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Τόμος 3

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Τόμος 2

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All tested probiotic substances enhanced the growth of L. 5 at different levels when they were compared with glucose. Besides having low growth effect, XOS also stimulated the growth of L. 5 much more than glucose. The initial numbers of bacteria in the media were between 6.38-6.59 log cfu/ml. After incubation, the numbers of bacteria in the media with probiotics reached levels between 7.45-8.55 log cfu/ml. These values were 6.70 and 7.59 log cfu/ml after incubation for negative and positive controls, respectively.

Acidifying activities were different according to types of probiotics and higher than that in the negative control medium. pH values of the culture media with probiotics varied between 5.74 and 4.12.

References


Thermal resistance, growth pattern and inactivation of Enterobacter sakazakii in powdered and reconstituted infant formula milk


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Abstract

Enterobacter sakazakii has recently been recognized as an opportunistic foodborne pathogen. Dry infant formula has been implicated as the mode of transmission for this microorganism, which may cause severe form of neonatal meningitis and high mortality rate among infants. The objectives of this study were to investigate the heat resistance, growth pattern and inactivation of E. sakazakii in dry and reconstituted infant formula milk (IFM) under room and refrigeration temperatures. E. sakazakii strains (5 strains) showed wide variability in heat resistance at different temperatures (25, 50, and 63 °C). The D-values at 55 °C ranged from 1.51-14.83 min, at 60 °C from 0.17 to 2.71 min and at 63 °C from 0.05 min to 0.88 min. The calculated z-values for the studied E. sakazakii strains ranged from 3.76-10.11 °C. Household microwave was used to heat 60 ml portions of reconstituted IFM. The reconstituted IFM was inoculated with 1 × 10^5 CFU/ml of a mixture of four of the most heat-resistant strains of E. sakazakii. Heating reconstituted IFM for 20-30 s was not effective in reducing E. sakazakii. However, heating for 40-50 s was effective in eradicating all inoculated E. sakazakii. Additionally, storing powdered IFM for 15 d at refrigeration resulted in at least 1 log unit reduction in inoculated E. sakazakii...
strains. Whereas storing reconstituted IFM at refrigeration for two weeks resulted in more than 2 log units reduction in *E. sakazakii*. However, keeping reconstituted IFM at room temperature resulted in a very sharp increase in *E. sakazakii* count. *Lactobacillus acidophilus* was examined for its antimicrobial activity against *E. sakazakii*. However, no antimicrobial effect for *Lactobacillus acidophilus* was observed.

Introduction

*Enterobacter sakazakii*, a Gram-negative, rod-shaped, motile bacterium that belongs to the family *Enterobacteriaceae*, has recently been implicated in several cases of fatal neonatal meningitis. 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, immuno-compromised adult people with underlying medical conditions may also be a target for *E. sakazakii* infections (Gurtler et al., 2005). The reported facility rate associated with *E. sakazakii* infections is 40–60% (Bower and Braden, 2006).

Iverien and Fersythe (2003) speculated that *E. sakazakii* has a considerably low infectious dose of 1000 CFU/ml of reconstituted IFM. However, realistically, 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 difficult since *E. sakazakii* exhibits a remarkable resistance for a wide range of antibiotics (Gurtler et al., 2005).

*E. sakazakii* is a widespread microorganism (Kandhai et al., 2004; Farber, 2004; Arts, 2005) and it does not stand the heat of pasteurization used during 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 from the addition of ingredients at the powder 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 long time periods (~2 years) which 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 phospholipid membranes and proteins (Boeueve et al., 2003). Therefore, inactivation of *E. sakazakii* prior to feeding infants reconstituted IFM is crucial.

Reconstituted IFM is a non-sterile product that should be prepared, handled and stored appropriately, especially for neonates and premature infants. Usually very low numbers of *E. sakazakii* are detected in dry IFM. However, *E. sakazakii* has the capability to proliferate quickly if kept for extended periods of time in bottle heaters (Nazarowec-White and Farber, 1997a). Heat treatment prior to consumption of food has long been used as a primary means for reducing the risk of foodborne illness. Hence, determination of the thermal inactivation kinetics for *E. sakazakii* is important to apply effective heat treatment to inactivate this organism in reconstituted IFM. 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 growth pattern of *E. sakazakii* in powdered and reconstituted IFM at room and refrigeration temperatures. 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* DSM 292, *E. sakazakii* DSM 287, *E. sakazakii* 2.39-1, *E. sakazakii* 2.68, *E. sakazakii* 3, and *E. sakazakii* 55. All the strains were maintained refrigerated in tryptic soy agar (TSA) (Difco, Becton Dickinson, Sparks, MD) slants and transferred prior the experiment to Brain Heart Infusion broth (BHI) (Difco, Becton Dickinson, Sparks, 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 compared to exponential phase cells (Breeuer et al., 2003). The strains were provided by the Food Microbiology lab at Washington State University.

