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Prevalence and Thermal Stability of *Enterobacter sakazakii* from Unprocessed Ready-to-Eat Agricultural Products and Powdered Infant Formulas

Mi-Kyoung Jung and Jong-Hyun Park*

Department of Food and Bioengineering, Kyungwon University, Seongnam, Kyonggi 461-701, Korea

Abstract *Enterobacter sakazakii*, designated as an unique microbial species in 1980, may cause bacteremia, necrotizing enterocolitis and infant meningitis. The distribution and the thermostability of *E. sakazakii* in unprocessed ready-to-eat (RTE) agricultural products of 252 and in 25 powdered infant formulas (PIF) were analyzed. Eighty one, 50, 43, and 47% of brown rice, pumpkin, potato, and carrot samples, respectively, had aerobic plate counts (ARC) in the range of 5 log CFU/g or more. Almost all the other products sampled had APC of approximately 2 log CFU/g. Fifty three, 75, 67, and 68% of banana, pumpkin, soybean, and carrot had Enterobacteriaceae counts approximating 3 log CFU/g. Sixty six percent of the brown rice tested had Enterobacteriaceae counts approximating 5-6 log CFU/g. *E. sakazakii* was isolated from 3/25(12%), 4/23(17%), 1/24(4%), and 1/27(4%) of PIF, brown rice, laver, and tomato samples, respectively. D-values were 3.52-4.79 min at 60 and D_{60}-values were similar as the isolates reported. Thermal inactivation of four thermovariant *E. sakazakii* strains during the rehydration of PIF with hot water were investigated. At 50°C, the levels of *E. sakazakii* decreased one log CFU/g for 4-6 min and thereafter the levels remained stable for 20 min. At 60°C, inactivation by about 2 log CFU/g occurred for 20 min. Therefore, the unprocessed agricultural products might be a source of contamination for PIF when used as an ingredient after drying and pulverization. Rehydration of PIF for infant feeding with a water temperature of 60°C rather than 50°C, as recommended by the manufacturers, may be helpful in the reduction of potential *E. sakazakii* risk.

Keywords: *E. sakazakii*, Enterobacteriaceae, prevalence, ready-to-eat agricultural products, infant formula food, D-value

Introduction

Enterobacter spp. of Enterobacteriaceae has been isolated from hospitalized patients to the point that they are recognized as the newly emerging pathogens (1). These pathogens were originally resistant to the antibiotics and in recent years developed resistance. Among the principal pathogens were *E. aerogenes, E. agglomerans, E. cloacae,* and *E. sakazakii,* which are broadly distributed throughout the environment.

E. sakazakii was initially known as 'yellow pigmented *Enterobacter cloacae*'. In 1980 Farmer *et al.* (2), however, reclassified *E. sakazakii* from *E. cloacae* according to DNA-DNA hybridization, biochemical characteristics, and yellow-pigmented colonies. *E. sakazakii* is known to cause neonatal infections such as necrotizing enterocolitis, bacteremia, and bacterial meningitis (3-7). An increasing number of cases of meningitis in infants and newborns have been reported for outbreaks and the symptoms since the first reported identification of a yellow pigmented *E. cloacae* in the UK in 1961 (8). Such infections among children caused poor health and a mortality prognosis as great as 40-80% (9). If not fatal, the effects on the nervous system can be semi-permanent.

The infective dose of *E. sakazakii* was estimated at 10^3-10^5 cell (10), which are similar to that for outbreaks of *Escherichia coli* O157, *Listeria monocytogenes* 4D, and the typical meningitis pathogen of *Neisseria menigitidis*. Infection and symptoms, however, vary depending upon the host cells, foods contaminated, and stress. There have been few reports on toxicity and pathogenicity, however, enterotoxins and polysaccharide capsule contributions, as per *N. meninfitidis* and *Streptococcus pneumoneae,* would be important virulence factors (10). According to a recent report, *E. sakazakii* could be resistant to compliment-mediated cytotoxicity and its motility might be critical for attachment to CaCo2 cells and infection (11).

