SURVIVAL AND GROWTH OF ENTEROBACTER SAKAZAKII IN POWDERED AND RECONSTITUTED INFANT FORMULAS, PERFORMANCE OF MEDIA FOR RECOVERING STRESSED CELLS, AND SENSITIVITY OF THE PATHOGEN TO THE LACTOPEROXIDASE SYSTEM

by

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(Under the direction of LARRY R. BEUCHAT)

ABSTRACT

Studies were done to examine the survival of Enterobacter sakazakii in powdered and reconstituted infant formulas and the performance of media for recovering stressed cells. Sensitivity of the pathogen to the lactoperoxidase system (LPOS) was determined. The ability of tryptic soy agar supplemented with 0.1% pyruvate (TSAP) (control medium), two new fluorogenic agars developed by Leuschner, Baird, Donald, and Cox (LBDC) and Oh and Kang (OK), fecal coliform agar (FCA), Druggan-Forsythe-Iversen medium (DFI), VRBG agar, and Enterobacteriaceae enrichment agar (EE), to support colony development by healthy and heat-, freeze-, acid-, alkaline-, and desiccation-stressed cells of Enterobacter sakazakii was examined. The general order of performance of media for recovering control and heat-, freeze-, acid-, and alkaline-stressed cells by spiral plating was TSAP > LBDC > FCA > OK > VRBG > DFI > EE, which is similar to results obtained from desiccated cells. Results indicate that differential, selective media vary greatly in their ability to support resuscitation and colony formation by stressed cells of E. sakazakii. The ability of E. sakazakii to survive in six commercially
manufactured powdered infant formulas formulas ($a_w$ 0.25 – 0.86) for up to 12 months at 4, 21, and 30°C was determined. Populations decreased significantly in all formulas at $a_w$ 0.25 – 0.50 during storage for 1 month at 21 or 30°C, and again between 1 and 6 months in most formulas; significant reductions occurred between 6 and 12 months in some formulas. At all storage temperatures, reductions in populations tended to be greater in formulas at $a_w$ 0.43 – 0.86 than in formulas at $a_w$ 0.25 – 0.30. Survival of the pathogen was generally unaffected by composition of powdered infant formula. *E. sakazakii*, initially at a population of 0.02 CFU/ml, grew to populations of ≥ 1 log CFU/ml in reconstituted infant formulas held at 12, 21, and 30°C for 48, 12, and 8 h, respectively. Initially at a population of 0.53 CFU/ml, the pathogen grew to populations of ≥ 1 log CFU/ml in reconstituted infant formulas held at 12 and 21°C for 24 and 8 h, respectively, and to populations of > 3 log CFU/ml when held at 30°C for 8 h. Growth of *E. sakazakii* was not greatly influenced by the composition of formula. The lactoperoxidase system (LPOS), when applied to reconstituted infant formula using lactoperoxidase (LPO) at concentrations of 10 – 30 µg/ml, inhibited growth of *E. sakazakii* initially at 0.03 CFU/ml (≤ 25 CFU/100 g of dry powder) to less than 1 CFU/ml for up to 24 h at 21 – 37°C. Treatment of reconstituted infant formula with LPO at concentrations of 10 – 30 µg/ml prevented the growth of *E. sakazakii* in formulas held at 21, 30, and 37°C. This research provides information on the recovery of healthy and stressed *E. sakazakii* cells on selective/differential media, survival and growth of the pathogen in powdered or reconstituted infant formula, and the use of LPOS in preventing the growth of *E. sakazakii* in reconstituted infant formula.

INDEX WORDS: *Enterobacter sakazakii*, Powdered infant formula, Lactoperoxidase
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DEDICATION

I dedicate this work to the three most important people in my life. My beloved wife Jana has given me loving and tireless support during the last eight years of my education. Without her, I would not be here today. To my son Tristan Alexander – my inspiration and joy. To my Lord and God, Jesus Christ.
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TABLE OF CONTENTS

ACKNOWLEDGMENTS ............................................................................................................... v

LIST OF TABLES ....................................................................................................................... viii

LIST OF FIGURES ..................................................................................................................... xiv

CHAPTER

1 INTRODUCTION AND LITERATURE REVIEW ..........................................................1

2 PERFORMANCE OF MEDIA FOR RECOVERING STRESSED CELLS OF
   ENTEROBACTER SAKAZAKII AS DETERMINED USING SPIRAL
   PLATING AND ECOMETRIC TECHNIQUES.................................................................117

3 SURVIVAL OF ENTEROBACTER SAKAZAKII IN POWDERED INFANT
   FORMULA AS AFFECTED BY FORMULA COMPOSITION, WATER
   ACTIVITY, AND TEMPERATURE..............................................................................174

4 GROWTH OF ENTEROBACTER SAKAZAKII IN RECONSTITUTED
   INFANT FORMULA AS AFFECTED BY FORMULA COMPOSITION
   AND TEMPERATURE..................................................................................................211

5 VIABILITY OF ENTEROBACTER SAKAZAKII IN RECONSTITUTED INFANT
   FORMULA CONTAINING THE LACTOPEROXIDASE SYSTEM..............................234

6 SUMMARY AND CONCLUSIONS ...............................................................................263
LIST OF TABLES

Table 1-1: Contents of modified Castenholtz medium.................................................................14
Table 1-2: Method for analyzing powdered infant formula for presumptive E. sakazakii ............15
Table 1-3: Symptoms of E. sakazakii infection...........................................................................20
Table 1-4: Observations from case reports describing E. sakazakii infections .........................24
Table 1-5: Concentrations of chemical components used to create the lactoperoxidase (LPO) system ..................................................................................................................................................61
Table 1-6: Guidelines for preparation and handling infant formula............................................74
Table 1-7: Joint FAO/WHO recommendations to the powdered infant formula industry and infant caregivers concerning processing, preparing and handling powdered and reconstituted products ..................................................................................................................................76
Table 2-1: Populations of control and heat-stressed cells of Enterobacter sakazakii recovered on TSAP and differential selective media ..........................................................................................128
Table 2-2: Populations of control, heat-stressed, freeze-stressed, acid-stressed, and alkaline-stressed cells of a composite of four strains of Enterobacter sakazakii recovered on TSAP and differential, selective media .............................................................................................130
Table 2-3: D values for various E. sakazakii heated at various temperatures as reported in various studies.................................................................................................................................131
Table 2-4: Populations of control and freeze-stressed cells of Enterobacter sakazakii recovered on TSAP and differential, selective media ..................................................................................134
Table 2-5: Populations of control and acid-stressed cells of *Enterobacter sakazakii* recovered on TSAP and differential selective media ................................................................. 135

Table 2-6: pH values of *E. sakazakii* suspensions before and after acid stress treatment ........ 137

Table 2-7: Populations of control and alkaline-stressed cells of *Enterobacter sakazakii* recovered on TSAP and differential selective media ................................................................. 138

Table 2-8: pH values of *E. sakazakii* cultures before and after alkaline stress treatment ........ 140

Table 2-9: Populations of composites of four strains of control and heat-, freeze-, acid-, and alkaline-stressed cells of *E. sakazakii* recovered on TSAP and composites of differential, selective media ................................................................. 145

Table 2-10: Populations of desiccation-stressed cells of *Enterobacter sakazakii* recovered from powdered infant formula .................................................................................................... 146

Table 2-11: Populations of desiccation-stressed cells of *Enterobacter sakazakii* recovered from powdered infant formula stored at 21°C for 31 days on TSAP and differential, selective media .................................................................................................... 147

Table 2-12: Populations of desiccation-stressed cells of a composite of four strains of *Enterobacter sakazakii* recovered on TSAP and differential, selective media ...... 149

Table 2-13: Growth indices of heat-stressed, freeze-stressed, acid-stressed, and alkaline-stressed cells of *Enterobacter sakazakii* recovered on TSAP and differential, selective media using the ecometric technique .................................................................................................... 151

