THERMAL RESISTANCE, SURVIVAL AND INACTIVATION OF
ENTEROBACTER SAKAZAKII IN POWDERED AND
RECONSTITUTED INFANT FORMULA

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ABSTRACT

Enterobacter sakazakii has recently been recognized as an opportunistic
foodborne pathogen with dry infant formula serves as the mode of transmis-
ション. The objectives of this study were to investigate the heat resistance,
survival and inactivation under room and refrigeration temperatures storage
of dry and reconstituted infant formula milk (IFM). E. sakazakii strains (eight
strains) showed a wide variability in heat resistance at different temperatures
(55, 60 and 63°C). The D-values at 55°C ranged from 1.51 to 14.83 min, at 60°C
from 0.17 to 2.71 min and at 63°C from 0.05 to 0.88 min. The calculated z
values for the studied E. sakazakii strains ranged from 3.76–10.11°C. Micro-
wave oven heating of 60 mL portions of reconstituted IFM for 40–50 s was

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Effective in eradicating inoculated E. sakazakii. Storing powdered IFM for 15 days at 4°C resulted in at least a 1-log reduction in E. sakazakii strains, whereas storing reconstituted IFM at 4°C for 2 weeks resulted in more than a 2-log reduction in E. sakazakii.

PRACTICAL APPLICATIONS

This study shows that E. sakazakii strains differ widely in their heat resistance. No differences were observed between biofilm formers and non-formers in terms of heat-resistance in thermal inactivation kinetics experiments. Conventional high temperature short-time pasteurization processes are considered sufficient to inactivate all E. sakazakii strains and household microwave oven (40–50 s for 60 mL portions) can be used to inactivate E. sakazakii if present in reconstituted infant formula milk (IFM). Growth of E. sakazakii can be inhibited in powdered and reconstituted IFM by refrigeration. Also, it is recommended that reconstituted IFM be discarded or refrigerated if not immediately consumed. The probiotic L. acidophilus ATCC 4356 was not effective in inhibiting E. sakazakii in powdered or reconstituted IFM.

INTRODUCTION

Enterobacter sakazakii is a Gram-negative, rod-shaped, motile bacterium that belongs to the family Enterobacteriaceae, and has recently been implicated in several cases of fatal neonatal meningitis (Bar-Oz et al. 2001; Gurtler et al. 2005; Bowen and Braden 2006). Reconstituted infant formula milk (IFM) has been involved as a mode of transmission in several outbreaks and sporadic cases of E. sakazakii infection (Nazarowec-White and Farber 1997a). Additionally, immunocompromised adult people with underlying medical conditions may also be a target for E. sakazakii infections (Gurtler et al. 2005). The reported fatality rate associated with E. sakazakii infections is 40–80% (Bowen and Braden 2006).

Iversen and Forsythe (2003) speculated that E. sakazakii has a low infectious dose of 1,000 cfu in reconstituted IFM. However, this dose is higher than what is normally found in powdered IFM. Nonetheless, gross temperature abuse or poor hygienic practices may prompt E. sakazakii growth and elicit such infectious dose. Prevention of the occurrence of E. sakazakii in IFM is extremely important, particularly because treatment is usually too late or may be difficult, since E. sakazakii exhibits a remarkable resistance for a wide range of antibiotics (Gurtler et al. 2005).
E. sakazakii is ubiquitous in the environment (Farber 2004; Kandhai et al. 2004; Arts 2005) and is usually inactivated during the pasteurization process used in the preparation of powdered IFM (Nazarowec-White and Farber 1997b). However, contamination of IFM with E. sakazakii occurs mostly as post-processing contamination from the processing environment, or through the addition of ingredients during the powder production stage (Nazarowec-White and Farber 1997a) or due to colonization of E. sakazakii in the IFM preparation utensils such as bottles, brushes and spoons. In an E. sakazakii outbreak, the source of infection was traced back to the blender used in the preparation of rehydrated IFM for neonates (Bar-Oz et al. 2001). Therefore, strict cleaning and hygienic practices are necessary to eliminate E. sakazakii and to prevent biofilm formation. Additionally, E. sakazakii was shown to have a remarkable capability to survive in a dry environment for a long time period (~2 years). This property gives it a competitive advantage to prevail in a dry environment such as powdered IFM (Edelson-Mammel et al. 2005) because of its capability to accumulate compatible solutes such as trehalose, that protect E. sakazakii against osmotic stress by stabilizing phospholipids membranes and proteins (Breeuwer et al. 2003). Therefore, inactivation of E. sakazakii prior to feeding infants reconstituted IFM is crucial to prevent illness.

