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Thermal Resistance and Inactivation of *Enterobacter sakazakii* Isolates during Rehydration of Powdered Infant Formula

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Abstract *Enterobacter sakazakii* may be related to outbreaks of meningitis, septicemia, and necrotizing enterocolitis, mainly in neonates. To reduce the risk of *E. sakazakii* in baby foods, thermal characteristics for Korean *E. sakazakii* isolates were determined at 52, 56, and 60°C in saline solution, rehydrated powdered infant formula, and dried baby food. In saline solution, their *D*-values were 12–16, 3–5, and 0.9–1 min for each temperature. *D*-values increased to 16–20, 4–5, and 2–4 min in rehydrated infant formula and 14–17, 5–6, and 2–3 min in dried baby food. The overall calculated *z*-value was 6–8 for saline, 8–10 for powdered infant formula, and 9–11 for dried baby food. Thermal inactivation of *E. sakazakii* during rehydration of powdered infant formula was investigated by viable counts. Inactivation of cultured *E. sakazakii* in infant formula milk did not occur for 20 min at room temperature after rehydration with the water at 50°C and their counts were reduced by about 1–2 log CFU/g at 60°C and 4–6 log CFU/ml with the water at 65 and 70°C. However, the thermostability of adapted *E. sakazakii* to the powdered infant formula increased more than two times. Considering that the levels of *E. sakzakii* observed in powdered infant formula have generally been 1 CFU/100 g of dry formula or less, contamination with *E. sakazakii* can be reduced or eliminated by rehydrating water with at least 10°C higher temperature than the manufacturer-recommended 50°C.

Key words: *Enterobacter sakazakii*, powdered infant formula, *D*-value, thermal inactivation, rehydration

Enterobacter sakazakii is a member of the family Enterobacteriaceae, genus *Enterobacter*, which has been recognized as one of the newly emerging pathogens [24]. *E. sakazakii* is a motile, peritrichous, and Gram-negative rod. It was first known as a yellow pigmented *Enterobacter cloacae*, but was newly classified from *E. cloacae* in 1980 by Farmer *et al.* [5] based on DNA-DNA hybridization, biochemical reactions, and its yellow pigment production. *E. sakazakii* was known to cause neonatal infections such as necrotizing enterocolitis, bacteremia, and bacterial meningitis [1, 12, 19, 23]. Although the vehicle for *E. sakazakii* has not been identified in all cases, powdered infant formula has been epidemiologically identified as the source of *E. sakazakii* in at least three outbreaks of neonatal meningitis and one outbreak of necrotizing enterocolitis [2, 6, 15]. Nazarowec-White and Farmer [16] reported that the prevalence of *E. sakazakii* in powdered infant formula available in Canadian retail markets varied between 0 and 12% among the five manufacturers examined. In addition, another study reported that *E. sakazakii* and other Enterobacteriaceae were detected and their levels determined in 20 of 141 infant formula foods obtained from 36 countries [14]. New regulations to reduce the risk of *E. sakazakii* in the EU urges for no dectection of *E. sakazakii* among 30 samples of every lot from the last year. Possible control measures in reducing the risk from *E. sakazakii* might be the low intrinsic contamination of powdered infant formula, reduction of contamination during reconstituting the formula for preparation, heating at the rehydration stage prior to use by the consumer, and its growth minimization after rehydration.