Table 2-14: Growth indices (GI) of a composite of four strains of control or heat-stressed, freeze-stressed, acid-stressed, and alkaline-stressed cells of *Enterobacter sakazakii* recovered on TSAP and differential, selective media using the ecometric technique .................................................................................................... 153
Table 2-15: Comparison of the performance of TSAP and differential, selective media in recovering Enterobacter sakazakii using spiral plating and ecometric evaluation

..................................................................................................................................................156

Table 2-16: Growth indices of a composite of four strains of heat-stressed, freeze-stressed, acid-stressed, and alkaline-stressed cells of Enterobacter sakazakii recovered on TSAP and a composite of differential, selective media determined using the ecometric technique ........................................................................................................................................157

Table 2-17: Differences in spiral plating and ecometric evaluation in the recovery of E. sakazakii plated on differential, selective media ........................................................................................................................................158

Table 2-18: Differences in spiral plating and ecometric evaluation in the recovery of E. sakazakii plated on TSAP ........................................................................................................................................159

Table 2-19: Morphological characterization of Enterobacter sakazakii strain ES132 on TSAP and differential, selective media ........................................................................................................................................161

Table 2-20: Morphological characterization of Enterobacter sakazakii strain 4921 on TSAP and differential, selective media ........................................................................................................................................162

Table 2-21: Morphological characterization of Enterobacter sakazakii strain 111389 on TSAP and differential, selective media ........................................................................................................................................163

Table 2-22: Morphological characterization of Enterobacter sakazakii strain FRM-TN on TSAP and differential, selective media ........................................................................................................................................164

Table 3-1: Commercially manufactured powdered infant formulas used ........................................................................................................................................179

Table 3-2: Detection of E. sakazakii by enrichment of powdered infant formulas inoculated with a low population of E. sakazakii (0.80 log CFU/g) and stored at 4, 21, or 30°C for up to 12 months........................................................................................................................................189
Table 3-3: Populations of *E. sakazakii* (log CFU/g) recovered from spray-inoculated powdered milk .................................................................198

Table 4-1: Commercially manufactured powdered infant formulas used ........................................246

Table 5-1: Survival of *Enterobacter sakazakii* recovered from in reconstituted infant formula inoculated with a low population (0.03 CFU/ml) and incubated for up to 24 h at 21, 30, or 37°C .................................................................................................................245

Table 5-2: Estimated generation times for *E. sakazakii* in infant formula inoculated with a high population of the pathogen (4.40 CFU/ml) for up to 24 h at 21, 30, or 37°C ..............247

Table 5-3: pH values of reconstituted powdered infant formulas inoculated with *E. sakazakii*, (0.03 CFU/ml) treated with the lactoperoxidase system, and stored for up to 24 h at 21, 30, or 37°C ...........................................................................................................250

Table 5-4: Populations of mesophilic aerobic bacteria in reconstituted infant formula inoculated with a high population of *E. sakazakii* (4.40 log CFU/ml), treated with the lactoperoxidase system, and incubated for up to 24 h at 21, 30, or 37°C ..............253

Table 5-5: pH values of reconstituted powdered infant formulas inoculated with *E. sakazakii* (4.40 log CFU/ml), treated with the lactoperoxidase system, and stored for up to 24 h at 21, 30, or 37°C ...........................................................................................................254
LIST OF FIGURES

Figure 3-1: Water activity of powdered infant formulas (codes A, B, and C) inoculated with *E. sakazakii* at 0.80 log CFU/g (low inoculum) and 4.69 – 4.86 log CFU/g (high inoculum) and stored at 4 (○), 21 (□), and 30ºC (△) for up to 12 months.................187

Figure 3-2: Water activity of powdered infant formulas (codes D, E, and F) inoculated with *E. sakazakii* at 0.80 log CFU/g (low inoculum) and 4.66 – 4.81 log CFU/g (high inoculum) and stored at 4 (○), 21 (□), and 30ºC (△) for up to 12 months.................188

Figure 3-3: Populations of *E. sakazakii* recovered from powdered infant formula (code A) as affected by initial aw 0.26 (○), 0.34 (□), and 0.49 (△), and storage temperature. Bars indicate standard deviations. Detection limit was 1 log CFU/g (10 CFU/g). Values less than 1 log CFU/g but more than 0 log CFU/g indicate that *E. sakazakii* was detected in one or more replicate samples.................................................................191

Figure 3-4: Populations of *E. sakazakii* recovered from powdered infant formula (code B) as affected by initial aw 0.27 (○), 0.33 (□), and 0.50 (△), and storage temperature. Bars indicate standard deviations. Detection limit was 1 log CFU/g (10 CFU/g). Values less than 1 log CFU/g but more than 0 log CFU/g indicate that *E. sakazakii* was detected in one or more replicate samples.................................................................192
Figure 3-5: Populations of \textit{E. sakazakii} recovered from powdered infant formula (code C) as affected by initial \(a_w\) 0.30 (○), 0.32 (□), and 0.44 (△), and storage temperature. Bars indicate standard deviations. Detection limit was 1 log CFU/g (10 CFU/g). Values less than 1 log CFU/g but more than 0 log CFU/g indicate that \textit{E. sakazakii} was detected in one or more replicate samples.................................................................193

Figure 3-6: Populations of \textit{E. sakazakii} recovered from powdered infant formula (code D) as affected by initial \(a_w\) 0.26 (○), 0.31 (□), and 0.44 (△), and storage temperature. Bars indicate standard deviations. Detection limit was 1 log CFU/g (10 CFU/g). Values less than 1 log CFU/g but more than 0 log CFU/g indicate that \textit{E. sakazakii} was detected in one or more replicate samples.................................................................194

Figure 3-7: Populations of \textit{E. sakazakii} recovered from powdered infant formula (code E) as affected by initial \(a_w\) 0.26 (○), 0.33 (□), and 0.44 (△), and storage temperature. Bars indicate standard deviations. Detection limit was 1 log CFU/g (10 CFU/g). Values less than 1 log CFU/g but more than 0 log CFU/g indicate that \textit{E. sakazakii} was detected in one or more replicate samples.................................................................195

Figure 3-8: Populations of \textit{E. sakazakii} recovered from powdered infant formula (code F) as affected by initial \(a_w\) 0.25 (○), 0.32 (□), and 0.43 (△), and storage temperature. Bars indicate standard deviations. Detection limit was 1 log CFU/g (10 CFU/g). Values less than 1 log CFU/g but more than 0 log CFU/g indicate that \textit{E. sakazakii} was detected in one or more replicate samples.................................................................196

Figure 3-9: Water activity of powdered infant formula (codes A and C) inoculated with \textit{E. sakazakii} (4.98 – 7.07 CFU/g) and stored at 4 (○), 21 (□), and 30°C (△) for up to 24 weeks .................................................................................................................................200
Figure 3-10: Population of *E. sakazakii* strain 2855 recovered from powdered infant formula (code A) as affected by initial *a*<sub>w</sub> 0.52 (●), 0.75 (□), 0.81 (△), and 0.86 (○) and storage temperature. Bars indicate standard deviations. Detection limit was 1 log CFU/g (10 CFU/g). Values less than 1 log CFU/g but more than 0 log CFU/g indicate that *E. sakazakii* was detected in one or more replicate samples.

Figure 3-11: Population of *E. sakazakii* strain 2855 recovered from powdered infant formula (code C) as affected by initial *a*<sub>w</sub> 0.61 (●), 0.72 (□), and 0.80 (△) and storage temperature. Bars indicate standard deviations. Detection limit was 1 log CFU/g (10 CFU/g). Values less than 1 log CFU/g but more than 0 log CFU/g indicate that *E. sakazakii* was detected in one or more replicate samples.

