheat (CS1) at the Dept. of Nutrition and Food Technology, Jordan Univ. of Science and Technology; Jordan were used in this study. All cultures were

MS 20070759 Submitted 10/9/2007, Accepted 12/20/2007. Authors Osaili, Shaker, Olaimat, and Al-Nabulsi are with Dept. of Nutrition and Food Technology, Faculty of Agriculture, Jordan Univ. of Science and Technology, PO. Box 3030 Irbid, 22110, Jordan. Author Al-Holy is with Dept. of Clinical Nutrition and Dietetics, Faculty of Allied Health Sciences, Hashemite Univ., PO. Box 150459, Zarqa-Jordan. Author Forsythe is with School of Science and Technology, Jordan were used in this study. All cultures were...
stored in brain heart infusion (BHI) (Oxoid Ltd., Basingstoke, U.K.) broth with 20% glycerol at −40 °C. To grow *E. sakazakii* cultures, a loop of each culture was grown individually at 37 °C for 24 h (stationary phase) in 15-mL tubes containing 10 mL of BHI. *E. sakazakii* cultures were subcultured in BHI 3 times before use.

**Preparation of unstressed *E. sakazakii* cell suspension**

Equal volumes (1 mL) of each *E. sakazakii* strain were combined to form a cocktail culture. The mixed culture was centrifuged (3000 × g, 20 min). The supernatant was discarded and the pellet was resuspended in 1 mL of 0.1% peptone water (Becton Dickinson, Sparks, Md., U.S.A.) to a concentration of approximately 10^{10} CFU/mL.

**Preparation of stressed *E. sakazakii* cell suspension**

Stress conditions (acid, alkaline, chlorine, or ethanol stresses) used in the present study were determined based on preliminary experiments and published studies. In preliminary studies (not shown), *E. sakazakii* cell suspensions were exposed to the previous stress conditions for different time intervals. The number of survivors was determined by plating samples on tryptic soy agar (TSA) (Oxoid) before and after treatment. Treatment conditions that reduced the numbers of cells by approximately ≤ 1 log were selected and used in the present study.

**Acid stress.** Acid-stressed cultures were prepared as described by Gurtler and Beuchat (2005) with minor modifications. One milliliter of each freshly prepared *E. sakazakii* cell suspension was added to 9 mL of potassium phosphate buffer adjusted to pH 3.5 with 85% lactic acid (Sigma, St. Louis, Mo., U.S.A.) and held at 21 °C for 30 min. Afterwards, the pH was adjusted to 6.4 by adding the treated suspension to 30 mL of potassium phosphate buffer.

**Alkaline stress.** Alkaline-stressed cultures were prepared as described by Gurtler and Beuchat (2005) with minor modifications. One milliliter of each freshly prepared *E. sakazakii* cell suspension was added to 2 mL of potassium phosphate buffer previously adjusted to pH 11.2 with sodium hydroxide (2M) (Fluka, Buchs, Switzerland) and held at 21 °C for 5 min. Subsequently, the pH was adjusted to 6.9 by adding the treated suspension to 8 mL of potassium phosphate buffer.

**Chlorine stress.** Chlorine-stressed cells were prepared as described by Taormina and Beuchat (2001) with minor modifications. Sodium hypochlorite (NaOCl) solution (5% available chlorine) (ACROS, Geel, Belgium) was used to prepare specific concentration of free available chlorine by dilution with potassium phosphate buffer. One milliliter of each freshly prepared *E. sakazakii* cell suspension was added to 9 mL of potassium phosphate buffer containing approximately 6 ppm active chlorine and held for 10 min. Subsequently, the solution was neutralized by adding the treated suspension to 30 mL of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (0.01 N) (s.d. fine-CHEM Ltd., Mumbai, India).

**Ethanol stress.** Ethanol-stressed cultures were prepared as described by Lou and Yousef (1996) with minor modifications. One milliliter of each freshly prepared *E. sakazakii* cell suspension was added to 9 mL of potassium phosphate buffer containing 12% (v/v) ethanol (99%) and held at 21 °C for 60 min. Afterwards, the suspension was pelleted and washed twice with 10 mL potassium phosphate buffer.

