Incidence, Survival, and Growth of *Enterobacter sakazakii* in Infant Formula

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**ABSTRACT**

*Enterobacter sakazakii* has been implicated in a severe form of neonatal meningitis. Although studies have failed to identify an environmental source for the organism, dried infant formula has been implicated in outbreaks and sporadic cases of *E. sakazakii* meningitis. The high mortality rate (50 to 75%), the severity of the infection in infants, and the lack of information on the incidence, survival, and growth of *E. sakazakii* in foods led to this study. Experiments were undertaken to determine the incidence of *E. sakazakii* in dried infant formula, the temperature range for growth, and the growth characteristics of *E. sakazakii* in reconstituted dried infant formula. Strains of *E. sakazakii* were isolated from dried infant formula available on the Canadian retail market. The prevalence varied from 0 to 12% in samples from five different companies. For both clinical and food isolates, minimum growth temperatures of 5.5 to 8.0°C were observed by using a temperature-gradient incubator. The potential growth of *E. sakazakii* was followed by using a mixture of food and clinical isolates in three different formulas incubated at 4, 10, and 23°C. Average generation times were 40 min at 23°C and 4.98 h at 10°C. *E. sakazakii* strains did not grow at 4°C and began to die off during storage at this temperature. The results of this study stress the importance of using aseptic methods and proper temperature control in the preparation, use, and storage of dried infant formula.

Key words: *Enterobacter sakazakii*, incidence, growth, infant formula

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...hers of the family *Enterobacteriaceae*. Species of *Enterobacteriaceae* were isolated from 52.2% of the 141 different samples, with the species most frequently isolated being *Enterobacter agglomerans*, *E. cloacae*, *E. sakazakii*, and *Klebsiella pneumonia*. *E. sakazakii* was isolated from 20 of the 141 samples, i.e., in samples from 13 of the 35 countries. The two strains of *E. sakazakii* were also cultured from powdered infant formula in Czechoslovakia (16). Simmons et al. (19) reported isolation of *E. sakazakii* from infant formula assumed to have been contaminated during the manufacturing process. Three cases of neonatal meningitis caused by *E. sakazakii* found in dried infant formula were reported by Bierling and coworkers (1) in Iceland. In the latter two reports, a relationship was established between isolates found in dried infant formula and clinical isolates from infected neonates (3). In 1990 two cases of *E. sakazakii* infection of unknown etiology were reported to Health Canada (T. Gleeson, personal communication). In one case, analysis of two cans of formula obtained from the home of a 1-month-old infant showed no microbiological contamination. However, the original can of formula had been discarded and therefore could not be evaluated. Skladal et al. (20) found *E. sakazakii* to be one of the major contaminating bacteria in ultra-high-temperature (UHT) milk cartons, implying that this organism may survive pasteurization temperatures. Little information exists with respect to the growth characteristics of this organism. Farmer et al. (5) examined 57 strains of *E. sakazakii* and reported growth of the organism at 25, 36, and 45°C. Fifty of the tested strains grew at 47°C but not at 4 or 50°C. There are no known literature reports describing the growth of *E. sakazakii* in reconstituted dried-infant formula.

Although the exact prevalence of neonatal meningitis caused by *E. sakazakii* is not known, the severity of the infection, the high mortality rate, and the lack of information on the growth characteristics of this organism made the need for this study evident. The objective was to determine the incidence and prevalence of *Enterobacter sakazakii* in dried infant formula on the Canadian market and to evaluate the growth characteristics of clinical and food isolates in laboratory medium and reconstituted dried infant formula.
**MATERIALS AND METHODS**

**Source of bacterial strains**

The clinical strains of *E. sakazakii* used in this study were obtained from culture collections of the following sources: one strain from St. Joseph’s Hospital, London, Ontario; one from the Montreal Children’s Hospital, Montreal, Quebec; and three strains from The Hospital for Sick Children, Toronto, Ontario, Canada. Five food strains were isolated from powdered infant formula.