Figure 3-12: Population of *E. sakazakii* strain 3396 recovered from powdered infant formula (code A) as affected by initial *a*<sub>w</sub> 0.43 (●), 0.50 (□), 0.65 (△), and 0.81 (○) and storage temperature. Bars indicate standard deviations. Detection limit was 1 log CFU/g (10 CFU/g). Values less than 1 log CFU/g but more than 0 log CFU/g indicate that *E. sakazakii* was detected in one or more replicate samples.

Figure 3-13: Population of *E. sakazakii* strain 3396 recovered from powdered infant formula (code C) as affected by initial *a*<sub>w</sub> 0.53 (●), 0.57 (□), and 0.72 (△) and storage temperature. Bars indicate standard deviations. Detection limit was 1 log CFU/g (10 CFU/g). Values less than 1 log CFU/g but more than 0 log CFU/g indicate that *E. sakazakii* was detected in one or more replicate samples.
Figure 4-1: Populations (log CFU/ml) of *E. sakazakii* recovered from reconstituted infant formulas (codes A – C) inoculated at low (0.02 CFU/ml) or high (0.53 CFU/ml) populations of the pathogen and stored at 12 (□), 21 (Δ), or 30°C ( ) for up to 72 h. Samples were surface plated on TSAP. Bars indicate standard deviations. The detection limit was 1 CFU/ml (0 log CFU/ml).................................220

Figure 4-2: Populations (log CFU/ml) of *E. sakazakii* recovered from reconstituted infant formulas (codes D – F) inoculated at low (0.02 CFU/ml) or high (0.53 CFU/ml) numbers of the pathogen and stored at 12 (□), 21 (Δ), or 30°C ( ) for up to 72 h. Samples were surface plated on TSAP. Bars indicate standard deviations. The detection limit was 1 CFU/ml (0 log CFU/ml).................................................................221

Figure 4-3: Change in pH of reconstituted infant formulas (codes A – C) inoculated with low (0.02 CFU/ml) or high (0.53 CFU/ml) numbers of *E. sakazakii* and stored at 4 (○), 12 (□), 21 (Δ), or 30°C ( ) for up to 72 h. Bars indicate standard deviations .............224

Figure 4-4: Change in pH of reconstituted infant formulas (codes D – F) inoculated with low (0.02 CFU/ml) or high (0.53 CFU/ml) numbers of *E. sakazakii* and stored at 4 (○), 12 (□), 21 (Δ), or 30°C ( ) for up to 72 h. Bars indicate standard deviations .............225

Figure 4-5: Populations of mesophilic aerobic microorganisms (total aerobic mesophilic plate counts) recovered from reconstituted infant formulas (codes A – C) inoculated with low (0.02 CFU/ml) or high (0.53 CFU/ml) numbers of *E. sakazakii* and stored at 4 (○), 12 (□), 21 (Δ), or 30°C ( ) for up to 72 h. Bars indicate standard deviations. The detection limit was 1 CFU/ml (0 log CFU/ml)..................................................226

Figure 4-6: Populations of mesophilic aerobic microorganisms (total aerobic mesophilic plate counts) recovered from reconstituted infant formulas (codes D – F) inoculated with
low (0.02 CFU/ml) or high 0.53 CFU/ml) numbers of *E. sakazakii* and stored at 4 (◦), 12 (□), 21 (Δ), or 30ºC ( ) for up to 72 h. Bars indicate standard deviations. The detection limit was 1 CFU/ml (0 log CFU/ml)..........................................................................................227

Figure 5-1: Populations (log CFU/ml) of *E. sakazakii* recovered from reconstituted infant formula inoculated with a low population of the pathogen (0.03 CFU/ml) and stored at 21 (◦), 30 (□), or 37ºC (Δ) for up to 24 h. Samples were surface plated on TSAP. Bars indicate standard deviations. The detection limit was 1 CFU/ml (0 log CFU/ml) .........................................................................................................................244

Figure 5-2: Populations (log CFU/ml) of *E. sakazakii* recovered from reconstituted infant formula inoculated at a high (4.40 log CFU/ml) population, treated with 0 (◦), 5 (□), or 10 µg/ml (Δ) of lactoperoxidase, and stored for up to 24 h. Samples were surface plated on TSAP. Bars indicate standard deviations..........................................................248

Figure 5-3: Populations (log CFU/ml) of mesophilic aerobic bacteria recovered from reconstituted infant formula inoculated with a low (0.03 CFU/ml) populations of *E. sakazakii*, and treated with 0 (◦), 10 (□), 20 (Δ) or 30 ( ) µg of lactoperoxidase and stored for up to 24 h. Samples were surface plated on TSAP. Bars indicate standard deviations. The detection limit was 1 CFU/ml (0 log CFU/ml) .............................................251
INTRODUCTION AND LITERATURE REVIEW

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INTRODUCTION

Pangalos (1929) reported that an unidentifiable yellow-pigmented coliform was the causative bacterium in a case of septicemia in an infant. The first reported account implicating a bacterium now known as Enterobacter sakazakii, taking its name from Riichi Sakazaki, a Japanese microbiologist, as the causative agent in meningitis, however, is that of Urmenyi and Franklin (1961). They described two cases of terminal neonatal meningitis that occurred in 1958 in St. Albans, England. The next definitive report of infection caused by *E. sakazakii* described a child in Denmark that survived meningitis but experienced severe mental and neurological impairment (Joker et al., 1965). Reports from both England and Denmark listed the causal agent as an atypical yellow-pigmented *Enterobacter cloacae*.

The first recorded use of the name *E. sakazakii* was by Farmer et al. (1977) and Brenner et al. (1977). *E. sakazakii* was later distinguished from *E. cloacae* based on differences in DNA relatedness, pigment production, biotyping, and antibiotic susceptibility patterns (Farmer et al., 1980 and Izard et al., 1983). Five names previously used for the coliform, including the Urmenyi and Franklin bacillus, yellow coliform, yellow *Enterobacter*, pigmented cloacae A organism, and most notably, yellow-pigmented *E. cloacae* were listed. Additionally, NTCC cultures previously delineated as *Serratia* and *Chromobacter typhiflavum* were confirmed to be *E. sakazakii*.

Infections caused by *E. sakazakii* have been rarely reported. The bacterium has been implicated most frequently in causing illness in neonates and children from 3 days to 4 years of age, with at least 76 cases of *E. sakazakii* infections and 19 deaths in infants and children being reported (Iversen and Forsythe, 2003). At least nine cases of *E. sakazakii* infections in adults have been documented. Infectious neonatal and childhood cases have been reported to occur in
at least eight countries and nine states in the United States. Studies involving \textit{E. sakazakii} have largely focused on methods to eradicate the coliform from powdered infant formula, thermal resistance, environmental reservoirs, pathogenicity, antibiotic resistance, development of rapid methods of identification, exopolysaccharide production, subtyping, and predictive modeling. Although one study utilizing the suckling mouse model to determine virulence mechanisms and minimum infectious dose has suggested the possibility of enterotoxin production by \textit{E. sakazakii} (Pagotto et al., 2003), other virulence factors associated with the organism remain unknown. Thus, further work should be undertaken with respect to potential correlations of pathogenicity with pigmentation, shape, and texture of colonies and the use of other animal models and cell cultures as enterotoxin assay systems (Pagotto et al., 2003). Studies to determine conditions that influence survival and growth or cause death of \textit{E. sakazakii} in dry and reconstituted infant formulas are needed, assuming post-process contamination occurs. Research has not fully explored traditional or technologically advanced treatments for their efficacy in eliminating the pathogen from the dry powder. Other areas in need of research attention include studies of conditions affecting biofilm formation by \textit{E. sakazakii} in processing plants and hospital settings, competitive exclusion to control or prevent growth, commercial sanitizer efficacy, methods to recover and resuscitate injured cells, evaluation of practices associated with preparing and feeding infant formula in hospitals, surveys of neonatal wards and neonatal intensive care units for the presence of \textit{E. sakazakii}, and an evaluation of hygienic practices in the home that may contribute to neonatal infections.