**Powdered infant milk formula**

Commercial PIMF (56.6% carbohydrate, 11.4% protein, and 25.4% fat) was obtained from a local processor. No *E. sakazakii* were detected in the formula (Iversen and others 2004a).

**Thermal inactivation of stressed *E. sakazakii***

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Prior to heat treatment, unstressed and stressed cell suspensions were centrifuged, as described before, and resuspended in 2 mL peptone water (0.1%) to be used in the thermal inactivation study.

**D- and z-value determination of *E. sakazakii***

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Thermal inactivation of stressed *E. sakazakii* in reconstituted PIMF. Fifty-milliliter volumes of reconstituted PIMF were prepared according to the manufacturer’s instruction in sterile 100-mL capacity Duran bottles. The formula was preheated to 52, 54, 56, or 58 °C in a temperature-controlled shaking water bath. A calibrated thermocouple was placed in a replicate diluent bottle to monitor the temperature profile over the experimental periods. One milliliter of the unstressed, acid-, alkaline-, chlorine-, and ethanol-stressed cell suspension was mixed with 50 mL reconstituted PIMF at each temperature. At timed intervals, depending on temperature, samples (1 mL) were transferred to sterile tubes and cooled in an ice-water bath. For unstressed samples, the timed intervals were 15, 5, 2, and 0.5 min at temperatures of 52, 54, 56, and 58 °C, respectively. For acid- and ethanol-stressed samples, the timed intervals were 10, 4, 1.5, and 0.42 min at temperatures of 52, 54, 56, and 58 °C, respectively. For alkaline-stressed samples, the timed intervals were 10, 4, 1, and 0.33 min at temperatures of 52, 54, 56, and 58 °C, respectively. For chlorine-stressed samples, the timed intervals were 10, 4, 1, and 0.42 min at temperatures of 52, 54, 56, and 58 °C, respectively.

**Thermal inactivation of stressed *E. sakazakii***

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Thermal inactivation of stressed *E. sakazakii* in PIMF with hot water added. Unstressed or stressed *E. sakazakii* cell suspension was mixed with PIMF as described by Osaili and others (2007). Briefly, 100 g of commercial PIMF were spread on the bottom of a sterile 50-cm-dia stainless steel bowl and 0.5 mL of each culture were separately sprayed on the powder using a chromatography reagent sprayer at a nitrogen pressure of 2 lb/in². To ensure homogeneous distribution of *E. sakazakii* cells, the treated powder was mixed by a sterile spatula and passed through a sterile screen with 0.5-mm pores. The inoculated formulas were then stored at 25 °C in 500-mL sterile, nontransparent screw-cap bottle for 24 h.

Nine grams of inoculated PIMF were transferred to sterilized 150-mL capacity plastic baby feeding bottles and reconstituted, based on the manufacturer’s recommendation, with 60-mL sterile water at 25 °C (control), 50, 60, 70, 80, 90, or 100 °C. The bottles were gently agitated by hand for 10 min at room temperature and samples were analyzed for *E. sakazakii*.

**Bacterial enumeration**

*E. sakazakii* survivors from thermal inactivation experiments were enumerated by spread plating aliquots of the samples and their appropriate dilutions in duplicate on TSA supplemented with 0.1% sodium pyruvate. After incubation aerobically at 37 °C for 24 h, survivor cells were enumerated. Triplicate thermal inactivation trials were performed at each studied temperature.

**D- and z-value determinations**

The D-value for the microorganism at each temperature was calculated from the linear regression model for the log<sub>10</sub> of surviving bacterial cells and heating time.

The z-values (°C) were calculated as the negative inverse slope of the linear regression line for the log D-values over the range of heating temperatures tested.

**Statistical analysis**

The means of the D- and z-values and survivors of stressed *E. sakazakii* were compared with those of unstressed *E. sakazakii*.
Thermal inactivation of stressed *E. sakazakii*

at each temperature using the Student’s *t*-test at 0.05 significant level.

