Detergent and sanitizer stresses decrease the thermal resistance of
Enterobacter sakazakii in infant milk formula

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Abstract

Infant milk formula has been identified as a potential source of *Enterobacter sakazakii*. This bacterium can cause a severe form of neonatal meningitis and necrotizing enterocolitis. This study determined the effect of acid, alkaline, chlorine and ethanol stresses on the thermal inactivation of *E. sakazakii* in infant milk formula. 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 water at temperatures between 50 and 100°C. The *D*- and *z*-values of the cells were determined using linear regression analysis. Detergent and sanitizer stresses decreased the thermal resistance of *E. sakazakii* in powdered and reconstituted infant milk formula. The *D*-values for acid, alkaline, chlorine and ethanol stressed *E. sakazakii* at 52-58°C were 14.57-0.54, 12.07-0.37, 10.08-0.40 and 11.61-0.50 min, respectively. The values of alkaline, chlorine and ethanol stressed cells were significantly lower than those of unstressed cells. Only the *z*-value (4.4°C) of ethanol stressed *E. sakazakii* was significantly different than that of unstressed cells (4.12°C). Reconstitution at 60°C did not significantly reduce the number of pre-stressed *E. sakazakii* cells compared with unstressed control cells, whereas significant decreases were obtained at 70°C. Using water at 70°C during the preparation of reconstituted PIMF before feeding infants, may be a suitable and applicable means of reducing the risk of *E. sakazakii* in the formula. The results of this study may be of use to regulatory agencies, infant milk producers and infant caregivers to design heating processes to eliminate *E. sakazakii* that may be present in infant milk formula.

Key words: *E. sakazakii*, Infant milk formula, Acid stress, Alkaline stress, Chlorine stress, Ethanol stress, Thermal inactivation
1. Introduction

*Enterobacter sakazakii* is a ubiquitous Gram-negative, facultatively anaerobic, rod, that belongs to *Enterobacteriaceae* family. *E. sakazakii* has been isolated from wide range of foods including powdered infant milk formula (PIMF) and food factory environments including 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 post-drying contamination during mixing with other ingredients, filling and packaging (FAO/WHO 2006). *E. sakazakii* can survive for at least 2.5 years in PIMF (Caubilla-Barron and Forsythe 2007a). The presence of *E. sakazakii* in PIMF has been associated with outbreaks of severe forms of neonatal meningitis, necrotizing enterocolitis, bacteraemia with a high mortality rate (Nazarowec-White and Farber 1997a; Simmons and others 1989; Lai 2001; van Acker and others 2001; Himelright and others 2002, Caubilla-Barron and others 2007b). 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 2004b).

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* was 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 56-70°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 environment, *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 pre-stressed *E. sakazakii* cells, as could occur prior to contamination of infant formula.

Osaili and others (2007b) 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 objective of the current study was to assess the effect of acid, alkaline, chlorine and ethanol stresses on the thermal inactivation (*D*- and *z*-values) of *E. sakazakii* in reconstituted PIMF. 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.
2. Materials and Methods

2.1. E. sakazakii strains

One ATCC (51329) 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 stored in brain heart infusion (BHI) (Oxoid Ltd., Basingstoke, UK) 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 three times before use.

2.2. Preparation of the unstressed E. sakazakii cells 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, Sparka, Md, USA) to a concentration of approximately $10^{10}$ CFU/ml.

2.3. 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 the 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 ca. \( \leq 1 \) log were selected and used in the present study.

2.3.1. Acid stress

Acid stressed cultures were prepared as described by Gurtler and Beuchat (2005) with minor modifications. One millilitre 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, MO, USA) 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.

2.3.2. Alkaline stress

Alkaline stressed cultures were prepared as described by Gurtler and Beuchat (2005) with minor modifications. One millilitre 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. After that the pH was adjusted to 6.9 by adding the treated suspension to 8 ml of potassium phosphate buffer.