Thermal treatment of foods just prior to consumption has long been used as a primary means of reducing the risks associated with foodborne pathogens. The infective dose for *E. sakazakii* was estimated at 10^3–10^5 cells [20]. The effective use of thermal treatment requires accurate information on the heat resistance of the target microorganism. Nazarowec-White and Farmer [17] used a submerged vessel method to evaluate the thermal resistance of a pooled isolate of *E. sakazakii* in reconstituted powdered infant formula. Edelson-Mammel and Buchanan [4] determined thermal resistance with the use of a submerged coil apparatus and evaluated the effect of rehydrating powdered infant

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formula with water at different temperatures in the survival of the microorganism. Nazarowec-White and Farmer [17] also reported that *E. sakazakii* was more thermotolerant than most other Enterobacteriaceae, which might contribute to its survival of heat treatments and subsequent presence in desiccated products. The lowest temperature of growth was 5.5°C, and therefore, potentially, the organism could grow during refrigerated storage. However, the rates of growth of the organism at ambient and subambient temperatures are required to predict possible ingested doses after the infant feed has been rehydrated and stored [8]. So far, there have been no reports on the thermal stability of the pathogen for Korean isolates. In this study, the heat resistance and thermal inactivation of Korean *E. sakazakii* isolates through heat, during rehydration of powdered infant formula as a breast milk alternative and dried baby food at the weaning stage, were evaluated.

The strains used for this study were *E. sakazakii* NCTC 11467, and two strains of *E. sakazakii*, KWBC 10309 and KWBC 10102, were isolated from powdered infant formula and vegetable [9]. The cultures were incubated using brain heart infusion broth (Difco Laboratory, Detroit MI, U.S.A.) at 37°C for 24 h from the stock culture at −70°C, and subcultured consecutively three times for the experiment.

For the determination of *D*-value at each heating menstrum of saline, powdered infant formula, and dried baby food, cell suspensions were used. Two ml of incubated BHI broth at 37°C for 18 h was centrifuged at 8,000 rpm for 10 min and then resuspended in 1 ml of 0.1% buffered peptone water. The initial level of *E. sakazakii* was approximately 10^6 CFU/ml. The glass centrifuge tubes containing 19 ml of saline solution, rehydrated infant formula, and baby food with a sterile magnetic stir bar were placed into a water bath. Prior to inoculation, the suspending medium was preheated to the test temperatures of 52, 56, and 60°C. At various time intervals, 1 ml samples were withdrawn and placed on an ice bath before spreading on the tryptone soya agar (TSA, Oxoid, Hampshire, England) plates. After inoculating, the plates were incubated for 18 h at 37°C and the colonies were counted. From the data, standard regression analysis was performed by log-linear models in Excel (Microsoft, Washington DC, U.S.A.) and the *D*-value was determined by taking the negative reciprocal of the slope. The *z*-value was found using a linear regression of log *D*-values of three temperatures: 52, 56, and 60°C.

To investigate the survival of *E. sakazakii* in the feeding bottles after rehydration with the waters at 50, 60, 65, and 70°C, 1 ml of the concentrated culture of *E. sakazakii* was added to 99 ml of rehydrated powdered infant formulas at each temperature in the baby bottle and counted on the tryptic soy agar plate [13, 21]. The concentrated culture was prepared by centrifuging a 5 ml overnight culture at 8,000 rpm for 10 min and resuspending the pellet in 1 ml of 0.1% buffered peptone water. The initial level of *E. sakazakii* was approximately 10^6 CFU/ml.

For heat inactivation after adaptation to the powdered infant formula, a 0.1-ml concentrated culture as an inoculum was added to 13 g of formula and dry mixed for an additional 15 min by hand. The inoculated formula was then stored for 1 week at room temperature and rehydrated in the baby feeding bottle by addition of 100 ml water with different temperatures. The bottles were then gently agitated by hand at room temperature and measured continuously every 2 min for 20 min. Viable counts were also determined on TSA after 18 h at 37°C.

In this study, *D*-values were calculated at three different temperatures in the suspending solutions of saline, powdered infant formula, and dried baby food (Table 1). In saline solution, *D*-values of 15, 4, and 1 min were obtained for 52, 56, and 60°C, respectively. *D*-values increased to 19, 4, and 3 min in rehydrated powdered infant formula and 16, 6, and 2 min in dried baby food. From the data, it appears that the thermal resistance of *E. sakazakii* increased in rehydrated powdered infant formula and dried baby food because the amount of fat each contained, 3.51 g per 100 ml in powdered infant formula and 2.37 g per 100 ml in dried baby food. It has been known that many factors can influence the heat resistance of bacteria. Some

Table 1. *D*-values (in minute) of Korean *E. sakazakii* isolates at each suspending medium.