**Incidence**

**Sampling.** The incidence of *E. sakazakii* in dried infant formula from five different companies that sell at the retail level on the Canadian market was determined. The protein source in each of the formulas was cow’s milk (whey protein) and the carbohydrate source, lactose. Samples (cans) from different lots manufactured on different days were obtained at the manufacturing or retail levels, with a total of 120 cans being evaluated, 24 from each manufacturer.

**Analysis.** The can lid margins and the spoons used for sampling the cans were sterilized in flames of burning ethanol before samples were withdrawn. Double-distilled water was autoclaved in flasks and cooled to 45°C. Dried infant formula powder (100-, 10-, and 1-g amounts) was added to the water (1:10), and shaken by hand until the powder was uniformly suspended. After overnight incubation at 36°C, 10 ml of each suspension was withdrawn from each flask and added to 90 ml of *Enterobacteriaceae* enrichment broth (buffered glucose, brilliant green, bile broth) (Oxoid, Unipath Inc., Nepean, Ontario, Canada). After overnight incubation (36°C), 1-ml amounts of broth were mixed with 20-ml amounts of fluid violet red bile glucose agar (Oxoid) in duplicate petri plates. After solidification of the agar, the plates were incubated overnight at 36°C. Presumptive *E. sakazakii* colonies were confirmed using the API 20E biochemical identification system (API System, Laval, Quebec, Canada) and estimates were determined by the most probable number technique (6).

**Minimum growth temperature experiments**

Ten clinical and food strains of *E. sakazakii* and the type strain, ATCC 29544 (ATCC, Rockville, MD, USA) were examined. Each strain was grown overnight (36°C) in 10 ml of brain heart infusion (BHI) (Difco Laboratories, Detroit, MI) broth and then diluted in fresh BHI broth to a final concentration of 10³ CFU/ml. The diluted broth was dispensed in 5-ml aliquots into L-shaped aerobic culture tubes (L-tubes) (Scientific Industries, Mineola, NY) and placed into a temperature-gradient incubator (Scientific Industries, Mineola, NY). In preliminary experiments, a constant temperature gradient was established in the incubator so that the temperature ranged from 4 to 50°C in 30 L-tubes, with the temperature differential between tubes ranging from 1 to 1.5°C. Thirty duplicate tubes were inoculated with *E. sakazakii* and then placed in the temperature-gradient incubator. Growth was assessed by visually examining the turbidity of each culture over a 20-day period.

**Growth study**

**Formula.** Infant formula powders from the three companies that have the highest market share of Canadian retail sales were selected to evaluate the growth of *E. sakazakii* in reconstituted infant formula. Formula was prepared according to the manufacturer’s directions. The powder was weighed (92, 82.5, and 93.5 g for formulas A, B and C, respectively) and added to 600 ml of sterile double-distilled water. The 600 ml of formula was divided into 2 flasks each containing 300 ml. One flask was inoculated with a mixture of five clinical strains of *E. sakazakii* and the other flask with a mixture of five food isolates. Each spiked 300 ml of formula was further divided into 50-ml portions. Experiments were done in duplicate.

**Inoculation of formula.** Each of the 10 test strains was incubated overnight at 36°C in 5 ml of BHI broth, and diluted to a final concentration of 1.1 × 10⁵ cells/ml of formula. The inoculated formulas were incubated at three different temperatures, namely, 4, 10 and 23°C. The first temperature (4°C) was selected as a “proper refrigeration temperature,” 10°C was considered a slightly abusive temperature, and 23°C a simulation of room-temperature abuse.

**Sampling.** At 23°C, samples were taken every 2 h over a 24-h period, while at 10°C samples were taken on day 0 and then every day for 10 d. Formula incubated at 4°C was sampled every other day for 20 days. At these timed intervals, samples were diluted in 0.1% (wt/vol) buffered peptone water, plated onto duplicate plates of VRBG agar using the pour plate technique, and incubated at 36°C for 24 h. Presumptive *E. sakazakii* colonies were confirmed using the API 20E test strip.