Heating regimen
IFM (Excamil with iron, Infant formula, Mead Johnson Nutritionals, Evansville, Ind) was purchased from 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. The tubes were inoculated with each one of the eight *E. sakazakii* strains (1 x 10^6 to 1 x 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 °C, 60 °C, and 63 °C, respectively. The temperature of the tubes was monitored by a type T thermocouple (Barnett 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 recovered at each treatment temperature immediately after reaching 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 submerging the tubes in the water bath (Al-Holy et al., 2004). The come-up-times were about 2.6 min. After completion of heating, the tubes were immersed promptly in a meshed ice bath at 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. The samples were spread-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, Sparks, 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 at least three replicate experiments. The standard deviations of the *D*-value and *z*-value were calculated.
Household microwave experiment

IFM was reconstituted in distilled water according to the manufacturer’s instruction. Sixty milliliters portion were dispensed in screw-capped dilution bottles, and then sterilized at 121 °C for 15 min. The bottles were inoculated with a mixture of four different strains of E. sakazakii, namely, E. sakazakii ATCC 12868, E. sakazakii ATCC 29004, E. sakazakii DSM 292, E. sakazakii DSM 287. These strains were selected because they exhibited the most heat resistance at the temperatures examined in the current study. The initial level of E. sakazakii was ca 1 x 10^5 CFU/ml. A household microwave (Sharp Carousel, Model No R-6209 HK, 60 Hz, Mahwah, 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 with a portable thermometer. The heated bottles were immersed immediately in crushed ice and tested for the presence of E. sakazakii survivors using the aforementioned overlay method. The experiment was repeated for at least three times.

Growth of capsulated and type strains of E. sakazakii at room and refrigeration temperatures

Two E. sakazakii strains (E. sakazakii DSM 292, E. sakazakii DSM 287) of strong biofilm (capsule) forming capability and two others (E. sakazakii DSM 239-1, E. sakazakii DSM 268) of weak biofilm forming capability were investigated in terms of their ability to grow in powdered and reconstituted IFM at room (21 °C) and refrigeration (4 °C) temperatures. The E. sakazakii strains were inoculated in 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 x 10^6 CFU/ml in the reconstituted IFM and ca 1 x 10^7 CFU/g in the dry IFM. Growth pattern of E. sakazakii was monitored by spread plating on VRBA every 2 d in the reconstituted IFM and every 3 d in the powdered IFM. The experiment was replicated three times.

Antimicrobial activity of L. acidophilus against E. sakazakii in reconstituted and powdered IFM

Lactobacillus acidophilus ATCC 4356 was activated by growing in MRS broth (Difco, Becton Dickinson, Sparks, MD) under aerobic conditions at 37 °C for 24 h. A loopful was streaked on 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 (ATCC 12868, ATCC 29004, DSM 292, and DSM 287) were grown for 24 h in BHI broth. Broths (15 ml) of E. sakazakii strains and of L. acidophilus ATCC 4356 were centrifuged by an RC-5 super-speed centrifuge (Dupont Instrument, Newtown, CT) at 6000 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. Thereafter, the bacterial/saline suspension (100 μl) was inoculated in either 10 ml tubes containing reconstituted IFM or to dry IFM (25g in sterile beaker.) L. acidophilus was added at a level of ca 1 x 10^6 CFU/ml or 1 x 10^5 CFU/g. Immediately after that the reconstituted IFM and the dry IFM were inoculated with a mixture of four different strains of E. sakazakii (ATCC 12868, ATCC 29004, DSM 292, and DSM 287) at a level of ca 1 x 10^6 CFU/ml or 1 x 10^5 CFU/g. The reconstituted and dry IFM were monitored for the presence of E. sakazakii by spread plating on VRBA every two days and incubation 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). The 2-phenyl ethanol was added because it inhibits 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 with Anaerogen sachet (Oxoid, Basingstoke, 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 (Accumen
Results and Discussion