Reconstituted IFM is a nonsterile product that should be prepared, handled and stored appropriately. Usually, very low numbers of E. sakazakii are detected in dry IFM. However, E. sakazakii has the capability to multiply quickly if kept for extended periods of time in bottle heaters or at room temperature (Nazarowec-White and Farber 1997a). Heat treatment prior to consumption of a food has long been used as the primary means for reducing the risk of foodborne illness. Hence, determination of the thermal inactivation kinetics for E. sakazakii is important if a sufficient heat treatment is to be applied to inactivate this organism in reconstituted IFM. This is particularly important because the reported thermal resistance parameters are quite variable. The objectives of this study were to determine the thermal inactivation kinetics for different strains of E. sakazakii in reconstituted IFM and to investigate the survival of E. sakazakii in powdered and reconstituted IFM at room and refrigeration temperatures. An additional objective was to study the antimicrobial effect of the probiotic Lactobacillus acidophilus ATCC 4356 against E. sakazakii.

MATERIALS AND METHODS

Bacterial Cultures

Eight strains of E. sakazakii were used for the evaluation of thermal resistance of E. sakazakii to heat inactivation. The cultures used were E.
sakazakii ATCC 12868, E. sakazakii ATCC 29004, E. sakazakii FSM 292, E. sakazakii FSM 287, E. sakazakii 2.39-1, E. sakazakii 2.68, E. sakazakii 3 and E. sakazakii 55. The strains were obtained form the Food Microbiology Laboratory at Washington State University. All the strains were maintained refrigerated in tryptic soy agar (TSA) (Difco, Becton Dickinson, Spark, MD) slants and transferred prior to the experiment to Brain Heart Infusion (BHI) broth (Difco, Becton Dickinson, Spark, MD) and grown for 24 h at 37°C to reach the stationary phase of growth. Stationary phase cells of E. sakazakii are more resistant to environmental stresses as compared to exponential phase cells (Breeuwer et al. 2003).

Heating Regimen

IFM (Enfamil with iron, Infant formula, Mead Johnson Nutritionals, Evansville, IN) was purchased from a local grocery store and reconstituted according to the manufacturer’s instructions in distilled water. One milliliter of the milk was dispensed into flat top microcentrifuge tubes (1.5 mL) (Fisher Scientific Inc., Pittsburgh, PA). The reconstituted IFM was sterilized at 121°C for 15 min to get rid of the background microorganisms. The tubes were inoculated with each one of the eight E. sakazakii strains (1 × 10^6 – 1 × 10^7 cfu/mL) individually. Heat treatment was conducted in a water bath (Iso temp 215, Fisher Scientific Inc., Pittsburgh, PA) for 5.00, 0.50 and 0.33 min at 55, 60 and 63°C, respectively. The temperature of the tubes was monitored by a type T thermocouple (Barnat Co, Barrington, IL) connected with a portable thermometer. The tubes were submerged completely in the water bath where the temperature was controlled at the target temperature ± 0.5°C. A sample was removed out of water bath and immersed into ice slush at each treatment temperature immediately after reaching the come-up-time; this time was designated as time zero. The come-up-time is defined as the time required to bring the material at the coldest point of the heating tubes to the specified heat treatment temperature after the tubes had been submerged in the water bath (Al-Holy et al. 2004). The initial number of E. sakazakii (at time zero), is the number of E. sakazakii survivors after the come-up-time has been reached. The come-up-time was approximately 1.6 min. After completion of heating, the tubes were immersed promptly in an ice slush bath at 0.0 ± 0.2°C before being tested for E. sakazakii survivors.