**Statistical analysis.** Data were analyzed by the Gompertz equation to produce fitted growth curves by using the Inplot® (Graph Pad, Inplot® ver. 4.04, GraphPad Software Inc., Sorento, California) statistical software package to obtain the generation and lag times for clinical and food isolates of *E. sakazakii* in each of the three formulas evaluated. Generation time and lag time results were further subjected to an analysis of variance (SAS Institute Inc., Cary, NC) in order to determine significant statistical differences among the formulas and between clinical and food isolates. The analysis included two experiments done in duplicate.

**RESULTS AND DISCUSSION**

**Incidence**

A total of 120 cans (from 5 different companies) of infant formula were examined for the presence of *E. sakazakii*. The microorganism was cultured from 8 cans of product (Table 1). The levels of *E. sakazakii* found in the positive samples was 0.36 CFU/100 g. These findings were similar to those of Muytjens et al. (13) who reported levels of *E. sakazakii* ranging from 0.36 to 66.0 CFU/100 g of dried infant formula in three of the cans of Canadian formula examined.

**Growth studies**

In a temperature-gradient incubator a linear temperature gradient is established, allowing an organism to be grown simultaneously over a wide temperature range (11). The

<table>
<thead>
<tr>
<th>Infant formula source</th>
<th>E. sakazakii in samples/total samples (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A..........................</td>
<td>3/24 (12)</td>
</tr>
<tr>
<td>B..........................</td>
<td>2/24 (8)</td>
</tr>
<tr>
<td>C..........................</td>
<td>2/24 (8)</td>
</tr>
<tr>
<td>D..........................</td>
<td>1/24 (4)</td>
</tr>
<tr>
<td>E..........................</td>
<td>0/24 (0)</td>
</tr>
<tr>
<td>Total .....................</td>
<td>8/120 (6.7)</td>
</tr>
</tbody>
</table>

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minimum growth temperatures of the clinical (C) and food (F) isolates of E. sakazakii in BHI broth ranged from 5.5°C for strains 4C, 7F, and 10F, to 8.0°C for strain 3C (Table 2). The minimum growth temperature of the E. sakazakii type strain (ATCC 29544) was 7°C. None of the strains grew below 5.5°C. Of the 57 E. sakazakii strains tested by Farmer and coworkers (5), none grew at 4°C.

Walker (23), using a temperature-gradient incubator, investigated the minimum growth of 29 bacterial strains representing six recognized pathogenic genera. The organisms were grown in BHI as well as in UHT milk, with results being similar for both growth media. It was concluded that the organisms could be divided into either mesophiles or psychrotrophs. The mesophiles, Salmonella spp., Staphylococcus aureus, and Escherichia coli, did not grow below 5°C, while the minimum growth temperature for the psychrotrophic pathogens, Listeria monocytogenes, Yersinia enterocolitica, and Aeromonas hydrophila, ranged from -0.9 to 1.2°C. The results of our study indicate that E. sakazakii falls into the mesophile category as defined by Walker (23). Raghubeer and Matches (17), also using a temperature-gradient incubator, evaluated the temperature range for growth of E. coli O157:H7, Klebsiella pneumoniae, and Enterobacter aerogenes, in E. coli medium (EC medium).

For K. pneumoniae, after a 24-h incubation, the temperature range for growth was 26 to 41°C, while at 36 and 48 h, the minimum temperature for growth decreased to 22.7°C. The minimum growth temperatures for E. aerogenes ranged from 24.3 to 27.6°C after 24 to 48 h of incubation, while the maximum growth temperature was 41°C. After 24 and 48 h, E. sakazakii initiated growth at 17 and 13.5°C, respectively. The much lower minimum growth temperature for E. sakazakii compared to those of E. coli, K. pneumoniae and E. aerogenes reported by Raghubeer and Matches (17) may be the result of differences in initial cell concentration, i.e., 10 cells per ml in the Raghubeer and Matches (17) study versus 1,000 cells per ml in the present study.