Sources of \textit{E. sakazakii} associated with infant infections have, in most cases, not been confirmed. Studies corroborated by epidemiologic investigations, however, have implicated rehydrated powdered infant formula (a non-sterile product) as well as equipment and utensils
used to prepare rehydrated formulae in hospital settings (Bar-Oz et al., 2001, Biering et al., 1989, Clark et al., 1990, Muytjens and Kollee, 1990, Muytjens et al., 1983, Noriega et al., 1990 and Simmons et al., 1989; Van Acker et al., 1990). Voluntary recalls of infant formula containing *E. sakazakii* have occurred in the United States, which have led to decisions by manufacturers, hospital staff, and the U.S. Food and Drug Administration (FDA) to pursue higher microbiological standards and hygienic practices. This has resulted in an increased effort to seek solutions to eliminate infections caused by a bacterium about which sources of contamination, ecology, and virulence characteristics are not well understood.

**TAXONOMY AND BIOCHEMICAL CHARACTERIZATION**

Taxonomy, classification and nomenclature of genera in the family Enterobacteriaceae have evolved over the years based on various distinctions in serology, morphology, biochemical traits and genetic characteristics. There are 14 species or biogroups in the genus *Enterobacter* (Farmer, 1995). *E. sakazakii*, a motile, peritrichous, Gram-negative, non-sporulating, straight rod, previously known as yellow-pigmented *E. cloacae*, is considered a biovar in the genus *Enterobacter* (Monroe and Tift, 1979, Muytjens et al., 1983 and Nazarowec-White and Farber, 1997b). Major differences between *E. sakazakii* and other *Enterobacter* species have been traditionally thought to be its inability to ferment D-sorbitol and its ability to produce an extracellular deoxyribonuclease (Farmer et al., 1980). However, some strains of *E. sakazakii* more recently have been shown to ferment D-sorbitol (Heuvelink et al., 2001). *Enterobacter sakazakii* is α-glucosidase positive, which can be demonstrated in media containing 4-nitrophenyl-α-D-glucopyranoside (Muytjens et al., 1984). This assay has been recommended as a supplementary confirmation test to avoid false-positives (Muytjens, 1985).
Based on DNA–DNA hybridization showing yellow-pigmented strains to have less than 50% homology with non-pigmented strains, Brenner (1974) suggested that yellow-pigmented *E. cloacae* should comprise a new species. Phenotypic characterization and differentiation based on biochemical traits, serotyping, bacteriophage typing and antibiotic resistance are frequently among the first steps used to distinguish characteristics of isolates (Arbeit, 1995, Einstein, 1990 and Nazarowec-White and Farber, 1999). Some have suggested using phenotype tests (e.g., biotyping, bacteriocin typing, serotyping and phage typing) to differentiate *Enterobacter* species; however, none of these tests has proven effective in distinguishing strains within the species, nor can they be used for all species of *Enterobacter* (Gaston, 1988, Grattard et al., 1994, Nazarowec-White and Farber, 1999 and Poilane et al., 1993). Iversen et al. (2004f) investigated the phylogenetic relationships of *E. sakazakii* using 16S ribosomal DNA and *hsp60* sequencing. They found that strains were distributed among four clusters, indicating taxonomic heterogeneity. The type strain 16S rDNA sequence was 97.8% similar to that of *Citrobacter roseri* and 97.0% similar to that of *E. cloacae*. Studies have shown that the *Enterobacter* genus is polyphyletic (Loc-Corrillo et al., 2004). Strains currently classified as *E. sakazakii* fall into two distinct groups which can be further subdivided based on *hsp60* sequences. Both genotypes include clinical strains and do not correspond to biochemical profiles.

Farmer et al. (1980) extended the work of Brenner (1974) and Brenner et al. (1977) by further distinguishing 57 strains of yellow-pigmented *E. sakazakii* based on DNA hybridization, antibiotic susceptibility and biochemical reactions. Other distinguishing characteristics of the bacterium include greater pigment production at temperatures less than 36°C, with optimum pigment production at 25°C, survival of cells in stock cultures stored at 17–30°C without transfer for up to 8 years, utilization of citrate as a sole carbon source, 31–49% DNA–DNA homology.
with *E. cloacae*, and 57% guanine + cytosine ratio (Farmer et al., 1980). Production of the diffusible yellow pigment is unstable with repeated subculturing. The organism is negative for acid production from D-adonitol, D-arabitol, dulcitol, erythritol and glycerol (Farmer et al., 1980).

The halotype strain of *E. sakazakii* ATCC 29544 was examined by Farmer et al. (1980) for biochemical characteristics. Positive biochemical reactions included Voges-Proskauer, citrate-Simmons, arginine-Moellers, ornithine-Moellers, motility at 36°C, growth in KCN, D-glucose gas production, esculin hydrolysis, acetate utilization, nitrate to nitrite reduction, motility at 22°C, and DNase on toluidine blue (5 days at 25°C and 3 days at 36°C). Iversen and Forsythe (2003) reported the delayed toluidine blue reaction taking up to 7 days. The halotype strain produces acid from D-arabinose, cellobiose, i-inositol, lactose, maltose, D-mannitol, D-mannose, melibiose, a-methyl-D-glucoside, raffinose, sucrose, L-rhamonose, sucrose, trehalose, and D-xylose (Farmer et al., 1980). Negative biochemical reactions and tests for *E. sakazakii* listed by Farmer et al. (1980) included indole production, methyl red, hydrogen sulfide on TSI, lysine-Moellers, malonate utilization, mucate-acid production, tartrate-Jordan, lipase-corn oil, pectate hydrolysis, and tyrosine clearing. Krieg and Holt (1984) later reported that ca. 10% of *E. sakazakii* strains tested positive for the production of indole.

Freshly isolated *E. sakazakii* may produce colonies with two distinct morphologies (Farmer et al., 1980). One colony type is described as being either dry or mucoid, crenated (notched or scalloped) and leathery or rubbery when touched with a wire loop, i.e., very little biomass adheres to the loop and the colony snaps back to the agar when touched. These characteristics may be attributable to the production of a heteropolysaccharide (Harris and Oriel, 1989). A second colony morphology has been described as smooth and more amenable to
removal of cells from colonies with a wire loop. Cells that produce leathery colonies when subcultured from stock cultures may revert to the production of typical smooth colonies, some of which exhibit very slight pigment production. This differentiation in phenotypicity has been more recently described by Iversen and Forsythe (2003) as being either “matt” or “glossy.” Farmer et al. (1980) observed that *E. sakazakii* grown in broth tends to clump and sediment, a characteristic that has been also noted by others (Nazarowec-White and Farber, 1997b). Approximately 58% (33 of 57) of the strains examined were considered to be fecal coliforms on the basis of gas production in *Escherichia coli* (EC) broth incubated at 44.5°C for 48 h. It is recommended that screening be done to confirm that isolates of *E. cloacae* presumptive for *E. sakazakii* be tested for yellow pigment production, D-sorbitol fermentation and a delayed-reaction (7 days) DNase test. It is of interest to note that, for some bacteria, an increased level of polysaccharide production often results in subsequent pigment production (Jay, 2000). The few pigmented species in the family Enterobacteriaceae produce non-diffusible and endocellular pigments (Vidon et al., 1987). The 1984 edition of Bergy’s manual lists five species in this category: *Enterobacter sakazakii*, *Enterobacter agglomerans*, *Leclercia adecarboxylata*, formerly known as *Escherichia adecarboxylata* (Tamura et al., 1986), *Xenorhabdus luminescens*, and *Xenorhabdus nematophilus* (Krieg and Holt, 1984). At least three other species of the family have since been recognized as pigment producers: *Escherichia hermanii*, *E. vulneris*, and a strain of *Yersinia enterocolitica* (biovar I, strain 195A14J) isolated from milk in France (Vidon et al., 1987; Brenner et al., 1982a, Brenner et al., 1982b). The latter strain expressed pigment when incubated at 28°C for 24 - 48 h; however, pigmentation around the edges of colonies disappeared after 3 or 4 days growth (Vidon et al., 1987). Sporadic formation of non-pigmented colonies on agarose gel at 38°C was observed. Pigment extracted with methanol was insoluble in
light petroleum, chloroform, and ethylacetate. Spectrophotometric analysis showed that the pigment was non-carotenoid, yet contained a polyenic chain of 9±1 double bonds. Electrophoresis of DNA from yellow-pigmented cultures revealed the presence of a 42-kb plasmid. Non-pigmented colonies, however, lacked this plasmid, leading the authors to conclude that synthesis of the yellow pigment is genetically controlled by the plasmid. Others have reported that the yellow pigment production by *Erwinia herbicola* is controlled by a 350-Md plasmid (Gantotti and Beer, 1982).