Enumeration of E. sakazakii

The survivors of E. sakazakii were enumerated by 10-fold serially diluting the heat-treated samples in 0.1% peptone water. The overlay method was used specifically for enumerating survivors because it improves the resuscitation of the heat-injured cells (Al-Holy et al. 2007).
plated on TSA supplemented with 0.1% (w/v) of sodium pyruvate (Acros Organics, Geel, Belgium) for the purpose of enhancing resuscitation of heat-stressed cells. Pyruvic acid improves recovering injured cells by degrading hydrogen peroxide or blocking its formation (McDonald et al. 1983). The plates were incubated for 2 h at 37°C. Thereafter, a thin layer (8 mL) of violet red bile agar (VRBA) (Difco, Becton Dickinson, Spark, MD) was overlaid onto TSA and the plates were incubated for additional 22 h at 37°C.

**D values and z Values Determination**

The number of survivors at each temperature was plotted against time. The best fit-line was extrapolated and the D values were determined \((-1/slope\) of the regression line). The z values were determined by plotting the calculated log D values against the corresponding temperatures \((-1/slope\) of the regression line). Each single number is an average of three replicate experiments. The standard deviations of the D value and z values were calculated.

**Household Microwave Oven Experiment**

IFM was reconstituted in distilled water according to the manufacturer’s instructions. Sixty-mL portions were dispensed in screw-capped dilution bottles, and then sterilized at 121°C for 15 min. The bottles were inoculated with 100 μL of each of four different strains of *E. sakazakii*, namely, *E. sakazakii* ATCC 12868, *E. sakazakii* ATCC 29004, *E. sakazakii* FSM 292 and *E. sakazakii* FSM 287. These strains were selected because they exhibited considerably high heat resistance at the temperatures examined in the current study. The initial level of *E. sakazakii* was ca 1 × 10^8 cfu/mL. A household microwave oven (Sharp Carousel, Model No R-0209 HK, 60 Hz, 1,000 W, Mahawah, NJ) was used to heat the bottles for 20, 30, 40, or 50 s. Inoculated but unheated reconstituted IFM served as control. After heating, the bottles were shaken vigorously and the temperature of the heated IFM was monitored using a sterile type-T thermocouple connected to a portable thermometer. The heated bottles were immersed immediately in ice slurry and tested for the presence of *E. sakazakii* survivors using the aforementioned overlay method. The experiment was repeated for three times.

**Survival of Capsulated and Uncapsulated Strains of *E. sakazakii* in IFM at Room and Refrigeration Temperatures**

Two *E. sakazakii* strains (*E. sakazakii* FSM 292, *E. sakazakii* FSM 287) of strong biofilm (capsule) forming capability and two others (*E. sakazakii* 2.39-1, *E. sakazakii* 2.68) of weak biofilm forming capability were investigated in terms of their ability to survive in powdered and reconstituted IFM at
room (21C) and refrigeration (4C) temperatures. *E. sakazakii* strains were inoculated into tubes containing 10 mL of sterile reconstituted IFM or in 50 g of powdered IFM. The initial levels of the *E. sakazakii* were *ca* $1 \times 10^6$ cfu/mL in the reconstituted IFM and *ca* $1 \times 10^5$ cfu/g in the dry IFM. An inoculum (100 µL) of stationary-phase cells of *E. sakazakii* was sprinkled onto the formula in a drop-wise manner. After inoculation, the IFM with the inoculum was mixed vigorously by a sterile spatula for 3 min. Growth of *E. sakazakii* was monitored by spread plating on VRBA every 2 days following inoculation in the reconstituted IFM for 14 days, and every 3 days for 25 days in the powdered IFM. The experiment was replicated three times.