Determination of 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 quality of the product. Fig. 1 shows first-order thermal inactivation kinetics survivor curves of an E. sakazakii strain 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-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 (Breeze et al., 2003) except for E. sakazakii ATCC 29004. Fig. 2 shows representative thermal death time curves for E. sakazakii 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). A pooled D-value equal to 10.30 min and 2.5 min at 60 °C for ten clinical and food isolates of E. sakazakii inoculated in reconstituted IFM was reported while the pooled z-value for those strains ranged between 3.8 and 6.9 °C (Nasr-Orge-White and Farber, 1997b). At 60 °C, the D-value for E. sakazakii ranged from 1.1-1.8 min and at 62 °C from 0.2-0.3 min, while a z-value of about 3.7 °C was obtained (Iversen et al., 2004). A very close z-value (5.6 °C) was also determined for E. sakazakii (Edelson-Mammel and Buchanan, 2004). However, it is worthwhile to mention that no obvious differences in D-values for the type E. sakazakii strains (2.39-1 and 2.68) and the capsule strains (FCS 292 and FCS 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 700014 (D-value = 0.88 min and z-value = 6.61 °C), the high temperature short time pasteurization (HTST) process will result in at least 6 log units reduction in the viable count of the most thermal-resistant strains 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.

Table 1. Decimal reduction times (D-values) and z-values (± standard deviation) of Enterobacter sakazakii strains inoculated in reconstituted infant formula

<table>
<thead>
<tr>
<th>Strain</th>
<th>D-value (min)</th>
<th>Z-value (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>55°C</td>
<td>60°C</td>
</tr>
<tr>
<td>E. sakazakii ATCC 12868</td>
<td>14.21±3.50</td>
<td>0.53±0.08</td>
</tr>
<tr>
<td>E. sakazakii ATCC 29004</td>
<td>14.83±3.50</td>
<td>2.71±0.32</td>
</tr>
<tr>
<td>E. sakazakii FSM 292</td>
<td>11.64±2.61</td>
<td>0.82±0.02</td>
</tr>
<tr>
<td>E. sakazakii FSM 287</td>
<td>1.85±0.03</td>
<td>0.45±0.03</td>
</tr>
<tr>
<td>E. sakazakii 2.39-1</td>
<td>3.93±0.34</td>
<td>0.45±0.09</td>
</tr>
<tr>
<td>E. sakazakii 2.68</td>
<td>5.02±1.00</td>
<td>0.45±0.01</td>
</tr>
</tbody>
</table>
*Values are average of at least three replicates*

![Graph](image_url)

**Fig. 1.** Survivor curves of *Enterobacter sakazakii* FSM 287 in reconstituted infant formula heated at 60 °C. Triplicate experiments were done.

![Graph](image_url)

**Fig. 2.** Thermal death time curves of *Enterobacter sakazakii* ATCC 12868 in reconstituted infant formula. Triplicate experiments were done.

A composite mixture of four of the most heat-resistant *E. sakazakii* strains were chosen for thermal treatment by a household microwave (Fig. 3). Despite the fact that scalding could be a problem when using microwave to heat reconstituted IFM, microwave is widely used to prepare reconstituted IFM to warm up formula before infant feeding because of the rapid heating pattern and the convenience of using microwave. Inoculated (about 5 log units of *E. sakazakii*)
reconstituted IFM was heated to different time intervals (0, 20, 30, 40, and 50 s). As expected, as the heating time increased, the extent of reduction proportionally increased. Microwaving for 40 s resulted in about 4 log units reduction in *E. sakazakii*, while heating for 50 s resulted in a complete elimination of *E. sakazakii* from heated reconstituted IFM. Therefore, apparently household microwave 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 more than 4 log units reduction in *E. sakazakii* count (Kindle et al., 1996).

![Graph](image)

Fig. 3. Thermal inactivation of a composite mixture of four strains of *Enterobacter sakazakii* (ATCC 12868, ATCC 29004, FSM 292, and FSM 287) in reconstituted infant formula heated by household microwave at different time intervals. The average temperatures measured immediately after heating were 21.0, 49.3, 65.3, 77.3, and 86.5 °C for the control (0 s), 20 s, 30 s, 40 s, and 50 s of microwave heating, respectively. Values are average of at least three replicates ± standard deviation. The samples were heated in 60 ml portions.

