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 entercolitis. 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)

78 indicated that there was about 20-fold divergence in thermal resistance between 12 strains
79 of *E. sakazakii* in reconstituted PIMF at 56-70°C.
80 Although the thermotolerance of microorganisms is affected by their physiological states
81 (Lou and Yousef 1996; Doyle and others 2001; Wesche and others 2005), all published
82 thermal inactivation studies of *E. sakazakii* in infant milk formula have used unstressed
83 cells, grown under optimal laboratory conditions (Nazarowec-White and Farber 1997b;
84 Breeuwer and others 2003; Edelson-Mammel and Buchanan 2004; Iversen and others
85 2004b). However, in infant formula processing environment, *E. sakazakii* may be
86 exposed to chemical stresses from the use of detergents and sanitizers in cleaning and
87 sanitizing equipment, pipes and floors. Therefore, it is appropriate to study the
88 thermotolerance properties of the pre-stressed *E. sakazakii* cells, as could occur prior to
89 contamination of infant formula.
90 Osaili and others (2007b) have already shown that desiccation and heat stresses caused
91 significant reduction in *D*-values of the same strains of *E. sakazakii* as used in the present
92 study.
93 To our knowledge, no information is available in the literature on the effect of detergent
94 and sanitizer stresses on the thermal resistance of *E. sakazakii* in infant milk formula.
95 Therefore, the objective of the current study was to assess the effect of acid, alkaline,
96 chlorine and ethanol stresses on the thermal inactivation (*D*- and *z*-values) of *E. sakazakii*
97 in reconstituted PIMF. Such information will be of interest to regulatory agencies, infant
98 formula producers and infant caregivers to design heating processes that are sufficient to
99 kill *E. sakazakii* that may be present in infant milk formula.
100

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

125 treatment. Treatment conditions that reduced the numbers of cells by ca. ≤ 1 log were
126 selected and used in the present study

127 *2.3.1. Acid stress*

128 Acid stressed cultures were prepared as described by Gurtler and Beuchat (2005) with
129 minor modifications. One millilitre of each freshly prepared *E. sakazakii* cell suspension
130 was added to 9 ml of potassium phosphate buffer adjusted to pH 3.5 with 85% lactic acid
131 (Sigma, MO, USA) and held at 21°C for 30 min. Afterwards, the pH was adjusted to 6.4
132 by adding the treated suspension to 30 ml of potassium phosphate buffer.

133 *2.3.2. Alkaline stress*

134 Alkaline stressed cultures were prepared as described by Gurtler and Beuchat (2005) with
135 minor modifications. One millilitre of each freshly prepared *E. sakazakii* cell suspension
136 was added to 2 ml of potassium phosphate buffer previously adjusted to pH 11.2 with
137 sodium hydroxide (2M) (Fluka, Buchs, Switzerland) and held at 21°C for 5 min. After
138 that the pH was adjusted to 6.9 by adding the treated suspension to 8 ml of potassium
139 phosphate buffer.

140 *2.3.3. Chlorine stress*

141 Chlorine stressed cells were prepared as described by Taormina and Beuchat (2001) with
142 minor modifications. Sodium hypochlorite (NaOCl) solution (5% available chlorine)
143 (ACROS, Geel, Belgium) was used to prepare specific concentration of free available
144 chlorine by dilution with potassium phosphate buffer. One millilitre of each freshly
145 prepared *E. sakazakii* cell suspension was added to 9 ml of potassium phosphate buffer
146 containing ca. 6 ppm active chlorine and held for 10 min. After that the solution was

147 neutralized by adding the treated suspension to 30 ml of $Na_2S_2O_3$ (0.01 N) (s.d. fine-
148 CHEM LTd., Mumbai, India).

149 *2.3.4. Ethanol stress*

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

155

156 ## 2.4. Powdered infant milk formula

157 Commercial PIMF (56.6% carbohydrate, 11.4% protein, and 25.4% fat) was obtained
158 from local processor. No *E. sakazakii* were detected in the formula (Iversen and others
159 2004a).