2.3.3. 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 millilitre of each freshly prepared *E. sakazakii* cell suspension was added to 9 ml of potassium phosphate buffer containing ca. 6 ppm active chlorine and held for 10 min. After that the solution was
neutralized by adding the treated suspension to 30 ml of Na$_2$S$_2$O$_3$ (0.01 N) (s.d. fine-
CHEM LTD., Mumbai, India).

2.3.4. Ethanol stress

Ethanol stressed cultures were prepared as described by Lou and Yousef (1996) with
minor modifications. One millilitre of each freshly prepared *E. sakazakii* cell suspension
was added to 9 ml of potassium phosphate buffer containing 12% (vol/vol) ethanol (99%)
and held at 21°C for 40 min. After that, the suspension was pelleted and washed twice
with 10 ml potassium phosphate buffer.

2.4. Powdered infant milk formula

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

2.5. Thermal inactivation of stressed *E. sakazakii*

2.5.1. Thermal inactivation (D- and *z*-values) of stressed *E. sakazakii* in reconstituted
PIMF

Fifty millilitre 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 millilitre of the unstressed, acid,
alkaline, chlorine and ethanol stressed cell suspension was mixed with 50 ml
reconstituted infant formula 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 temperature of 52, 54, 56 and 58°C, respectively. For chlorine stressed samples, the timed intervals were 10, 4, 1 and 0.42 min at temperature of 52, 54, 56 and 58°C, respectively.

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

Unstressed or stressed E. sakazakii cell suspension was mixed with PIMF as described by Osaili and others (2007a). Briefly, 100 g commercial PIMF was spread on the bottom of a sterile 50 cm diameter stainless steel bowl and 0.5 ml of each culture was 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 strains, 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, non transparent 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 (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.
2.6. 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.

2.7. *D*- and *z*-value determinations

The *D*-value for the microorganism at each temperature was calculated from the linear regression model for the log₁₀ 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.

2.8. Statistical analysis

The means of the *D*- and *z*-values of stressed *E. sakazakii* were compared with unstressed *E. sakazakii* in relevant products using the student’s t-test at 0.05 significant level.

3. Results

3.1. *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 four temperatures. Table 1 shows the survivor curves of unstressed and acid, alkaline,
chlorine and ethanol stressed *E. sakazakii* at 52 to 58°C in reconstituted PIMF. The *D*-values of unstressed and acid, alkaline, chlorine and ethanol stressed *E. sakazakii* at 52-58°C ranged from 16.40-0.56, 14.57-0.54, 12.07-0.37, 10.08-0.40 and 11.61-0.50 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 16-46%, 16-49% and 11-39%, respectively. In addition, the *D*-values of acid stressed *E. sakazakii* were significantly lower than that of unstressed cells at 52°C and not significantly lower at 54, 56 and 58°C in the range of 4-11%.

The *z*-values of unstressed and acid, alkaline, chlorine and ethanol stressed *E. sakazakii* were 4.12±0.03, 4.24±0.07, 3.9±0.18, 4.16±0.08, 4.4±0.13°C, respectively. Only the *z*-value of ethanol stressed *E. sakazakii* was significantly different than that of unstressed cells.

3.2 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 PIMF, detergent and sanitizer stresses 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 log_{10}, respectively, compared with 1.2 log_{10} reduction in the unstressed cells. Although the survivors of stressed *E. sakazakii* from reconstituted formula at 60°C were lower than survivor of the unstressed cells, the reduction was only significant 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 thermotolerance of pre-stressed *E. sakazakii*. Two scenarios were studied. Firstly, the D- and z-values of cells pre-stressed due to exposure to detergents, etc. was calculated. Secondly, the recovery of cells from the desiccated condition following reconstitution at different temperatures. Exposure of *E. sakazakii* to environmental stresses, including acid, alkaline, chlorine and ethanol, may occur in a variety of situations 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 literature. Lou and Yousef (1996) studied the thermotolerance of 1 hour acid stressed *Listeria monocytogenes* and reported that acid stress at pH 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. In agreement with our results, Folsom and Frank (2000) reported that chlorine treatment decreased the heat resistance of *Escherichia 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 $D_{50}$ of the microbe by 50% (from 1.59 to 0.8 min) and 70% (from 5.45 to 1.65 min) in apple juice and phosphate buffer, respectively. Our results agree with Lou and Yousef (1996) who reported that ethanol stress, at same concentration level used in the current study, decreased the $D_{50}$ of *L. monocytogenes*, but at 2-8% the thermal resistance increased. The high level of ethanol in culture media may cause a structural damage to the cells. *Staphylococcus aureus* exposed to 5 to 6.5% ethanol showed plasmolysis, cell wall rupture, losses in the cell wall, septum widening, and frequent mesosome formation (Ballesteros and others, 1992).