*E. sakazakii* does not tolerate chilling and appear to decrease gradually when stored under refrigeration conditions (4 °C) (Nazarowec-White and Farber, 1997a). Fig. 4 compares the capability of two strains of *E. sakazakii* that are known to form exopolysaccharide capsules and two non-capsule forms to grow in powdered IFM at room and refrigeration temperatures. After inoculation of both types in powdered IFM, capsaubl.Usageles strains increased by about 1.5 log units by day 6, and the same pattern was exhibited by the type strains till day 9 at room temperature. However, both types decreased dramatically thereafter and decreased by about 1 log unit after 15 d of storage compared to the initial *E. sakazakii* count at day 0. In comparison, both types (capsulated and type 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 reduction was more pronounced in the capsulated (−1.5 log units) strains compared to the type (−0.7 log units) strains of *E. sakazakii*. 
Fig. 4. Growth pattern of capsulated (Caps) (E. sakazakii FSM 252 and E. sakazakii FSM 287)
and type (E. sakazakii 2.39-1. and E. sakazakii 2.68) strains of Enterobacter sakazakii in
powdered infant formula stored at room (RT) (21 °C) and refrigeration (Ref) (4 °C)
temperatures.

Fig. 5 shows the growth pattern of capsulated and type strains of E. sakazakii in reconstituted
IFM at room (21 °C) and refrigeration (4 °C) temperatures. Both types increased dramatically at
room temperature until the end of the storage period where the numbers of E. sakazakii exceeded
9 log units. The viscosity of reconstituted IFM increased dramatically with time due to
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 log units) compared to the type (3.3 log
units) 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 growth. Prolonged periods of
storage or administration at room temperature might lead to proliferating the numbers of E. sakazakii that may be present. This is because E. sakazakii has a relatively short generation time at room temperature (~40 min) (Richards et al., 2005). These results suggest that, if not immediately consumed, storing powdered and reconstituted IFM under refrigeration is important to impede the growth of E. sakazakii.

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 some strains of E. coli and
E. sakazakii (Hayes et al., 2006). As shown in Fig. 6, L. acidophilus ATCC 4356 persists in
powdered IFM with low water activity (αw=0.29). However, it did not pose an antimicrobial
activity against E. sakazakii. In Fig. 7, the growth of L. acidophilus ATCC 4356 was
accompanied by a drop in the pH from ~6.5 to 3.0, nonetheless, E. sakazakii numbers increased
sharply, suggesting that this probiotic does not exhibit an inhibitory activity against E.
sakazakii.
Fig. 5. Growth pattern of capsulated (Caps) \textit{(E. sakazakii FSM 292 and \textit{E. sakazakii FSM} 287)} and type \textit{(E. sakazakii 2.39-1, \textit{E. sakazakii} 2.68)} strains of \textit{Enterobacter sakazakii} in reconstituted infant formula stored at room (RT) \textit{(21 °C)} and refrigeration (Ref) \textit{(4 °C)} temperatures.

Fig. 6. Growth pattern of a composite mixture of four strains of \textit{Enterobacter sakazakii} (ATCC 12868, ATCC 29004, FSM 292, and FSM 287) in powdered infant formula inoculated with \textit{L. acidophilus} ATCC 4356 and kept at room temperature \textit{(21 °C)} for 14 d.
Fig. 7. Growth pattern of a composite mixture of four strains of Enterobacter sakazakii (ATCC 12888, ATCC 29004, FSM 292, and FSM 287) and pH changes in reconstituted infant formula inoculated with L. acidophilus ATCC 4356 and kept at room temperature (21 °C) for 10 d.

Conclusions:
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 as revealed in their thermal inactivation kinetics. However, HTST pasteurization process is considered sufficient to inactivate all E. sakazakii strains. Household microwave (40-50 s for 60 ml portions) can be used to inactivate E. sakazakii if present in reconstituted 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.

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References
Comparative study between overlay method and selective-differential media for recovery of stressed *Enterobacter sakazakii* cells from infant formula milk

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Abstract

This study was done to compare the performance of different selective-differential media with the overlay method for recovery of stressed cells of *Enterobacter sakazakii* from infant formula milk (IFM). Five different selective-differential media were used in this study, namely: OK medium, violet red bile agar (VRBA), Druggan-Forsythe-Iversen agar (DFI), Enterobacteriaceae enrichment (EE) agar, and fecal coliform agar (FCA). Tryptic soy agar supplemented with 0.1% sodium pyruvate (TSAP) was used as a control. The overlay method involved adding a thin layer (8 ml) of each of the selective media onto TSAP. Reconstituted IFM was inoculated by ca 1 × 10^7 CFU/ml of a mixture of four strains of E. sakazakii and