Strains	Suspending medium	*D*-values (min) at		
		52 (°C)	56 (°C)	60 (°C)
E. sakazakii NCTC 11467	Saline	12.02	3.42	1.24
	Infant formula	16.43	4.67	2.78
	Baby food	14.18	5.17	2.76
E. sakazakii KWBC 10309	Saline	16.18	4.67	0.92
	Infant formula	19.92	3.91	2.08
	Baby food	17.48	6.32	2.38
E. sakazakii KWBC 10102	Saline	16.08	3.36	0.87
	Infant formula	20.08	4.02	2.43
	Baby food	17.18	5.32	2.09

Table 2. Calculated *z*-value (°C) of Korean *E. sakazakii* isolates at each suspending medium.

Strains	*z*-value (°C)		
	Saline	Powdered infant formula	Dried baby food
E. sakazakii NCTC 11467	8.11	10.37	11.26
E. sakazakii KWBC 10309	6.40	8.15	9.24
E. sakazakii KWBC 10102	6.32	8.73	9.62

of these include the physiological state of the organism, growth temperature of the inoculum [11], the heating menstrum including fat concentration, amount of solids, and sugar concentrations, as well as the methodology used for bacterial recovery; all make it difficult to compare *D*-values directly among different research reports. Nazarowec-White and Farmer [17] reported a similar *D*-value of 2.43 min for the *E. sakazakii* isolated from food at 60°C; however, others [4, 13, 15] reported different *D*-values like 2.57 min and 4.41 min. Iversen *et al.* [7] also reported a lower *D*-value of 1 min than that in this study. Roughly, Korean isolates showed a higher *D*-values in comparison with these reports. Table 2 shows the *z*-value calculated in our study for *E. sakazakii*. The *z*-value of 8–10°C for *E. sakazakii* in rehydrated powdered infant formula was greater than the other reported *z*-values 5.82°C [14], 5.6°C [15], and 5.8°C [16]. Nazarowec-White and Farmer [17] also reported *z*-values of 5.6 and 6.0°C for pooled food and pooled clinical isolates. The *z*-value of the most heat-resistant strain was 6.0°C; however, in our study, *z*-values were higher and differed from each medium. Using the decimal reduction time for *E. sakazakii* in rehydrated powdered infant