**Results**

**D- and z-values of stressed *E. sakazakii***

The *E. sakazakii* death kinetics were modeled using linear regression analysis. The regression curves were fitted with *R*² values (coefficient of determination) of >0.90 for all 4 temperatures. Table 1 shows the D-values of unstressed and stressed *E. sakazakii* in reconstituted PIME. The D-values of unstressed and acid-, alkaline-, chlorine- and, ethanol-stressed *E. sakazakii* at 52 to 58 °C ranged from 16.40 to 0.56, 14.57 to 0.54, 12.07 to 0.37, 10.08 to 0.40, and 11.61 to 0.50 min, respectively. The D-values of alkaline-, chlorine-, and ethanol-stressed *E. sakazakii* were significantly (*P* < 0.05) lower at all temperatures than those of unstressed cells in the range of 16% to 46%, 20% to 49%, and 11% to 29%, respectively. In addition, the D-values of acid-stressed *E. sakazakii* were significantly lower than that of unstressed cells at 52 °C and were not significantly lower at 54, 56, and 58 °C in the range of 4% to 11%.

The *z*-values of unstressed and acid-, alkaline-, chlorine-, and ethanol-stressed *E. sakazakii* were 4.12 ± 0.03, 4.24 ± 0.07, 4.16 ± 0.08, and 4.4 ± 0.13 °C, respectively. Only the *z*-value of ethanol-stressed *E. sakazakii* was significantly different than that of unstressed cells.

**Thermal inactivation of stressed *E. sakazakii* in PIMF with hot water**

Table 2 shows the survivors of unstressed and stressed *E. sakazakii* after reconstituting PIMF in baby feeding bottles with water at various temperatures. Similar to the results obtained from the thermal inactivation experiments of stressed *E. sakazakii* in reconstituted PIME detergent and sanitizer stressed sensitized *E. sakazakii* in PIMF to heat treatment. Reconstitution of PIMF with water at 60 °C decreased the level of acid-, alkaline-, chlorine-, and ethanol-stressed *E. sakazakii* by 1.7, 1.8, 1.8, and 1.9 log₁₀, respectively, compared with 1.2 log₁₀ reduction in the unstressed cells. Although the survivors of stressed *E. sakazakii* from reconstituted formula at 60 °C were lower than survivors of the unstressed cells, the reduction was significant only in ethanol-stressed cells. Increasing the temperature of water to 70 °C caused a significant reduction in stressed cells compared with the unstressed cells by approximately 1 log₁₀. There were no significant differences between the populations of stressed and unstressed *E. sakazakii* when PIMF was reconstituted with water at 80, 90, and 100 °C where the populations were < 1 log₁₀.

**Discussion**

The present work determined the effect of acid, alkaline, chlorine, and ethanol stresses on the thermotolerance of stationary phase *E. sakazakii*. Exposure of *E. sakazakii* to environmental stresses, including acid, alkaline, chlorine, and ethanol, may occur in a variety of situations that could have implications on food safety. For instance, exposure of *E. sakazakii* to these chemical stresses may occur frequently in milk-processing facilities through the use of detergents to remove milk residues from equipment and floors and through the use of sanitizers to sanitize equipment after cleaning.

Information on the thermotolerance properties of *E. sakazakii* pre-exposed to chemical detergents and sanitizers is not found in the literature. Lou and Yousef (1996) studied the thermotolerance of 1 h acid-stressed *L. monocytogenes* and reported that acid stress at pHs 4.5 and 5.0 increased the heat resistance of the microbe in phosphate buffer by up to 10-fold while at pH 4 decreased its thermal resistance in the medium.

Our results showed that sublethal exposure to alkaline stress reduced the thermal resistance of *E. sakazakii* in infant milk formula. However, Taormina and Buechat (2001) reported that alkaline-stressed *L. monocytogenes* were more heat resistant in tryptose phosphate broth than the unstressed cells. The differences between