160

161 ## 2.5. Thermal inactivation of stressed *E. sakazakii*

162 *2.5.1. Thermal inactivation (D- and z-values) of stressed E. sakazakii in reconstituted*
163 *PIMF*
164

165 Fifty millilitre volumes of reconstituted PIMF were prepared according to the
166 manufacturer's instruction in sterile 100-ml capacity Duran bottles. The formula was
167 preheated to 52, 54, 56 or 58°C in a temperature-controlled shaking water bath. A
168 calibrated thermocouple was placed in a replicate diluent bottle to monitor the
169 temperature profile over the experimental periods. One millilitre of the unstressed, acid,
170 alkaline, chlorine and ethanol stressed cell suspension was mixed with 50 ml
171 reconstituted infant formula at each temperature. At timed intervals, depending on

172 temperature, samples (1 ml) were transferred to sterile tubes and cooled in an ice-water
173 bath. For unstressed samples, the timed intervals were 15, 5, 2 and 0.5 min at
174 temperatures of 52, 54, 56 and 58°C, respectively. For acid and ethanol stressed samples,
175 the timed intervals were 10, 4, 1.5 and 0.42 min at temperatures of 52, 54, 56 and 58°C,
176 respectively. For alkaline stressed samples, the timed intervals were 10, 4, 1 and 0.33 min
177 at temperature of 52, 54, 56 and 58°C, respectively. For chlorine stressed samples, the
178 timed intervals were 10, 4, 1 and 0.42 min at temperature of 52, 54, 56 and 58°C,
179 respectively.
180
181 *2.5.2. Thermal inactivation of stressed E. sakazakii in PIMF with hot water*
182 Unstressed or stressed *E. sakazakii* cell suspension was mixed with PIMF as described by
183 Osaili and others (2007a). Briefly, 100 g commercial PIMF was spread on the bottom of a
184 sterile 50 cm diameter stainless steel bowl and 0.5 ml of each culture was separately
185 sprayed on the powder using a chromatography reagent sprayer at a nitrogen pressure of
186 2 lb/in^2. To ensure homogeneous distribution of *E. sakazakii* strains, the treated powder
187 was mixed by a sterile spatula and passed through a sterile screen with 0.5 mm pores.
188 The inoculated formulas were then stored at 25 °C in 500-ml sterile, non transparent
189 screw-cap bottle for 24 h.
190 Nine grams of inoculated PIMF were transferred to sterilized 150-ml capacity plastic
191 baby feeding bottles and reconstituted, based on the manufacturer's recommendation,
192 with 60 ml sterile water at 25 (control), 50, 60, 70, 80, 90 or 100°C. The bottles were
193 gently agitated by hand for 10 min at room temperature and samples were analyzed for *E.*
194 *sakazakii*.

195

196 2.6. Bacterial enumeration

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

202

203 2.7. *D*- and *z*-value determinations

204 The *D*-value for the microorganism at each temperature was calculated from the linear
205 regression model for the $\log_{10}$ of surviving bacterial cells and heating time.
206 The *z*-values (°C) were calculated as the negative inverse slope of the linear regression
207 line for the log *D*-values over the range of heating temperatures tested.

208

209 2.8. Statistical analysis

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

212

213 3. Results

214 *3.1. D- and z-values of stressed E. sakazakii*

215 The *E. sakazakii* death kinetics were modeled using linear regression analysis. The
216 regression curves were fitted with R^2 values (coefficient of determination) of > 0.90 for
217 all four temperatures. Table 1 shows the survivor curves of unstressed and acid, alkaline,