Contaminations by *E. sakazakii* and other Enterobacteriaceae were detected and their levels determined in 20 of 141 infant formula foods obtained from 36 countries (12). Also, another study reported that the five of 24 infant formula foods were contaminated and at a rate of 0-12% per product (13). Among 486 agricultural products examined, for example, *E. sakazakii* was isolated from 67 samples where the main sources were 2 of 62 cheeses, 40 of 122 spicy seasonings, and 15 of 66 dried agricultural products (14). Other investigators have reported *E. sakazakii* isolation from cheeses, meats (pork and sausage) and vegetables (15), as well as cabbages (16). Data from the World Health Organization (WHO) supports these findings and the increasing trend of cases. To control *E. sakazakii* outbreaks from these food products, sterilization is an effective process, however, these foods may continue to be sources for cross-contamination during processing if the initial contamination levels were extremely high. The heat resistance of *E. sazkazakii* was similar to other Enterobacteriaceae (17) such that standard pasteurization practices effectively destroy *E. sakazakii* (18).

There have been no reports on food contamination by *E. sakazakii* or the thermal stability of the pathogen for Korean isolates. In this study, the prevalence of *E.*

*Corresponding author: Tel: 82-31-750-5523; Fax: 82-31-750-5273
E-mail: p5062@kyungwon.ac.kr
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sakazakii in reconstituted powdered infant formulas and association with unprocessed ready-to-eat (RTE) agricultural products has been determined along with its heat resistance properties .

Materials and Methods

Bacterial strains and food samples The standard strains used for biochemical characterization were *E. sakazakii* NCTC11467, *E. sakazakii* KCTC2949, *E. cloacae* ATCC13047, and *E. cloacae* ATCC11438. All cultures were achieved using the tryptone soya broth, trytone soya agar (Oxoid, Hampshire, England) at 37°C. 252 agricultural samples tested included white rice, brown rice, soybeans, bananas, oranges, carrots, pumpkins, potatoes, tomatoes, and laver, which could be an ingredient used for powdered infant formula (PIF) after drying and pulverizing. Twenty five PIFs as examples of baby weaning foods and for the infant formula milk as substitute of breast milk were purchased from 29 markets in the Seoul and Kyonggi areas of Korea. The agricultural samples were purchased and transported on ice. Washing for 5 min with running cold tap water and without a sanitizing agent before the bacterial tests was conducted. The samples were prepared using a sterilized knives, scissors, and spatula on a clean bench. Each sample (approximately 25 g each) was homogenized for 120 sec in the 225 mL buffered peptone water (Oxoid) by the stomacher (IUL, Barcelona, Spain). The instruments were resterilized and the clean bench area was also recleaned after each preparation.

Aerobic plate coun (APC) and Enterobacteriaceae count Bacterial counting was made using the modified methods of Iversen *et al.* (14). One mL each of the prepared samples was added to 9 mL of the saline solution and serially diluted. The diluted samples were spread out on the aerobic plate count agar (Difco Laboratory, Detroit MI, USA) and violet red bile glucose agar (VRVGA) (Difco), and any formed colonies were counted after 48 hr culture at 37°C.

Isolation and identification of *E. sakazakii* The modified methods of Meytjens *et al.* (12) and Nazarowec-White *et al.* (13) were followed in isolating and identifying *E. sakazakii*. The samples homogenized by the stomacher incubated for 24 hr at 37°C followed by transfer (10 mL) to 100 mL of a GMP-plus enrichment medium (bioMerieux, Marcy I'Etoile, France) for an additional 24 hr at 37°C. The enriched culture of 10 mL was transferred to the 100 mL of EE broth Mossel (Difco Laboratory, MI, USA) and incubated for 24 hr at 37°C, where the bile salt and the brilliant green were present as inhibitors to the growth of non-Enterobacteriaceae. The enriched culture was streaked on VRBGA (Difco Laboratory) and 5 typical red colonies were selected for culture on trytone soya agar for 48-72 hr at 25°C. Any colony with a yellow pigment was selected again and identified by using an API20E kit (bioMerieux) and an ID32E kit (bioMerieux). *E. sakazakii* NCTC 11467 was used as the positive control organism.

Determination of D-value for *E.sakazakii* in rehydrated infant formula milks The powdered infant formula milk was rehydrated with sterilized water (100 mL) according to the manufacturer's instructions for reconstitution prior to infant feeding. The rehydrated milk preparations were kept in the water baths of each temperature (50 or 60°C). To analyze any reduction of *E. sakazakii* in the rehydrated milks, the enriched culture (0.1 mL) was added to a microtube (Axygen, Union City, CA) with each 0.9 mL of rehydrated milk at 60°C. The microtube with *E. sakazakii* rehydrated milk was kept in the water bath and taken out every 5 min. The tubes were kept in the ice-bath before streaking was carried out on the trytone soya agar plates. Plates were incubated for 18 hr at 37°C and the colonies were counted.