Our results showed that sub-lethal exposure to alkaline stress reduced the thermal resistance of *E. sakazakii* in infant milk formula. However, Taormina and Buechat (2001) reported that alkaline stressed *Listeria monocytogenes* were more heat resistant in tryptose phosphate broth than the unstressed cells. The differences in our results and the results of Taormina and Beuchat (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 9.0-12.0 and cells retained their shape while Gram-negative cells appeared collapsed and wrinkled.

The effect of desiccation, starvation, heat and cold stresses on the thermal inactivation of *E. sakazakii* in infant milk formula has been studied. Osaili and others (2007b) reported that desiccation and heat stresses caused a significant reduction in $D$-values of a cocktail of *E. sakazakii* strains at 52-58°C in reconstituted PIMF.

Osaili and other (2007b) reported that there were no significant differences between the $z$-values of unstressed and desiccated, starved, heat or cold stressed *E. sakazakii* in
reconstituted infant milk formula. The calculated z-values for alkaline and ethanol stressed *E. sakazakii* are generally lower and higher, respectively, than those observed by Osaili and others (2007b) for desiccated (4.20°C), starved (4.23°C), heat shocked (4.22°C) and cold shocked (4.12°C) *E. sakazakii*. Higher z-values mean more temperature is required to achieve 1 decimal reduction in the D-values.

Osaili and others (2007c) studied the thermal inactivation of desiccated *E. sakazakii* strains in PIMF reconstituted with water pre-equilibrated to 60-100°C and obtained similar results to those in the current study. WHO/FAO (2007) has recommended reconstitution PIMF with water at 70°C to reduce the potential risk of *E. sakazakii* in the formula.

The sensitivity of acid, alkaline, chlorine and ethanol stressed *E. sakazakii* in powdered and reconstituted infant milk formula is probably due to sub-lethal 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.

**Conclusion**

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 infant milk formula 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 manufacturing and reconstitution PFMF.

Acknowledgment

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Reference


Table 1. *D*-values of acid, alkaline, chlorine and ethanol stressed *E. sakazakii* in reconstituted infant milk formula

<table>
<thead>
<tr>
<th>mpTemperature(°C)</th>
<th>Control</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±0.46</td>
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<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>1.08±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>

*D*-values (min)

*Arithmetic mean of three 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 acid, alkaline, chlorine and ethanol stressed \emph{E. sakazakii} from reconstitution of PIMF with water at different temperatures.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Treatment</th>
<th>Control</th>
<th>Acid stressed</th>
<th>Alkaline stressed</th>
<th>Chlorine stressed</th>
<th>Ethanol stressed</th>
</tr>
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<tr>
<td>25</td>
<td></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>
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<tr>
<td>50</td>
<td></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>
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<tr>
<td>60</td>
<td></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></td>
<td>1.76±0.80</td>
<td>ND*</td>
<td>ND*</td>
<td>ND*</td>
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<tr>
<td>80</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Reconstitution of PIMF was agitated for 10 min at room temperature.

† Arithmetic mean of three replications ± standard deviation.

§ The value is significantly different (\( P < 0.05 \)) compared with that of unstressed cells at the same temperature.

§ ND: None detectable (log\(_{10}\) CFU/g) of \emph{E. sakazakii} was < 1