218 chlorine and ethanol stressed *E. sakazakii* at 52 to 58°C in reconstituted PIMF. The *D*-
219 values of unstressed and acid, alkaline, chlorine and ethanol stressed *E. sakazakii* at 52-
220 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,
221 respectively. The *D*-values of alkaline, chlorine and ethanol stressed *E. sakazakii* were
222 significantly ($P < 0.05$) lower at all temperatures than those of unstressed cells in the
223 range of 16-46%, 16-49% and 11-39%, respectively. In addition, the *D*-values of acid
224 stressed *E. sakazakii* were significantly lower than that of unstressed cells at 52°C and
225 not significantly lower at 54, 56 and 58°C in the range of 4-11%.
226 The z-values of unstressed and acid, alkaline, chlorine and ethanol stressed *E. sakazakii*
227 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*-
228 value of ethanol stressed *E. sakazakii* was significantly different than that of unstressed
229 cells.
230
231 *3.2 Thermal inactivation of stressed E. sakazakii in PIMF with hot water*
232 Table 2 shows the survivors of unstressed and stressed *E. sakazakii* after reconstituting
233 PIMF in baby feeding bottles with water at various temperatures. Similar to the results
234 obtained from the thermal inactivation experiments of stressed *E. sakazakii* in
235 reconstituted PIMF, detergent and sanitizer stresses sensitized *E. sakazakii* in PIMF to
236 heat treatment. Reconstitution of PIMF with water at 60°C decreased the level of acid,
237 alkaline, chlorine and ethanol stressed *E. sakazakii* by 1.7, 1.8, 1.8 and 1.9 $\log_{10}$,
238 respectively, compared with 1.2 $\log_{10}$ reduction in the unstressed cells. Although the
239 survivors of stressed *E. sakazakii* from reconstituted formula at 60°C were lower than
240 survivor of the unstressed cells, the reduction was only significant in ethanol stressed

241 cells. Increasing the temperature of water to 70°C caused a significant reduction in
242 stressed cells compared with the unstressed cells by approximately 1 $\log_{10}$. There were
243 no significant differences between the populations of stressed and unstressed *E. sakazakii*
244 when PIMF was reconstituted with water at 80, 90 and 100°C where the populations were
245 $< 1 \log_{10}$.

246

247 ## Discussion

248

249 The present work determined the thermotolerance of pre-stressed *E. sakazakii*. Two
250 scenarios were studied. Firstly, the D- and z-values of cells pre-stressed due to exposure
251 to detergents. etc. was calculated. Secondly, the recovery of cells from the desiccated
252 condition following reconstitution at different temperatures. Exposure of *E. sakazakii* to
253 environmental stresses, including acid, alkaline, chlorine and ethanol, may occur in a
254 variety of situations could have implications on food safety. For instance, exposure of *E.*
255 *sakazakii* to these chemical stresses may occur frequently in milk-processing facilities
256 through the use of detergents to remove milk residues from equipment and floors and
257 through the use of sanitizers to sanitize equipment after cleaning.

258 Information on the thermotolerance properties of *E. sakazakii* pre-exposed to chemical
259 detergents and sanitizers is not found in literature. Lou and Yousef (1996) studied the
260 thermotolerance of 1 hour acid stressed *Listeria monocytogenes* and reported that acid
261 stress at pH 4.5 and 5.0 increased the heat resistance of the microbe in phosphate buffer
262 by up to l0-fold while at pH 4 decreased its thermal resistance in the medium. In
263 agreement with our results, Folsom and Frank (2000) reported that chlorine treatment
264 decreased the heat resistance of *Escherichia coli* O157:H7 in buffer and apple juice. They
265 reported that exposure of *E. coli* O157:H7 to chlorine (0.6 ppm) for 20 min before heat

266 treatment decreased the D_{58} of the microbe by 50% (from 1.59 to 0.8 min) and 70% (from
267 5.45 to 1.65 min) in apple juice and phosphate buffer, respectively. Our results agree with
268 Lou and Yousef (1996) who reported that ethanol stress, at same concentration level used
269 in the current study, decreased the D_{56} of *L. monocytogenes*, but at 2-8% the thermal
270 resistance increased. The high level of ethanol in culture media may cause a structural
271 damage to the cells. *Staphylococcus aureus* exposed to 5 to 6.5% ethanol showed
272 plasmolysis, cell wall rupture, losses in the cell wall, septum widening, and frequent
273 mesosome formation (Ballesteros and others, 1992).
274 Our results showed that sub-lethal exposure to alkaline stress reduced the thermal
275 resistance of *E. sakazakii* in infant milk formula. However, Taormina and Buechat (2001)
276 reported that alkaline stressed *Listeria monocytogenes* were more heat resistant in
277 tryptose phosphate broth than the unstressed cells. The differences in our results and the
278 results of Taormina and Beuchat (2001) may be due to the differences in the cell wall
279 composition of Gram positive and Gram negative bacteria. Mendonca and others (1994)
280 found that Gram positive bacteria did not leak cell constituents following exposure to pH
281 9.0-12.0 and cells retained their shape while Gram-negative cells appeared collapsed and
282 wrinkled.
283 The effect of desiccation, starvation, heat and cold stresses on the thermal inactivation of
284 *E. sakazakii* in infant milk formula has been studied. Osaili and others (2007b) reported
285 that desiccation and heat stresses caused a significant reduction in D-values of a cocktail
286 of *E. sakazakii* strains at 52-58°C in reconstituted PIMF.
287 Osaili and other (2007b) reported that there were no significant differences between the z-
288 values of unstressed and desiccated, starved, heat or cold stressed *E. sakazakii* in