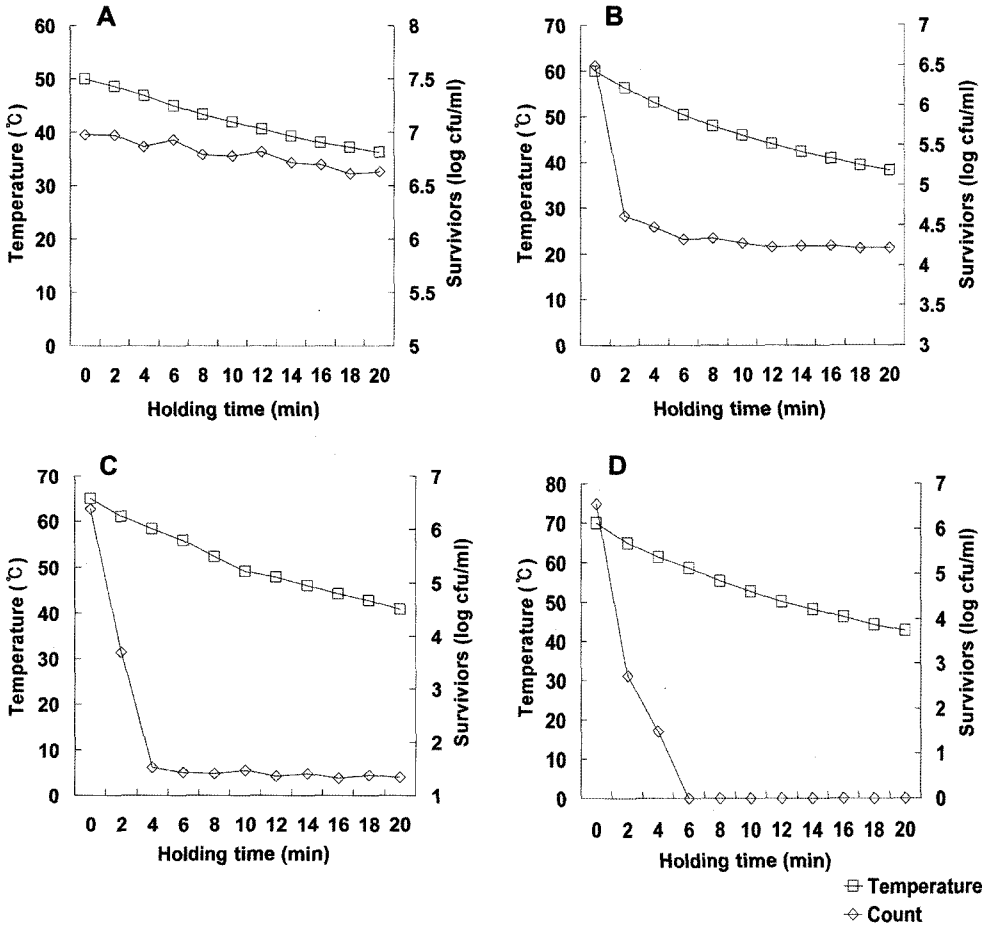

Fig. 1. Viable count of *E. sakazakii* KWBC 10102 and temperature profile of rehydrated infant formula at various hot water temperatures in the baby feeding bottles.
A. 50°C; **B.** 60°C; **C.** 65°C; **D.** 70°C.

formula at 60°C of 2.43 min and the mean *z*-value of 9.08°C, it is predictable that the decimal reduction time at 72°C is 5.7 s. Therefore, the standard high temperature short treatment (HTST) pasteurization process of 15 s at 72°C will result in about a 3-log reduction for the viable count of *E. sakazakii*. Standard pasteurization practice has also been reported to be effective for the destruction of *E. sakazakii* [18]. However, those baby foods are not sterile products.

The viable count profiles of three *E. sakazakii* strains in feeding bottles of infant formula milk were determined during rehydration with the waters of different temperatures at ambient temperature. When rehydrating with the water of 50°C, the counts remained unchanged for 20 min. With the water of 60°C, the counts decreased at a rate approximately 1–2 log CFU/ml during 20 min and 4–6 log CFU/ml decreased at 65 and 70°C. Such thermal inactivations were almost the same for those three strains. Edelson-Mammel and Buchanan [4] reported that no inactivation occurred with 50°C water, 1-D of inactivation was observed with 60°C, and 4-D of more inactivation was observed with the water temperatures ≥70°C. Kindle *et al.* [10] reported that over 4 log CFU/ml reduction was inactivated at 82–93°C for 85–93 sec. When powdered infant formula was rehydrated for infant feeding with the water of 50°C as recommended by the manufacturers, inactivation of *E. sakazakii* did not occur for 20 min. Another study suggested that preparing the formula with the water at 70°C [4] might help to reduce the contaminated *E. sakazaii*. When *E. sakazakii* adapted in the dry condition of powdered infant formula, however, the inactivation was so different that the adapted *E. sakazakii* showed higher thermostability (Fig. 2). It is probably due to biofilm formation of *E. sakazakii* adapted to the dry environment [8].