**Antimicrobial Activity of *L. acidophilus* Against *E. sakazakii* in Powdered and Reconstituted IFM**

*Lactobacillus acidophilus* ATCC 4356 was activated by growing it in MRS broth (Difco, Becton Dickinson, Spark, MD) under aerobic conditions at 37°C for 24 h. A loopful was streaked onto MRS agar and incubated aerobically at 37°C for 24 h. Thereafter, *L. acidophilus* ATCC 4356 was grown again aerobically at 37°C for 24 h in MRS broth. Four different strains of *E. sakazakii* (*E. sakazakii* ATCC 12868, *E. sakazakii* ATCC 29004, *E. sakazakii* FSM 292, *E. sakazakii* FSM 287) were grown for 24 h in BHI broth. Broths (15 mL) of *E. sakazakii* strains and of *L. acidophilus* ATCC 4356 were centrifuged using an RC-S super-speed centrifuge (Dupont Instrument, Newtown, CT) at 6,000 rpm for 5 min. The supernatant was discarded, and the precipitate (wet pellet) was resuspended and vortexed in 10 mL of sterile saline (0.9% NaCl) solution. This procedure was repeated again to thoroughly remove media components and to harvest pure cells. Then the bacterial/saline suspension (100 µL) was inoculated in either 10-mL tubes containing reconstituted IFM or to dry IFM (25 g in a sterile beaker.) *L. acidophilus* was added at a level of *ca* $1 \times 10^6$ cfu/mL or $1 \times 10^6$ cfu/g. Immediately after that, the reconstituted IFM and the dry IFM were inoculated with a mixture of four different strains of *E. sakazakii* (*E. sakazakii* ATCC 12868, *E. sakazakii* ATCC 29004, *E. sakazakii* FSM 292, *E. sakazakii* FSM 287) at a level of *ca* $1 \times 10^6$ cfu/mL or $1 \times 10^6$ cfu/g. The inoculated dry IFM was shaken vigorously for 3 min to ensure uniform distribution of the probiotic and *E. sakazakii* strains in the dry IFM. The reconstituted and dry IFM were monitored for the presence of *E. sakazakii* by spread plating on VRBA every 2 days and incubating at 37°C for 24 h. The growth of *L. acidophilus* was followed by plating on MRS agar supplemented with 2-phenyl ethanol (3 mL/L) (Sigma, St. Louis, MO), which was added to inhibit the growth of Gram-negative bacteria, including *E. sakazakii*. MRS plates were subsequently incubated under anaerobic conditions. The anaerobic condition was generated by placing MRS plates in an
anaerobic jar containing an Anaerogen sachet (Oxoid, Bastingstoke, Hampshire, England). The plates were enumerated after being incubated for 48 h at 37°C. Also the pH of the inoculated reconstituted IFM was measured throughout the experiment (Accument AB pH meter, Fisher Scientific Inc., Pittsburgh, PA). Water activity of the inoculated and the noninoculated powdered IFM was also determined (Aqualab water activity meter (Model 3TE), Decagon Inc., Pullman, WA).

RESULTS AND DISCUSSION

Determination of the thermal resistance parameters is important to design a thermal process sufficient to inactivate a target microorganism of concern without affecting the nutritional or the organoleptic qualities of the product. Figure 1 shows first-order thermal inactivation kinetics survivor curves of *E. sakazakii* FSM 287 in reconstituted IFM. Considerable variations in the thermal resistance of the studied *E. sakazakii* strains were observed in this study (Table 1). For instance, *E. sakazakii* ATCC 29004 exhibited a $D_{55}$ value of 14.8 min compared to 1.5 min for *E. sakazakii* 55. Nonetheless, $D$ values found for *E. sakazakii* strains in this study are comparable to $D$ values for other members of Enterobacteriaceae reported in the literature (Breeuwer et al. 2003) except for *E. sakazakii* ATCC 29004. Figure 2 shows representative thermal death time curves for *E. sakazakii* ATCC 12868 in reconstituted IFM. The $z$ values reported in this study ranged from 3.8 to 10.1°C, suggesting a great difference in the capability of different *E. sakazakii* strains to resist change in temperature. Widely differing values of thermal resistance for *E. sakazakii* were also reported (Edelson-Mammel and Buchanan 2004). Pooled $D_{56}$ value
for 10 clinical and food isolates of *E. sakazakii* inoculated in reconstituted IFM equal to 10.30 and 2.5 min at 60°C have been reported, while the pooled z value for those strains ranged between 5.8 and 6.0°C (Nazarowec-White and Farber 1997b). At 60°C, D values for *E. sakazakii* ranging from 1.1 to 1.8 min and at 62°C from 0.2 to 0.3 min and a z value of about 5.7°C have been reported (Iversen et al. 2004). A very close z value (5.6°C) was also reported for *E. sakazakii* (Edelson-Mammel and Buchanan 2004). However, it is noteworthy to mention that no obvious differences in D values for noncapsulated

### TABLE 1.

DECIMAL REDUCTION TIMES (D VALUES) AND Z VALUES (± STANDARD DEVIATION) OF ENTEROBACTER SAKAZAKII STRAINS INOCULATED IN RECONSTITUTED INFANT FORMULA