The results of our study, as well as those of Farmer et al. (5), indicate that E. sakazakii would not grow at a refrigeration temperature of 4°C. However, the temperatures of many home refrigerators range from 7 to 10°C (18). Harris (8) reported that 20% of the home refrigerators surveyed were found to be between 5 and 10°C, however, none were found above 10°C. In contrast, Van Garde and Woodburn (22) found that refrigerator temperatures in 21% of the households surveyed were ≥10°C, and Daniels (4) reported that more than 1 of 4 home refrigerators were above 7.2°C and almost 1 of 10 above 10°C. These commonly found but potentially abusive temperatures would allow E. sakazakii to grow. In terms of maximum growth temperature, Farmer and coworkers (5) found that none of the 57 strains tested grew at 50°C. In the present study, the maximum temperature at which visible growth of E. sakazakii was observed ranged from 41 to 45°C (Table 2).

There were no statistically significant differences found either among the tested infant formulas or between strains at 4, 10, and 23°C (P > 0.05). At 4°C in all three formulas and for both clinical and food strains, the concentration of E. sakazakii remained at the initial inoculum levels (1.1 x 10^3 CFU/ml) or declined with time (data not shown). These findings confirm the importance of proper refrigeration storage temperatures after reconstitution of infant formula powders to ensure that this organism does not grow.

The generation and lag times of E. sakazakii grown in the three formulas at 10 and 23°C were evaluated (Table 3).
Table 4. Some reported generation and lag times for various species of bacteria in BHI broth and milk products

<table>
<thead>
<tr>
<th>Organism</th>
<th>Growth medium</th>
<th>Temperature (°C)</th>
<th>Generation time (h)</th>
<th>Lag time (h)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterobacter sakazakii</td>
<td>Infant formula</td>
<td>23</td>
<td>0.67</td>
<td>2.76</td>
<td>Present study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>4.64</td>
<td>32.8</td>
<td>Present study</td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>BHI broth</td>
<td>23</td>
<td>0.74</td>
<td>5.10</td>
<td>USDA Pathogen Modeling Program version 4.0, PMP^b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>10.36</td>
<td>74.8</td>
<td>USDA Pathogen Modeling Program version 4.0, PMP</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>BHI broth</td>
<td>23</td>
<td>0.74</td>
<td>5.56</td>
<td>USDA Pathogen Modeling Program version 4.0, PMP</td>
</tr>
<tr>
<td></td>
<td>UHT milk</td>
<td>19</td>
<td>1.90</td>
<td>6.30</td>
<td>USDA Pathogen Modeling Program version 4.0, PMP</td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
<td>Whole milk</td>
<td>23</td>
<td>0.80</td>
<td>NR^b</td>
<td>(10)</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>Whole milk</td>
<td>10</td>
<td>4.20</td>
<td>NR</td>
<td>(10)</td>
</tr>
<tr>
<td>Enterobacter hafnia</td>
<td>Whole milk</td>
<td>23</td>
<td>0.69</td>
<td>NR</td>
<td>(10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>2.70</td>
<td>NR</td>
<td>(10)</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>Whole milk</td>
<td>10</td>
<td>4.00</td>
<td>24.0</td>
<td>(10)</td>
</tr>
<tr>
<td>Listeria monocytogenes</td>
<td>Whole milk</td>
<td>10</td>
<td>7.30</td>
<td>25.0</td>
<td>(9)</td>
</tr>
<tr>
<td>Shigella flexneri</td>
<td>Whole milk</td>
<td>28</td>
<td>0.80</td>
<td>1.90</td>
<td>(26)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>19</td>
<td>4.20</td>
<td>11.1</td>
<td>(26)</td>
</tr>
<tr>
<td></td>
<td>UHT milk</td>
<td>28</td>
<td>0.70</td>
<td>3.20</td>
<td>(27)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>19</td>
<td>3.30</td>
<td>14.7</td>
<td>(27)</td>
</tr>
<tr>
<td>Yersinia enterocolitica</td>
<td>Whole milk</td>
<td>25</td>
<td>1.30</td>
<td>NR</td>
<td>(21)</td>
</tr>
<tr>
<td></td>
<td>UHT milk</td>
<td>8.4</td>
<td>7.18</td>
<td>&lt;24</td>
<td>(24)</td>
</tr>
</tbody>
</table>

^a USDA Pathogen Modeling Program using pH, aw, and temperature from the present study.