To reduce the risk of *E. sakazakii* contamination from powdered infant formula, it is recommended that the water at the appropriate temperature for rehydration be used. *E. sakazakii* in powdered infant formula was easily inactivated by pasteurization; however, powdered infant formula is a non-pasteurization product [17]. The levels of *E. sakazakii* observed in powdered infant formula were reported to be

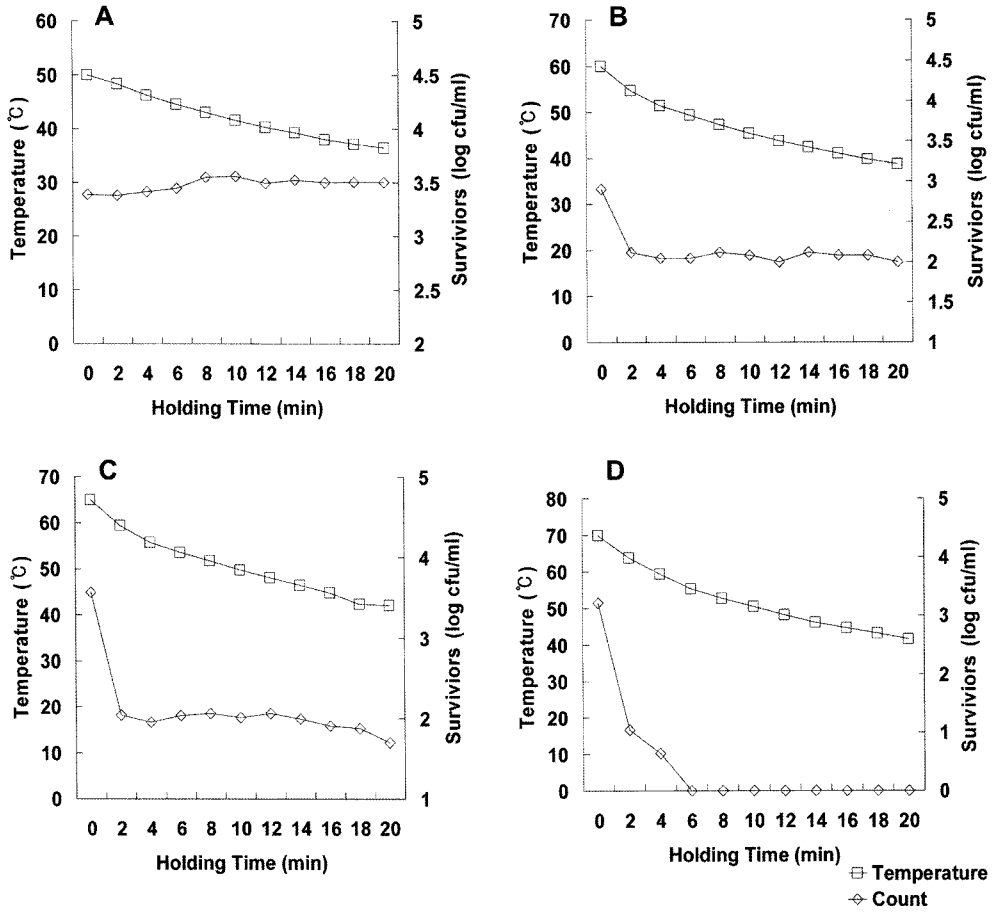

Fig. 2. Viable count of adapted *E. sakazakii* KWBC 10102 and temperature profile of rehydrated infant formula at various hot water temperatures in the baby feeding bottles.
A. 50°C; **B**. 60°C; **C**. 65°C; **D**. 70°C.

generally less than 1 CFU/100 g of dried formula [4], and the thermostability of *E. sakazakii* increased when adapted to dry condition. Therefore, in considering the thermal characteristics of *E. sakazakii*, rehydration of powdered infant formula for infant feeding with water of more than 60°C may be more helpful for the reduction of *E. sakazakii* with minimal nutrient reduction than water of 50°C as recommended by the manufacturers.

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