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<tr>
<th>Strain</th>
<th>D value (min)*</th>
<th>z value (°C)</th>
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<tr>
<td></td>
<td>55°C</td>
<td>60°C</td>
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<tr>
<td><em>E. sakazakii</em> ATCC 12868</td>
<td>14.21 ± 3.58</td>
<td>0.53 ± 0.08</td>
</tr>
<tr>
<td><em>E. sakazakii</em> ATCC 29004</td>
<td>14.83 ± 3.50</td>
<td>2.71 ± 0.32</td>
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<tr>
<td><em>E. sakazakii</em> FSM 292</td>
<td>11.64 ± 2.01</td>
<td>0.82 ± 0.02</td>
</tr>
<tr>
<td><em>E. sakazakii</em> FSM 287</td>
<td>1.85 ± 0.03</td>
<td>0.45 ± 0.03</td>
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<tr>
<td><em>E. sakazakii</em> 2.39-1</td>
<td>3.93 ± 0.34</td>
<td>0.45 ± 0.09</td>
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<tr>
<td><em>E. sakazakii</em> 2.68</td>
<td>5.02 ± 1.00</td>
<td>0.45 ± 0.01</td>
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<tr>
<td><em>E. sakazakii</em> 3</td>
<td>6.95 ± 2.00</td>
<td>0.38 ± 0.02</td>
</tr>
<tr>
<td><em>E. sakazakii</em> 55</td>
<td>1.51 ± 0.03</td>
<td>0.17 ± 0.01</td>
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* Values are average of at least three replicate experiments.

**FIG. 2. THERMAL DEATH TIME CURVES OF ENTEROBACTER SAKAZAKII ATCC 12868 IN RECONSTITUTED INFANT FORMULA (N = 3)**
E. sakazakii (2.39–1, 2.68) and the capsulated strains (FSM 292, 287) were observed. These results agree with the results obtained by Iversen et al. (2004). Adopting the most conservative thermal resistance values for E. sakazakii ATCC 29004 ($D_{63C} = 0.88 \text{ min}$ and $z$ value = 6.61°C), the high temperature short-time pasteurization process will result in at least a 6-log reduction in viable count of E. sakazakii. Therefore, this confirms that the contamination by E. sakazakii mostly occurs as a result of post-pasteurization contamination from poor hygienic practices in the processing or preparation environment or from bottles and utensils used in the preparation of the infant formula.

A composite mixture of four strains of E. sakazakii was chosen for thermal treatment by a household microwave oven (Fig. 3). Despite the fact that scalding could be a problem when using microwave oven to heat reconstituted IFM, it is widely used to prepare reconstituted IFM to warm up the formula before infant feeding because of the rapid heating pattern and convenience of using a microwave oven for this purpose. Though, cooling the reconstituted IFM to an appropriate temperature before infant feeding is indispensable to prevent scalding. Inoculated reconstituted IFM containing about 5 logs of E. sakazakii was heated to different time intervals (0, 20, 30, 40 and 50 s). As expected, as the heating time increased, reduction in cell numbers proportionally increased. Microwaving for 40 s resulted in about a 4-log reduction in E. sakazakii, while heating for 50 s resulted in a complete
elimination of E. sakazakii from heated reconstituted IFM. Even though, the temperature of the rehydrated IFM reached 77.3°C after microwaving for 40 s, about 0.8 logs of E. sakazakii were detected, most probably because of the uneven heating pattern given by the microwave heat. Therefore, apparently, household microwave oven can be used to prepare an E. sakazakii-free reconstituted IFM without scalding. It was reported that microwaving 150-mL portions of infant formula for 85 to 100 s lead to greater than a 4-log reduction in E. sakazakii count (Kindle et al. 1996).