The D-value of *E. sakazakii* according to the viable count and time at each temperature was determined. Linear regression of the thermal death curve was prepared and their correlation coefficients (R^2) were ranged from 0.9 to 1.0.

Survival of *E.sakazakii* in the feeding bottles after rehydration with waters at 50 and 60°C One mL of the cultures of *E. sakazakii* NCTC 11467 and 3 thermovarient *E.sakazakii* isolates were added to each of 99 mL rehydrated infant formula milks at 50 and 60°C in the feeding bottles. The bottles were kept at room temperature which was measured continuously every 2 min for 20 min. Viable counts were also determined on a trytose soy agar at the end of 18 hr at 37°C.

Results and Discussion

Aerobic plate and Enterobacteriaceae counts determined from unprocessed ready-to-eat agricultural food products The prevalence and the frequency of microbial flora for 252 foods (white rice, brown rice, soybean, banana, orange, laver, carrot, pumpkin, potato, and tomato) were determined. These foods could be expected as the ingredients of an infant's food consumption at weaning e.g. after drying and pulverization under non-heat treated conditions and without any sterilization. The APC showed as less than 2 log CFU/g as follows: 43% white rice, 33% soybean, 38% banana, 33% orange, 36% carrot, and 52% of the tomato samples, whereas the APC ranged from 2 log CFU/g for laver. The APC indicated a high level of contamination of more than 5 log CFU/g at 81% brown-rice, 50% pumpkin, 43% potato, and 47% carrot. Chang *et al.* (19) reported similar APC results as in Table 1, except that the levels of contamination for brown-rice and laver were higher in the present study. Enterobacteriaceae counts were 2 log CFU/g or less for 80% orange, 71% laver, 76% rice, 86% potato, and 78% tomato samples and were 3 log CFU/g or more for 53% banana, 75% pumpkin, 67% soybean, and 68% carrot. Brown-rice had the highest contamination (66%) at a level of 5-6 log CFU/g. For PIF, 16% for milk and 26% for weaning foods were intermediate at more than 4 log CFU/g. The level of less than 4 log CFU/g is required for dried instant foods according to CODEX (20) and less than 2 log CFU/g for Enterobacteriaceae (21). Iversen *et al.* (14) have reported that no Enterobacteriaceae was detected following direct plating of the rehydrated milk powders while 16 out of

Table 1. Prevalence and frequency of aerobic bacteria and Enterobacteriaceae from ready-to-eat agricultural products and powdered infant formulas

Classification	Sample (No.)	Number percentage at each aerobic plate count (CFU/g)						Number percentage at each Enterobacteriaceae count (CFU/g)						
		$\leq 10^2$	10^3	10^4	10^5	10^6	10^7	$\leq 10^1$	10^1	10^2	10^3	10^4	10^5	10^6
Grain	Rice (24)[1)]	43[2)]	29	18	12	0	0	16	48	12	12	8	4	0
	Brown rice (23)	0	0	19	47	22	12	0	0	3	12	19	44	22
	Soybean (12)	33	17	33	17	0	0	0	8	25	17	42	8	0
Fruit	Banana (23)	38	17	13	14	9	13	8	16	24	37	16	0	0
	Orange (18)	33	22	17	17	11	0	42	14	24	13	8	0	0
Seaweed	Laver (24)	17	13	17	17	13	23	33	21	17	21	8	0	0
Vegetable	Carrot (58)	36	9	9	24	16	7	14	7	10	18	22	21	7
	Pumpkin (20)	0	0	50	20	20	10	0	5	15	65	10	5	0
	Potato (23)	0	0	57	26	4	13	30	39	17	13	0	0	0
	Tomato (27)	52	7	7	22	11	0	52	11	15	7	7	7	0
Powdered infant formula	Milk(12)	68	18	8	0	8	0	0	0	0	0	0	0	0
	Food(8)	62	13	0	13	13	0	0	0	0	0	0	0	0

[1)]Number of samples analyzed is given in parentheses.
[2)]Percentage of samples. The samples were placed in ice boxes after purchase and transported to the laboratory. Samples were washed for 5 min with running tap water before the bacterial test was conducted.