Nazarowec-White and Farber (1999) tested nine clinical and eight food isolates of *E. sakazakii* in addition to the ATCC 29544 type strain (also a clinical isolate) for biochemical characterization as well as for antibiotic susceptibility. Using the API 20E® test, the seventeen isolates fell into three biotypes. Biotype 1 contained eight isolates, identical to the type culture, while biotypes 2 and three contained six and three isolates, respectively. Thus, six of the seventeen isolates (35%) that were biotype 2, which is inositol negative, are similar to 28% of the API database strains that are inositol negative. Biotype 3 was composed of three isolates that tested positive for the production of acetoin from glucose, i.e., the Voges-Proskeur test. These results are similar to those of Postupa and Aldova (1984) who found six of six strains to be Voges-Proskeur positive and four of six strains to be inositol negative, which agreed with the results of Farmer et al. (1980). Postupa and Aldova (1984) reported that 75% of 57 *E. sakazakii* strains were *i*-inositol positive while 98% were Voges-Proskeur positive. They also observed that inositol-negative strains increase the pH of growth media and inositol-positive strains cause media to be more acidic. Muytjens et al. (1984) reported *E. sakazakii* as unique in lacking the enzyme phosphoamidase. When cultures are treated ultrasonically, positive reactions became negative for leucine arylamidase, β-galactosidase, α-glucosidase, acid phosphatase, and N-acetyl-
β-glucosaminidase. Over 97% of 73 *E. sakazakii* isolates were found to produce Tween 80 esterase (Aldova et al., 1983). A later study confirmed these results (Postupa and Aldova, 1984).

In addition to phenotypic characterization of *E. sakazakii*, advances have been made in fingerprinting DNA and RNA by several techniques, e.g., PCR, randomly amplified polymorphic DNA (RAPD) PCR, pulsed-field gel electrophoresis (PFGE), chromosomal DNA restriction analysis, ribotyping and plasmid typing (Grant and Kroll, 1993, Farber, 1996 and Nazarowec-White and Farber, 1999). Nazarowec-White and Farber (1999) ribotyped *E. sakazakii* with the *Eco*RI restriction endonuclease and found that 18 isolates were represented by 10 ribotypes. This analysis has been determined to be more discriminatory than that of restriction endonuclease analysis (REA) (Clark et al., 1990). In another study, 30 *E. sakazakii* isolates from an infant formula factory comprised only 8 ribotypes (Anonymous, 1996). Kornacki (1998) isolated 17 *Eco*RI ribotypes from a factory environment. Nazarowec-White and Farber (1999) analyzed 18 isolates by PFGE using the restriction endonuclease *Xba*I and found each to have a distinct pattern. Characterization was superior to ribogrouping in that two sets of three isolates, comprising only two ribogroups, were distinguishable as six distinct pulsovars.

Farmer et al. (1980) examined 57 isolates of *E. sakazakii* and observed that all grew at 25°C and 45°C. None of the isolates grew at 4°C or 50°C, whereas at 47°C, 7 (12%) of the isolates failed to grow. Breeuwer et al. (2003) examined 22 strains of *E. sakazakii*, all capable of growing in brain heart infusion broth at 47°C. Nazarowec-White and Farber (1997c) reported that 12 strains of *E. sakazakii* grew at temperatures between 41 and 45°C. *E. sakazakii* and *Klebsiella pneumoniae* have been reported to grow more rapidly than *Pseudomonas aeruginosa*, *E. coli*, *Staphylococcus aureus*, *Mycobacterium terrae* and *Candida albicans* in rehydrated infant formula (Kindle et al., 1996). It was surmised that rapid growth of *E. sakazakii* might account
for nosocomial neonatal infections associated with the pathogen. Colonies formed on trypticase soy agar were reported to be 2–3 mm in diameter after 24 h at 36°C and 1–1.5 and 2–3 mm after 24 h and 48 h of incubation, respectively, at 25°C.

*E. sakazakii* has been shown to exhibit substantial resistance to acidic pH. Edelson-Mammel and Buchanan (2004a) examined survival characteristics of 12 strains of *E. sakazakii* in tryptic soy broth adjusted to pH 3.0 and 3.5 with HCl. Ten of twelve strains showed less than a 1-log decline over a 5-h period at 37°C; reductions in TSB at pH 3.0 were 4.9 to > 6.3 log CFU/ml. There was no correlation in acid resistance based on 1-h/pH 3.0 results and previously determined heat resistance of test strains (Edelson-Mammel and Buchanan, 2004c). Skladal et al. (1993) examined the fermentation of milk inoculated with 10 – 15 CFU of *E. sakazakii* per 500 ml and incubated at 30°C. Changes in pH and the production of L-lactate and D-lactate were monitored. *E. sakazakii* fermented milk rapidly, reducing the pH from 6.6 to 5.6 in less than 20 h. Concentrations of L-lactate and D-lactate reached 0.40 mM and 10.7 mM, respectively.

Conditions influencing the production of an exopolysaccharide (EPS) by *E. sakazakii* have been studied (Scheepe-Leberkuhne and Wagner, 1986). Distinguishing features of the complex were its high viscosity and gel formation in solution by means of trivalent cations. Attempts were made to optimize the production of EPS by increasing the carbon/nitrogen ratio in the growth medium. Production was highest in media supplemented with glucose and at an incubation temperature of 27°C. Maximum amounts of EPS produced by *E. sakazakii* in nutrient broth were obtained with a carbon/nitrogen ratio of 20.2:1 when glucose (15 g/l) was the carbon source (Scheepe-Leberkuhne and Wagner, 1986). The type of carbon source also affects EPS production, with glycerol being the most stimulatory and maltose, fructose, sucrose, glucose, mannose, galactose, ethanol, and xylose, in descending order, being less stimulatory. Nitrogen
sources that are most effective in stimulating polysaccharide production, in descending order were L-glutamine, D,L-alanine, yeast extract + casein hydrolysate, casein hydrolysate, urea, NO₃⁻, and NH₄⁺. Polysaccharide production by *E. sakazakii* began 4 h into the exponential growth stage and peaked at 18 h as measured by an increase in the viscosity of the growth medium. When compared to *A. viscosus*, *E. sakazakii* had greater viscosity at heteropolysaccharide concentrations greater than 0.25%. At a heteropolysaccharide concentration of 1%, *E. sakazakii* produced polysaccharide resulting in apparent viscosities of ca. 2,500 mPa·s vs. 1,000 mPa·s for the *A. viscosus* polysaccharide (Scheepe-Leberkuhne and Wagner, 1986).