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**Table 1** — D-values of unstressed and acid-, alkaline-, chlorine-, and ethanol-stressed *E. sakazakii* in reconstituted PIMF.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Unstressed</th>
<th>Acid stressed</th>
<th>Alkaline stressed</th>
<th>Chlorine stressed</th>
<th>Ethanol stressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>52</td>
<td>16.40 ± 0.19</td>
<td>14.57 ± 0.17</td>
<td>12.07 ± 0.85</td>
<td>10.08 ± 0.71</td>
<td>11.61 ± 0.48</td>
</tr>
<tr>
<td>54</td>
<td>5.34 ± 0.01</td>
<td>5.11 ± 0.17</td>
<td>4.47 ± 0.05</td>
<td>4.25 ± 0.22</td>
<td>4.74 ± 0.12</td>
</tr>
<tr>
<td>56</td>
<td>2.12 ± 0.14</td>
<td>2.01 ± 0.03</td>
<td>1.14 ± 0.10</td>
<td>1.06 ± 0.01</td>
<td>1.73 ± 0.06</td>
</tr>
<tr>
<td>58</td>
<td>0.56 ± 0.01</td>
<td>0.54 ± 0.03</td>
<td>0.37 ± 0.04</td>
<td>0.40 ± 0.01</td>
<td>0.50 ± 0.03</td>
</tr>
</tbody>
</table>

*Arithmetic mean of 3 replications ± standard deviation.
*The value is significantly different (*P* < 0.05) compared with that of unstressed cells at the same temperature.

**Table 2** — Survivors of unstressed and acid-, alkaline-, chlorine-, and ethanol-stressed *E. sakazakii* from reconstitution of PIMF with water at different temperatures.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Unstressed</th>
<th>Acid stressed</th>
<th>Alkaline stressed</th>
<th>Chlorine stressed</th>
<th>Ethanol stressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>7.02 ± 0.12</td>
<td>7.18 ± 0.09</td>
<td>7.21 ± 0.07</td>
<td>7.20 ± 0.06</td>
<td>7.06 ± 0.12</td>
</tr>
<tr>
<td>50</td>
<td>7.05 ± 0.04</td>
<td>7.11 ± 0.05</td>
<td>7.15 ± 0.06</td>
<td>7.11 ± 0.05</td>
<td>7.08 ± 0.05</td>
</tr>
<tr>
<td>60</td>
<td>5.79 ± 0.12</td>
<td>5.42 ± 0.64</td>
<td>5.41 ± 0.39</td>
<td>5.41 ± 0.24</td>
<td>5.13 ± 0.38</td>
</tr>
<tr>
<td>70</td>
<td>1.76 ± 0.80</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>80</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>90</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>100</td>
<td>ND</td>
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</table>

*Reconstitution of PIMF was agitated for 10 min at room temperature.
*Arithmetic mean of 3 replications ± standard deviation.
*The value is significantly different (*P* < 0.05) compared with that of unstressed cells at the same temperature.
*ND = not detectable (log₁₀ CFU/g) of *E. sakazakii* was < 1.
our results and those of Taormina and Buechta (2001) may be due to the differences in the cell wall composition of Gram-positive and Gram-negative bacteria. Mendonca and others (1994) found that Gram-positive bacteria did not leak cell constituents following exposure to pH 9.0 to 12.0 and cells retained their shape while Gram-negative cells appeared collapsed and wrinkled.

In agreement with our results, Folsom and Frank (2000) reported that chlorine treatment decreased the heat resistance of E. coli O157:H7 in buffer and apple juice. They reported that exposure of E. coli O157:H7 to chlorine (0.6 ppm) for 20 min before heat treatment decreased the D50 of the microbe by 50% (from 1.59 to 0.8 in rehydrated infant formula. J Food Prot 67:60–3.

The sensitivity of acid-, alkaline-, chlorine-, and ethanol-stressed E. sakazakii in powdered and reconstituted infant milk formula was probably due to sublethal injury. This would decrease the ability of the cells to resist the additional heat stress, resulting in lower D-values. The level of cell injury was not measured in this study; therefore, further research would be necessary to confirm this hypothesis.

During the manufacturing of PIMF, E. sakazakii may be exposed to a variety of environmental stresses, which will consequently sensitize the organism to later temperature treatments. The use of heat treatment during the preparation of reconstituted PIMF through the use of hot water (≥ 70°C) to reconstitute PIMF may be an effective means to reduce the possible risk of E. sakazakii in the infant milk formula. The use of heat should not substitute good manufacturing and hygienic practices during the manufacturing and reconstitution of PIMF.

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References