Table 2. Enterobacteriaceae isolated from powdered infant formulas, grains, fruits, laver, and vegetables after enrichment on DFI agar

Organism	Number of Enterobacteriaceae isolated from each food						
	Powdered infant formula (25)[1)]		Grain (59)	Fruit (41)	Laver (24)	Vegetable (128)	Total (277)
	Milk(9)[2)]	Food(16)[3)]					
E. cloacae	0	4	1	0	0	0	5
E. sakazakii	0	3	4	0	1	1	9
E. aerogens	0	0	0	0	0	1	1
Proteus vularis	0	0	0	0	0	1	1
Acinetobacter baumannii	0	0	0	0	0	1	1
Leuconostoc adecarboxylata	0	0	0	2	0	3	5
Klebsiella planticola	0	0	0	0	0	1	1
K. pneumoniae	0	0	1	0	0	2	3

[1)]Number of samples analyzed in parentheses.
[2)]The substitute for breast milk.
[3)]Weaning foods for infants.

122 herbs and spices contained Enterobacteriaceae at greater than 4 log CFU/g, thereby exceeding the maximum acceptable level (4 log CFU/g) during the self-life of the product. No Enterobacteriaceae was detected for the PIF. Therefore, our results indicate that the agricultural products contained a high level of the bacterial contamination despite washing under running water. Special preparations before using these ingredients (non-sterilized foods) are needed if the contamination levels are to be reduced.

Isolation and identification of *E. sakazakii* From the experiments with DFI agar and the biochemical methods of API20E and ID32E, three *E. sakazakii* and four *E. cloacae* strains were isolated and identified in 25 PIF. All three *E. sakazakii* isolates came from the weaning food of PIF. Four *E. sakazakii*, one *E. cloacae*, and one *Klebsiella pneumoniae* bacterial strains were isolated from 59 grain samples. All four *E. sakazakii* were isolated from the brown-rice sample. One strain of *E. sakazakii* was isolated from 24 laver samples and two *Leuconostoc adecarboxylata* strains were isolated from 41 fruit samples. Each one of *E. sakazakii, E. aerogens, P. vularis, Acinetobacter baumannii,* and *K. planticila*, three *L. adecarboxylata*, and two *K. pneumoniae* were isolated from 128 vegetable samples. One *E. sakazakii* was from the tomato samples. In addition to PIF, *E. sakazakii* has been isolated from a wide range of foods including cheeses, meats, vegetables, grains, herbs and spices as seen by DFI plating (14). The prevalence of *E. sakazakii* was 12% (3/25), 17% (4/23), 4% (1/24), and 4% (1/27) of

Table 3. D_{60}-values of *Enterobacter sakazakii* strains isolated from powdered infant formula food

Range (min)	Strain (Origin)[1)]
3.52-3.58	KWBC10309 (PIF[2)]), KWBC11314 (PIF), KWBC10132 (brown rice),
3.79-3.86	KWBC11213 (PIF), KWBC10102 (brown rice), KWBC10222 (brown rice),
4.40-4.79	NCTC11467, KCTC2949, KWBC10152 (brown rice), KWBC11413 (tomato), KWBC12314 (laver)

The strains were added to the infant formulas and which were then rehydrated for D-value determination at 60°C.
High correlation coefficients (R^2) ranging from 1.0 to 0.9 were estimated for all strains.
[1)]Parentheses indicates the origin for the isolation.
[2)] PIF£¾powdered infant formula

PIF, brown rice, laver, and tomato samples, respectively. The contamination by *E. sakazakii* on the total agricultural products 3.2% (9/277) was not especially high in comparison to 12% reported in other countries (12-16). Special products like brown rice and the weaning infant formulas showed the highest contamination levels with *E. sakazakii*.