^b NR, not reported.

There were no statistically significant differences found among the three formulas tested for both clinical and food strains. The lag time at 10°C varied from 19 h for formula A (food) to 47 h for formula B (clinical). Although lag times were generally shorter for the food, compared to the clinical isolates, the differences were not statistically significant (P > 0.05). Generation times for E. sakazakii at 10°C varied from 4.18 to 5.52 h. Again, the generation times for the food isolates were shorter than for the clinical strains, although the differences were not statistically significant (P > 0.05). Generation and lag times were calculated for Salmonella spp. and E. coli (Table 4) using the USDA Pathogen Modeling Program version 4.0, and the water activity and pH values of the reconstituted dried infant formula used in the present study. The predicted generation time for E. coli and Salmonella spp. at 10°C was 6.4 and 10.4 h, respectively; this compares with 4.6 h for E. sakazakii observed in the present study. The predicted lag time for Salmonella spp. and E. coli at 10°C was 74.8 and 47.0 h, respectively (Table 4); the mean lag time for E. sakazakii (all formulas, all strains) was the shortest of all three organisms, 32.8 h.

At 23°C, both E. coli and Salmonella spp. had a predicted generation time of 44.4 min (0.74 h), compared to a mean generation time of 40 min (0.67 h) for E. sakazakii (Table 4). A much greater difference was found when comparing lag times at 23°C, where predicted values for E. coli and Salmonella spp. were 5.7 and 5.1 h, respectively, compared to 2.7 h for E. sakazakii.

Phillips and Griffiths (15) found generation times of Pseudomonas fluorescens at 10°C to be 4.9 and 3.5 h in raw milk and pasteurized cream, respectively; values similar to those of E. sakazakii. Longeveld and Cuperus (10) evaluated the relationship between temperature and growth rate in pasteurized milk of various bacteria. As can be seen in Table 4, organisms from the same genus as E. sakazakii: E. cloacae, E. hafnia, and E. aerogenes, grown in pasteurized whole milk have similar generation and lag times at both 23 and 10°C. The generation and lag times reported in the literature for other organisms found in milk or milk products are also shown in Table 4. It appears that both the generation and lag times for these organisms are longer than for E. sakazakii.

Although low levels of E. sakazakii were found in dried infant formula, the incidence (6.7%) found in this study and in the study of Muytjens et al. (13) (52%) in combination with its relatively short lag time and generation time of E. sakazakii may be a cause for concern. Improper storage of reconstituted dried infant formula at ambient temperatures, e.g., on a bedside table for night feedings, or during shopping, may permit growth of E. sakazakii.

We are unaware of any literature reports describing the competitiveness of E. sakazakii and other organisms. Simmons et al. (19) demonstrated that when dried infant formula was mixed with nutrient broth, E. sakazakii grew well and survived better than the only other contaminant found, E. cloacae. The competitiveness of E. sakazakii with other organisms found in dried infant formula needs to be evaluated.

Results obtained from this study indicate the importance of proper preparation and storage of reconstituted dried-infant formula with respect to the survival and growth of E. sakazakii. This is the first study to demonstrate that, although at low levels, E. sakazakii can be found in dried infant formula present in the Canadian marketplace. Al-
though *E. sakazakii* does not grow at proper refrigeration temperatures of 4°C, it can grow slowly at slightly abusive temperatures (5.5°C). The short generation time (40 min) of *E. sakazakii* in reconstituted dried infant formula at room temperature shows how quickly this organism can grow. Further research is required to determine the infectious dose, growth competitiveness, and assessment of the hazard of *E. sakazakii* with respect to dried infant formula, as well as other food products.

REFERENCES