289 reconstituted infant milk formula. The calculated z-values for alkaline and ethanol
290 stressed *E. sakazakii* are generally lower and higher, respectively, than those observed by
291 Osaili and others (2007b) for desiccated (4.20°C), starved (4.23°C), heat shocked
292 (4.22°C) and cold shocked (4.12°C) *E. sakazakii*. Higher *z*-values mean more
293 temperature is required to achieve 1 decimal reduction in the *D*-values.
294 Osaili and others (2007c) studied the thermal inactivation of desiccated *E. sakazakii*
295 strains in PIMF reconstituted with water pre-equilibrated to 60-100°C and obtained
296 similar results to those in the current study. WHO/FAO (2007) has recommended
297 reconstitution PIMF with water at 70°C to reduce the potential risk of *E. sakazakii* in the
298 formula.
299 The sensitivity of acid, alkaline, chlorine and ethanol stressed *E. sakazakii* in powdered
300 and reconstituted infant milk formula is probably due to sub-lethal injury. This would
301 decrease the ability of the cells to resist the additional heat stress, resulting in lower *D*-
302 values. The level of cell injury was not measured in this study; therefore, further research
303 would be necessary to confirm this hypothesis.
304

305 ## Conclusion

306 During the manufacturing of PIMF, *E. sakazakii* may be exposed to a variety of
307 environmental stresses which will consequently sensitize the organism to later
308 temperature treatments. The use of heat treatment during the preparation of reconstituted
309 infant milk formula through the use of hot water (≥ 70°C) to reconstitute PIMF may be an
310 effective means to reduce the possible risk of *E. sakazakii* in the infant milk formula.

311 The use of heat should not substitute good manufacturing and hygienic practices during
312 manufacturing and reconstitution PIMF.

313

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317

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

mpTeerature(°C)	***D*-values (min)** †				
	Treatment				
	Control	Acid stressed	Alkaline stressed	Chlorine stressed	Ethanol stressed
52	16.40±0.19	14.57±0.17*	12.07±0.85*	10.08±0.71*	11.61±0.46*
54	5.34±0.01	5.11±0.17	4.47±0.05*	4.25±0.22*	4.74±0.12*
56	2.12±0.14	2.01±0.03	1.14±0.10*	1.08±0.01*	1.73±0.06*
58	0.56±0.01	0.54±0.03	0.37±0.04*	0.40±0.01*	0.50±0.03*

† 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 *E. sakazakii* from reconstitution of PIMF with water at different temperatures [+]

Temperature (°C)	Survivors of *E. sakazakii* (log_{10} cfu/g) [†]				
	Treatment				
	Control	Acid stressed	Alkaline stressed	Chlorine stressed	Ethanol stressed
25	7.02±0.12	7.18±0.09	7.21±0.07	7.20±0.06	7.06±0.12
50	7.05±0.04	7.11±0.05	7.15±0.06	7.11±0.05	7.08±0.05
60	5.79±0.12	5.42±0.64	5.41±0.39	5.41±0.24	5.13±0.38[*]
70	1.76±0.80	ND[*]	ND[*]	ND[*]	ND[*]
80	ND[§]	ND	ND	ND	ND
90	ND	ND	ND	ND	ND
100	ND	ND	ND	ND	ND

[†] 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 *E. sakazakii* was < 1