Determination of D-value of *E. sakazakii* in rehydrated infant formula milks To analyze thermostability of *E. sakazakii* isolates, D-values were determined in the rehydrated infant formula milk as shown in Table 3. D_{60}-values of *E. sakazakii* KWBC10309, *E. sakazakii* KWBC11314, and *E. sakazakii* KWBC11213 from PIF were from 3.52-3.79 min. and slightly less than those of the type strains whose D_{60}-values were 4.69 min and 4.79 min. *E. sakazakii* KWBC10102, *E. sakazakii* KWBC10132, and *E. sakazakii* KWBC10222 from brown-rice were midway between PIF isolates and the isolates from tomato and laver. *E. sakazakii* KWBC11413 from tomato and *E. sakazakii* KWBC12314 from laver showed the similar value to the type strains. Edelson-Mammel *et al.* (17) reported a similar D-value (4.41 min) for the *E. sakazakii* strain 607, however, Nazarowec-White *et al.* (18) reported very different D_{58}-values depending on the strains from the heat-labile and heat stable groups, presumably because of genetic diversity. The thermostability of *E. sakazakii* was higher than other Enterobacteriaceae and lower than *L. monocytogenes* (18). Our results indicate a very close D-value relationship among the isolates reported at 60°C.

Inactivation profile of *E. sakazakii* for the rehydrated infant formula milks in the feeding bottle The viable count profiles of four *E. sakazakii* strains in the feeding bottles of the infant formula milks were determined during rehydration at ambient temperature (Fig. 1 and 2). The *E. sakazakii* were selected based on their D_{60}-value indicated in Table 3. Both the milk samples showed the temperature of about 37°C in 15 min after rehydration with the waters of 50 and 60°C. After reaching 50°C, the counts decreased at a rate approximating 1 log CFU/mL at 4-6 min and then remained stable for an additional 20 min. After reaching 60°C, the counts decreased at a constant rate of approximately 2 log CFU/mL for 20 min. One or more log CFU/mL reduction was confirmed when the water of 60°C was used for rehydration at the ambient temperature. Iversen *et al.* (22) reported the doubling time of *E. sakazakii* at 21°C was at about 75 min and the growth might occur with the feeding bottles at temperatures of 35

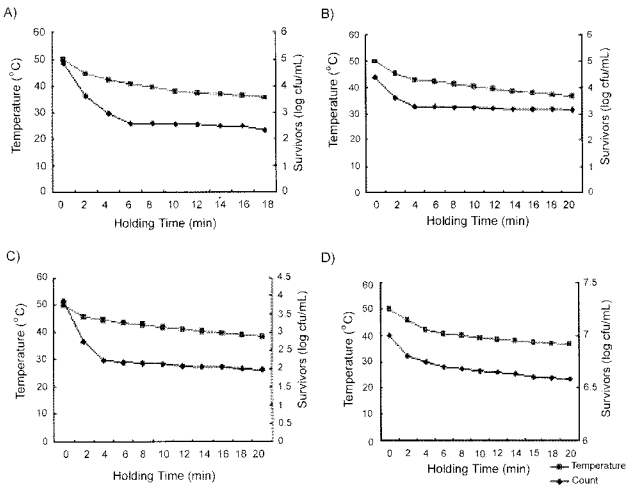

Fig. 1. Viable counts and temperature profiles of the rehydrated infant milk formulas with hot water at 50°C in the feeding bottles during cooling under room temperature conditions. A): *E. sakazakii* NCTC11467, B): *E. sakazakii* KWBC10309, C): *E. sakazakii* KWBC11314, D): *E. sakazakii* WBBC11213

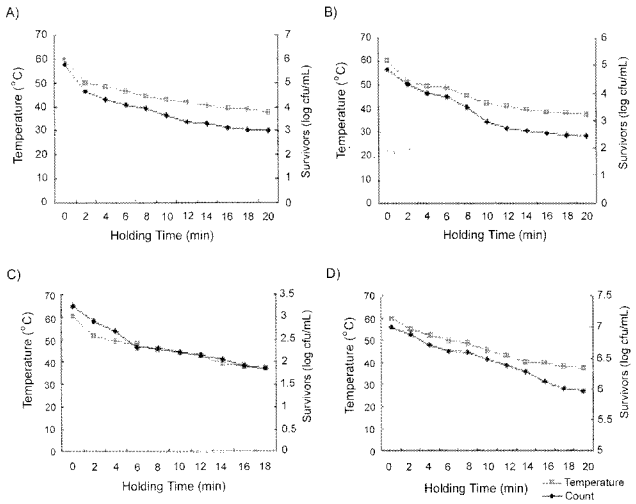

Fig. 2. Viable counts and temperature profiles of the rehydrated infant milk formulas with hot water at 60°C in the feeding bottles during cooling under room temperature conditions. A): *E. sakazakii* NCTC 11467, B): *E. sakazakii* KWBC 10309, C): *E. sakazakii* KWBC 11314, D): *E. sakazakii* KWBC 11213