A patent for a unique heteropolysaccharide produced by *E. sakazakii* has been awarded (Harris and Oriel, 1989). The invention was described by Harris and Oriel (1989) in the patent filing as a novel heteropolysaccharide produced by strains of *E. sakazakii*, such as *E. sakazakii* ATCC 53017, *E. sakazakii* ATCC 29004, and *E. sakazakii* ATCC12868. The patent filing went on to explain that the heteropolysaccharide is made up of 13 to 22% by weight of L-fucose, 19 to 24% by weight of D-galactose, 23 to 30% by weight of D-glucose, 0 to 8% by weight of D-mannose, and 29 to 32% by weight of glucuronic acid based on percentage of total carbohydrate. The heteropolysaccharide is purported to have many uses, e.g., as a suspending, thickening, or stabilizing agent, as well as a frictional drag reductant in aqueous systems.

Heteropolysaccharides such as xanthan gum produced by *Xanthomonas* are used in a wide array of applications, including gelling agents for detergents, sanitizers, and explosives, as suspending agents in starch, petroleum, and paints, and in the food industry as a friction reducing or stabilizing agent. Other heteropolysaccharides with patents include those produced by *K. pneumoniae* and *Bacillus polymyxa*. However, the heteropolysaccharide produced by *E.
sakazakii is unique as a viscoelastic reducer of the frictional drag of fluids. This property is similar yet superior to the heteropolysaccharide in okra mucilage. A concentration of only 0.01 to 2.0% is needed to reduce frictional drag for aqueous systems such as spray drift control of extinguishing fluids used in forest fires, for drinking or irrigation water, or for spray drift containment in the application of herbicides and pesticides in food crops (Harris and Oriel, 1989).

The patented E. sakazakii heteropolysaccharide was first found in tea and was subsequently referred to by its patent-holders as “tea-polysaccharide.” Of the strains tested, the one most preferred for the production of the polysaccharide was the ATCC strain 53017 first isolated from solar brewed tea held warm for several days. After first discovering and analyzing this organism, the researchers determined that it was about 3 microns in length, could grow from 25 - 45ºC, could carry out physiological and reproductive processes both aerobically and anaerobically, had a pH growth range of 5.0 – 9.0, and produced colonies characterized as either dry or mucoid (Harris and Oriel, 1989). The organism was observed to produce colonies that were yellow and leathery with a rubber-like texture. The patent holders stated that the bacillus produced the heteropolysaccharide polymer at temperatures between 20ºC and 40ºC and requires a simple carbohydrate source such as glucose or sucrose, and the polymer can be isolated from the growth medium by water miscible solvents such as ethanol, 2-propanol, or acetone. Perceived uses could be as a thickener in foods such as syrups, salad dressings, juices, puddings, and bakery fillings as well as in many industrial products such as paints, pastes, inks, adhesives, explosives, cleaners, polishes, and gels (Harris and Oriel, 1989). However, the polymer loses its unique rheological properties at pH higher than 10. The heteropolysaccharide was produced most efficiently in a modification of Castenholtz medium at a final pH of 7.2. To produce the
polymer, the modified Castenholtz medium (Table 1-1) is inoculated with *E. sakazakii* and incubated for 24 h at 30°C, after which the medium becomes gel-like, displaying typical viscoelastic characteristics by “snapping back” when swirled.

Others have shown that glucose enhances EPS production by *Enterobacter aerogenes*, *Xanthomonas campestris* (Sutherland, 1983) and *Arthrobacter viscosus* (Gasdorf et al., 1965), and patents have been filed for the production of gums from *E. sakazakii* (Harris and Oriel, 1989 and Yang, 2002).

**ISOLATION, IDENTIFICATION AND TYPING**

In addition to phenotypic characterization of bacterial isolates, advances have been made in fingerprinting DNA and RNA by multiple techniques, viz., PCR, randomly amplified polymorphic DNA (RAPD) PCR, pulsed-field gel electrophoresis (PFGE), chromosomal DNA restriction analysis, ribotyping, and plasmid typing (Nazarowec-White and Farber, 1999; Grant and Kroll, 1993; Farber, 1996). Nazarowec-White and Farber (1999) ribotyped *E. sakazakii* with the EcoR1 restriction endonuclease and found that 18 isolates represented 10 ribotypes. This analysis has been determined to be more discriminatory than that of restriction endonuclease analysis (REA) of *E. sakazakii* strains alone (Clark et al., 1990). In a similar study, 30 *E. sakazakii* isolates from one infant formula factory showed only 8 ribotypes (Anon., 1996). Nazarowec-White and Farber (1999), in analyzing 18 isolates by PFGE with the restriction endonuclease Xba1, found each to have a distinct PFGE pattern. Characterization was superior to ribogrouping in that two sets of three isolates, comprising only two ribogroups, were distinguishable as six distinct pulsovars. This is not surprising, as another study of 70 *Vibrio cholerae* isolates revealed 14 PFGE patterns comprising one ribogroup (Cameron et al., 1994). The 18 isolates were also analyzed via RAPD with one primer, resulting in 18 molecularly
Table 1-1. Contents of modified Castenholtz medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Ferric ammonium citrate</td>
<td>0.02 g</td>
</tr>
<tr>
<td>Nitrilotriacetic acid</td>
<td>0.04 g</td>
</tr>
<tr>
<td>Trace metal solution</td>
<td>4.0 g</td>
</tr>
<tr>
<td>CaCl$^2$</td>
<td>0.2 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.04 g</td>
</tr>
<tr>
<td>MgSO$^4$</td>
<td>0.40 g</td>
</tr>
<tr>
<td>NaNO$^3$</td>
<td>2.80 g</td>
</tr>
<tr>
<td>NaHPO$^4$</td>
<td>0.44 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>4.0 g</td>
</tr>
<tr>
<td>Tryptone</td>
<td>4.0 g</td>
</tr>
<tr>
<td>D-glucose</td>
<td>100 g</td>
</tr>
</tbody>
</table>

distinguishable patterns. Although both PFGE and RAPD were the most sensitive of the five discriminatory methods, which also included biotyping, antibiotic susceptibility testing, and ribotyping, the authors concluded that RAPD is the preferred technique based on simplicity, cost feasibility, and same-day results.

The U.S. Food and Drug Administration, 2002d and U.S. Food and Drug Administration, 2002f developed a method to isolate and enumerate *E. sakazakii* in dehydrated powdered infant formula (Table 1-2). This method is the same as that proposed by Muynjens et al. (1988) and Nazarowec-White and Farber (1997c), with the exceptions that the powdered infant formula is
Table 1-2. Method for analyzing powdered infant formula for presumptive E. sakazakia

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Sterilize can lid margins and sampling spoons</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>• Dilute 100 g, 10 g, and 1 g, of powdered infant formula with pre-warmed sterile water at 1:10 ratio, mix and incubate.</td>
<td>Overnight</td>
<td>36°C</td>
</tr>
<tr>
<td>• Add 10 ml of each suspension to 90 ml of Enterobacteriaceae enrichment broth and incubate.</td>
<td>Overnight</td>
<td>36°C</td>
</tr>
<tr>
<td>• Mix suspensions and surface plate 0.1 ml on VRBG agar, streak on VRBG agar with a 10 μl inoculating loop onto three quadrants for isolation and incubate.</td>
<td>Overnight</td>
<td>36°C</td>
</tr>
<tr>
<td>• Pick 5 presumptive positive E. sakazakii colonies from both sets of VRBG plates and subculture by streaking onto TSA and incubate</td>
<td>48-72 h</td>
<td>25°C</td>
</tr>
<tr>
<td>• Select yellow-pigmented colonies only and confirm per manufacturers instructions for the API 20E® biochemical confirmation system.</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>• Calculate the Most Probable Number (MPN) by the number of positive tubes at each dilution</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

a From FDA (2002d).

rehydrated with distilled water in the FDA method rather than with buffered peptone water, and enriched samples (0.1 ml) are streaked on violet red bile glucose (VRBG) agar rather than pour-plating 1.0 ml in VRBG agar. Leclercq et al. (2002) examined the growth of E. sakazakii on fecal coliform agar, a medium developed by Hsing-Chen and Wu (1992), and found the medium to support slightly higher recovery than violet red bile lactose agar. It has been suggested that the distinguishing quality of Tween 80 esterase production by most strains of E. sakazakii could be used to help confirm presumptive isolates (Aldova et al., 1983 and Postupa and Aldová, 1984).