E. sakazakii does not tolerate chilling and appears to decrease gradually in numbers when stored under refrigeration conditions (4°C) (Nazarowec-White and Farber 1997a). Figure 4 shows the capability of two strains of E. sakazakii that are known to form exopolysaccharide capsules and two non-capsule formers to grow in powdered IFM at room and refrigeration temperatures. After inoculation of both types of strains in powdered IFM, the capsulated strains increased by about 1.5 logs by day 6, and the same pattern was exhibited by the noncapsulated strains till day 9 at room temperature. Nonetheless, both types of strains decreased by about 1 log after 15 days of storage compared to the initial E. sakazakii count at day 0. In comparison, both types of strains (capsulated and noncapsulated strains) did not grow entirely when the inoculated powdered IFM was stored at refrigeration temperature (4°C) and decreased gradually till the end of the 15-day storage time. Nonetheless, the magnitude of the reduction was more pronounced in the capsulated (~1.5 logs) strains compared to the non capsulated (~0.7 logs) strains of E.
*E. sakazakii* is more tolerant to desiccation stress compared to other members of Enterobacteriaceae. Barron and Beuchat (2007) indicated that capsulated strains of *E. sakazakii* were still recoverable from dry IFM after 2.5 years. The extent of *E. sakazakii* reduction in dry IFM was more pronounced in formulas with *a_w* between 0.43–0.50 compared with formulas with *a_w* between 0.25–0.30 and the microbe was more persistent in formulas with lower *a_w* for long periods of storage (Gurtlet and Beuchat 2007a). *E. sakazakii* can also survive in dry infant cereals with a wide range of initial *a_w* (0.30–0.69) and storage temperature (4–30°C) for at least 12 months and the retention of viability is enhanced at lower *a_w* (Lin and Beuchat 2007).

The growth of *E. sakazakii* in reconstituted IFM stored for long periods of time under room and refrigeration conditions was studied. Figure 5 shows the growth pattern of capsulated and non-capsulated strains of *E. sakazakii* in reconstituted IFM at room (21°C) and refrigeration (4°C) temperatures. Both types of strains increased dramatically at room temperature until the end of the storage period, where the numbers of *E. sakazakii* exceeded 9-logs in number. There was a visible dramatic increase in the viscosity of reconstituted IFM with time, due to the exopolysaccharide production of the capsulated strains. In comparison, when the reconstituted IFM were stored at 4°C, both types decreased considerably. However, once more the extent of reduction was more pronounced in the capsulated (~4.7 logs) compared to the noncapsulated (3.3 logs) strains of *E. sakazakii*. This result agrees with the finding that *E. sakazakii* did not grow in infant cereal reconstituted with water or milk stored at 4°C (Richards *et al.* 2005). Reconstituted IFM is not a sterile product and can
provide a good medium for bacterial growth. Keeping reconstituted uninoculated IFM at ambient temperature (~25°C) may increase the potential for *E. sakazakii* growth (Rosset *et al.* 2007). Prolonged periods of storage or administration at room temperature might lead to proliferating numbers of *E. sakazakii* that may be present, as *E. sakazakii* has a relatively short generation time at room temperature (~40 min) (Richards *et al.* 2005). Gurtler and Beuchat (2007b) reported that *E. sakazakii* did not grow in reconstituted soy-based and milk-based infant formulas stored at 4°C for 72 h. On the contrary, keeping reconstituted formulas at an abused temperature (12°C) incurred a noticeable increase in *E. sakazakii* count. These results suggest that if not immediately consumed, storing powdered and reconstituted IFM under proper refrigeration is important to inhibit the growth of *E. sakazakii*. Nonetheless, it is recommended that infant formula be freshly prepared with the remaining milk should be discarded. However, if the formula needs to be prepared in advance, it should be kept at 4°C or below (Agostoni *et al.* 2004).

The antibacterial activity of the probiotic *L. acidophilus* was examined against *E. sakazakii* in powdered (Fig. 6) and reconstituted IFM (Fig. 7). A probiotic may pose antimicrobial activity against a pathogen by lowering the medium pH or by generating antimicrobial bioactive peptides (Hayes *et al.* 2006). For example, *L. acidophilus* DPC 6026 produces casein-derived proteinaceous substances that inhibit the growth of bacteria such as *E. coli* and *E. sakazakii* (Hayes *et al.* 2006). As shown in Fig. 6, *L. acidophilus* ATCC 4356 persists in powdered IFM with a low water activity (a_w = 0.29). However, it did
not pose any antimicrobial activity against *E. sakazakii*. The growth of *L. acidophilus* ATCC 4356 was accompanied by a drop in the pH of the infant formula from ~6.5 to 5.0 (Fig. 7). Nonetheless, *E. sakazakii* numbers increased sharply, suggesting that this probiotic does not exhibit any inhibitory activity against *E. sakazakii*.

**ACKNOWLEDGMENT**

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**REFERENCES**


INACTIVATION OF *E. sakazakii* IN INFANT FORMULA
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