-37°C before feeding. Skladal *et al.* (23) reported that its growth was very slow when kept in the refrigerator and yielded a doubling time at 10 hr at 10°C. Furthermore,

Kindle *et al.* (24) reported that over 4 log CFU/mL reduction was inactivated at 82-93°C for 85-93 sec. Therefore, to minimize the contamination of PIF from *E. sakazakii* it is recommended that rehydration use water at the appropriate elevated temperature and be with minimal nutrient reduction. Rehydration of PIF for infant feeding with the water of 60°C may be helpful to the reduction of *E. sakazakii* instead of the water of 50°C as recommended by the manufacturers.

It has been known that species of Enterobacteriaceae isolated from powdered milks substituting for breast milk are the cause of bacterial disease in infants. As a result, the United Nations FAO has recommended that the bacterial counts for coliform organisms in the acceptable powdered infant milk formulas be less than 3 CFU/g. According to a U.S. FoodNet 2002 survey of invasive infections by *E. sakazakii*, the frequency of the cases in infants is low with an infection rate of 1 per 100,000 infants under one year of age (25).

International Commission on Microbiological Specifications for Foods, however, reported that *E. sakazakii* would also be very dangerous to the neonates and infants. The acute bacterial infections of meningitis, necrotizing enterocolitis, and bacteremia would cause health problems for a long time, possibly throughout life. Therefore, the FAO/WHO recommended creation of a panel of experts to collect and evaluate data and information on powdered infant formulas (26, 27). The advisory panels focused mainly on the safe production of the powdered substitutes for breast milk. *E. sakazakii* is distributed broadly throughout nature, however, and can contaminate in the foods in agriculture through inappropriate heat treatment, or cross-contamination during food processing. Fortunately, we were unable to isolate *E. sakazakii* from any of the powdered infant formula milks, however, we did isolate four *E. sakazakii* from the powdered infant formulas used for weaning and determined the prevalence of *E. sakazakii* at 19% of the food products, which was a little high contamination in comparison with Yoo *et al.* (28). According to an earlier epidemiological study (12), target ages of the critical diseases ranged from birth to three years and mostly under one year. These foods are usually recommended by the manufacturers (in Korea) for the weaning of infants from six months onward. Therefore, especial care is needed to control for *E. sakazakii* outbreaks since such products are not sterile.

In general, these pathogens are controlled by heat treatment. Such the endeavours on *E. sakazakii* in the foods were focused on the control of maufacturing precess and storage after rehydrating the foods. Even though the level of contamination was low, growth would be easily anticipated at 35-37°C of the feeding bottles to an infective dose of 10^3-10^5 CFU/mL (29). Heat resistance analysis indicated *E. sakazakii* was the most thermotolerant among the Enterobacteriaceae (D_{72}=1.30088 min) from the infant formula milk (30). Heat treatment of 82-93°C for 85-100sec reportedly reduced, at a 4-log scale at various infant formula milks (24). Considering that the levels of *E. sakazakii* observed in dried infant formula are generally less than 1 CPU per 100 g of dry formula (17), the 4-log scale reduction treatment would virtually assure minimal or no enteric bacteria. The destruction of other nutrients in infant formula foods by elevated temperatures, however, must be considered.

Until now, an efficient way to decontaminate and to prevent *E. sakazakii* in and on foods has been essentially unknown (31). Faber (32) has recommended a strategy for the food industries to reduce *E. sakazakii* occurrence by improving hygienic practices in, and the monitoring of, the food processing environment, and further that end product testing be obligatory. Control of the storage and handling periods for rehydrated infant formulas would also be a means for reducing potential health risks. We suggest a rehydration water temperature of 60°C with a 20 min waiting period at room temperature as this procedure was shown to reduce up to a maximum two log scale *E. sakazakii* growth rather than the 50°C, recommended by the manufacturers. More research is urgently needed to quantify *E. sakazakii* in and on our Korean foods. Further studies on decontamination methods for our agricultural products, on the prevention of cross-contamination during food processing, as well as bacterial growth in reconstituted infant formulas is needed.

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