E. sakazakii is α-glucosidase positive, which can be demonstrated in media containing 4-nitrophenyl-α-D-glucopyranoside (Muytjens et al., 1984). This assay has been recommended as a supplementary confirmation test to avoid false-positive test conclusions (Muytjens, 1985) and
is a basis for the differential reaction on Druggan, Forsythe, and Iversen agar, a medium developed for isolating *E. sakazakii* (Iversen et al., 2004c). A 4-h α-glucosidase reaction was used to differentiate 29 presumptive *E. sakazakii* isolates from 152 environmental samples originating from three milk powder factories from other coliforms, two of which produced α-glucosidase, but within 24 h and not 4 h (Kandhai et al., 2004b).

A medium for presumptive detection of *E. sakazakii* in infant formula was developed by Leuscher et al. (2004). It is based on the production of α-glucosidase, which differentiates *E. sakazakii* from other species in the family Enterobacteriaceae. *E. sakazakii* forms yellow colonies that fluoresce under UV light on nutrient agar supplemented with 4-methyl-umbelliferyl α-D-glucoside (α-MUG). Other representatives of Enterobacteriaceae and non-Enterobacteriaceae, e.g., *Acinetobacter* sp., *Escherichia hermanii*, *Cedaceae lepagii*, *Leclercia acecarboxylata* and *Enterobacter agglomerans* isolated from infant formulae, also produced yellow pigment, but colonies did not fluoresce under UV light. The medium performed well in an interlaboratory study designed to detect *E. sakazakii* in powdered infant formula (Leuscher and Bew, 2004). In an independent study, another medium containing α-MUG was also shown to be reliable for differentiation and isolation of *E. sakazakii* (Oh and Kang, 2004 and Oh et al., 2004).

Kandhai et al. (2004b) developed a 4-h colorimetric screening assay based on α-glucosidase production by cells in yellow-pigmented colonies formed on tryptic soy agar. The colony is dispersed in saline containing paranitrophenyl-α-D-glucopyranoside, incubated at 37°C and examined spectrophotometrically for the formation of yellow-colored paranitrophenyl hydrolysate. Using this method, *E. sakazakii* was isolated from 18 of 152 environmental samples collected from three milk powder production plants.
A chromogenic medium (Druggan–Forsythe–Iversen agar, DFI) was formulated for selective detection of *E. sakazakii* (Druggan et al., 2004 and Iversen et al., 2004c). The medium is based on the α-glucosidase reaction, which is detected using 5-bromo-4-chloro-3-indolyl-α,D-glucopyranoside (XαGlc). Ninety-five clinical and food isolates of *E. sakazakii* were detected on DFI agar 2 days sooner than using the FDA (2002d) method. Characteristics of 148 strains representing 17 genera of Enterobacteraceae other than *E. sakazakii* were compared using DFI agar and VRBG agar. Only 19 strains representing three genera gave false-positive results on DFI agar. This compares to 31 strains giving false-positive results using the standard method.

There is a need for improvement in the current, largely phenotypically based, approach for detecting and confirming presumptive *E. sakazakii* isolates. The Enterotube II® as well as the API 20E® systems have been used for presumptive-positive confirmations of *E. sakazakii* via biochemical characteristics (Biering et al., 1989, Cottyn et al., 2001, Gassem, 1999, Kandhai et al., 2004b, Monroe and Tift, 1979, Mosso et al., 1994, Muytjens et al., 1983, Nazarowec-White and Farber, 1999, No et al., 2002, Seo et al., 2003, Simmons et al., 1989, Van Os et al., 1996 and Willis and Robinson, 1988). Recent findings, however, indicate that API 20E biochemical test strips can give false-negative as well as false-positive results (Iversen et al., 2004b). It was concluded that further biochemical characterization is needed to determine the traits most strongly associated with strains of *E. sakazakii* falling into two distinct genotypes.

Seo et al. (2003) reported confirming *E. sakazakii* via API ZYM® and Vitek® assays. Cottyn et al. (2001) used fatty acid methylester (FAME) analysis as well as API 20E® strips and Biolog® strips as confirmation assays. Lee and Kim (2003) recovered and identified *E. sakazakii* from a semi-pilot potable water system via membrane filtration, m-Endo agar, API 20E® kits, and the Microbial Identification (MIDI) system in which whole-cell fatty acid methyl ester profiles were
analyzed. Muytjens et al. (1983) identified *E. sakazakii* by DNase reactions after incubating cultures for 2 and 7 days, in addition to using the API 20E® assay.

Selection of an appropriate screening approach has an important bearing on the validity of the identification. Iversen and Forsythe (2004a) reported that three strains identified as *E. sakazakii* by the API 20E® assay were identified as *E. cloacae, Enterobacter amnigenus, and Enterobacter cloacae/gergoviae* by API 32E®, whereas 10 strains identified as *E. sakazakii* by API 32E® gave patterns consistent with *Pantoea* species by API 20E®. The close phenotypic similarities between *E. sakazakii* and *E. cloacae* and the existence of some sorbitol-positive *E. sakazakii* as well as some non-pigmented *E. sakazakii* strains causes one to wonder about the true incidence of *E. sakazakii* infections, given that *E. cloacae* is commonly reported in neonatal, nosocomial infections and has been traced to contaminated enteral feeding (Levy et al., 1989).

Seo et al. (2003) developed a real-time PCR assay to detect *E. sakazakii* by designing primers and probes using the partial macromolecular synthesis (MMS) operon of *E. sakazakii*. The assay was specific for differentiating *E. sakazakii* and *E. cloacae*, and almost 50 other genera of Enterobacteriaceae, allowing detection of as few as 100 CFU of *E. sakazakii* per ml of infant formula without enrichment. In a limited study of *E. sakazakii*-contaminated product (5 of 22 samples), the population was about 4 cells in 1000 g (Zink, 2003). Consequently, enrichment procedures would be necessary to increase the population to about 100 CFU/ml to enable detection. Given that the protocol for analyzing dry infant formula requires a 1:10 dilution in enrichment broth, about 15 generations or approximately 4–5 h at an optimal growth temperature would be required to reach this population.

Further characterization of *E. sakazakii* isolated from food and environmental samples can be accomplished using pulsed field gel electrophoresis (PFGE) restriction fragment length
polymorphism (RFLP) testing, multilocus enzyme electrophoresis tests, or ribotyping. Other potential methods of analyses include testing for antibiotic resistance patterns (antibiograms), toxin assays, hemagglutination, serotyping and phage typing. Nazarowec-White et al. (2003) and Farber (2004) recommended that laboratories type all \( E. \ sakazakii \) isolates for molecular characteristics to facilitate epidemiologic investigations and as a means to identify new infection vehicles. Williams et al. (2004) recently described a method to differentiate strains of \( E. \ sakazakii \) based on protein biomarkers. The biomarkers were sequenced to provide insight into why certain strains were more thermo-tolerant than others.

**CLINICAL ETIOLOGY AND PATHOGENICITY**

\( Enterobacter \) species can create community infections, are responsible for approximately half of all nosocomially acquired infections and are often implicated in co-infections (Borderon et al., 1996, Chang et al., 2000, Hervas et al., 2001, Huang et al., 2001, Leclerc et al., 2001, Nazarowec-White and Farber, 1997b and Wenger et al., 1997). Symptoms of \( E. \ sakazakii \) infections in infants, adults and elderly patients are listed in Table 1-3. Studies have shown that patients with extended hospital visits, especially in intensive care units, are at heightened risk for contracting infections from \( Enterobacter \) species (Al Ansari et al., 1994, Burchard et al., 1986, Flynn et al., 1987, Gallagher, 1990, Georghiou et al., 1995, Kuhn et al., 1991, McConkey et al., 1989, Pitout et al., 1997 and Sanders and Sanders, 1997). Others who may be at heightened risk include those previously treated with antibiotics, the immunocompromised, the elderly, patients with medical implants and those with acute, chronic, or serious illnesses (Pitout et al., 1997 and Sanders and Sanders, 1997). In 1992, \( Enterobacter \) species were reported as being the fifth and third most common among those recovered from the urinary and respiratory tracts, respectively, of patients in intensive care units (Jarvis and Martone, 1992). At least one report suggests that
<table>
<thead>
<tr>
<th>Symptom</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Infections reported in infants include</strong></td>
<td></td>
</tr>
<tr>
<td>formation</td>
<td></td>
</tr>
<tr>
<td>Conjunctivitis</td>
<td>Bar-Oz et al., 2001, Block et al., 2002 and Reina et al., 1989</td>
</tr>
<tr>
<td>Bulging fontanelles, destruction of the frontal lobes of the brain,</td>
<td>Willis and Robinson, 1988</td>
</tr>
<tr>
<td>seizures, spastic quadriplegia, hypothermia, fever, Cheyne-Stokes</td>
<td></td>
</tr>
<tr>
<td>respirations, bradycardia, poor feeding, irritability, jaundice,</td>
<td></td>
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<tr>
<td>grunting respirations, instability of body temperature, hemorrhagic</td>
<td></td>
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<tr>
<td>cerebral necrosis, meningoencephalitis, necrotic softened brain,</td>
<td></td>
</tr>
<tr>
<td>cyst formation, liquefaction of cerebral white matter and severe</td>
<td></td>
</tr>
<tr>
<td>neurologic complications</td>
<td></td>
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<tr>
<td><strong>Infections associated with catheters</strong></td>
<td>Arseni et al., 1985 and Block et al., 2002</td>
</tr>
<tr>
<td>Meningital patients displaying early signs of illness such as grunting,</td>
<td>Muytjens et al., 1983</td>
</tr>
<tr>
<td>pallor, cyanosis and collapse, bulging fontanelle, convulsions,</td>
<td></td>
</tr>
<tr>
<td>twitching and hypertonia</td>
<td></td>
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<tr>
<td>Necrotizing enterocolitis in neonates</td>
<td>Muytjens et al., 1983 and Van Acker et al., 2001</td>
</tr>
<tr>
<td>Brain abscesses and meningitis in neonates</td>
<td>Burdette and Santos, 2000, Kleiman et al., 1981, Muytjens et al., 1983 and Ries et al., 1994</td>
</tr>
<tr>
<td>Ventriculitis, cerebral infarction, brain cysts, late development of</td>
<td>Bar-Oz et al., 2001, Burdette and Santos, 2000 and Gallagher and Ball, 1991</td>
</tr>
<tr>
<td>hydrocephalus and brain abscesses</td>
<td></td>
</tr>
<tr>
<td>Bacteremia</td>
<td>Block et al., 2002, Burdette and Santos, 2000, Monroe and Tift, 1979, Nazarowec-White and Farber, 1997b and Noriega et al., 1990</td>
</tr>
</tbody>
</table>

(continued)

<table>
<thead>
<tr>
<th>Infections reported in adults include</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coinfective agent (among three bacteria) isolated from an ulceration in the foot of a diabetic</td>
<td>Pribyl et al., 1985</td>
</tr>
<tr>
<td>Appendicitis</td>
<td>Reina et al., 1989</td>
</tr>
<tr>
<td>Biliary sepsis</td>
<td>Lai, 2001</td>
</tr>
<tr>
<td>Infections in elderly patients include</td>
<td>Hawkins et al., 1991 and Nazarowec-White and Farber, 1997b</td>
</tr>
<tr>
<td>Urosepsis</td>
<td>Jimenez and Gimenez, 1982</td>
</tr>
<tr>
<td>Bacteremia</td>
<td>Hawkins et al., 1991</td>
</tr>
<tr>
<td>Infection in a vascular graft and a thigh wound</td>
<td>Dennison and Morris, 2002</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>Lai, 2001</td>
</tr>
</tbody>
</table>

*Table 1-3. (Continued)*
Young, 1993). Only one case of *E. sakazakii* sepsis was identified among 10,660 very low-birth-weight infants, suggesting that outside of an epidemic situation, *E. sakazakii* infection is very rare in these infants (Stoll et al., 2004). At least nine cases have also been reported among adults, all immunocompromised with underlying medical conditions (Dennison and Morris, 2002, Emery and Weymouth, 1997, Hawkins et al., 1991, Jimenez and Gimenez, 1982, Pribyl et al., 1985 and Lai, 2001).

The first reported cases of *E. sakazakii* were in 1958 in England (Urmenyi and Franklin, 1961). Additional cases of neonatal and infant *E. sakazakii* infections have been reported and further described in Canada and Belgium (Van Acker et al., 2001), Denmark (Joker et al., 1965), Iceland (Biering et al., 1989), Germany (Ries et al., 1994), Greece (Arseni et al., 1985), Israel (Bar-Oz et al., 2001 and Block et al., 2002), The Netherlands (Muytjens et al., 1983) Spain (Reina et al., 1989), and the United States (Burdette and Santos, 2000, Gallagher and Ball, 1991, Himelright et al., 2002, Kleiman et al., 1981, Lai, 2001, Monroe and Tift, 1979, Noriega et al., 1990, Simmons et al., 1989, Weir, 2002 and Willis and Robinson, 1988). In the United States cases, neonatal *E. sakazakii* infections have been reported in at least nine states. Iversen and Forsythe (2003) reported that at least 76 cases of neonatal *E. sakazakii* infections were documented to occur worldwide between 1958 and 2003. Contributing factors in neonates that might increase the risk of infection include immunosuppression, premature birth and low birth weight (Health Canada, 2002 and Himelright et al., 2002). One of the first two reported cases of neonatal *E. sakakzakii* infection involved a full-term newborn, although premature birth often accompanies and appears to result in a predilection to infection (Urmenyi and Franklin, 1961). The International Commission for Microbiological Specifications for Foods (2002) classified *E.*
sakazakii as a severe hazard for restricted populations, causing life-threatening or substantial chronic sequelae or illness of long duration.

Some common infective species of Enterobacter, particularly in newborns, include meningitis, cloaceae, aerogenes, agglomerans, hormechie, gergovial and sakazakii (Nazarowec-White and Farber, 1997b, Weir, 2002 and Willis and Robinson, 1988). Muyltjens et al. (1983) estimated, however, that 50–75% of septicemia and meningitis cases in neonates are attributable to Streptococcus agalactiae and E. coli infections. E. sakazakii is an opportunistic pathogen most commonly affecting immunocompromised neonates (Naqvi et al., 1985 and Willis and Robinson, 1988). The bacterium has been isolated from or implicated in numerous types of infections (Table 1-4). It is of interest that a review of 17 cases of neonatal meningitis revealed that patients with E. sakazakii infections fared worse than those with more frequently occurring meningitis caused by other Gram-negative bacteria, including E. cloaceae (Willis and Robinson, 1988). Lai (2001) reviewed reported cases of E. sakazakii infections and outbreaks, eliminating those lacking relevant or clinical information. Observations in these reports as well as those in hospital records are summarized in Table 1-4.

Very few cases of E. sakazakii infections have been reported in adults, who are considered a low-risk group (Burdette and Santos, 2000, Gallagher and Ball, 1991, Kleiman et al., 1981, Muyltjens et al., 1983 and Ries et al., 1994). Dennison and Morris (2002) described a case in which the patient required extended treatment with broad-spectrum antibiotics due to multi-antibiotic resistance of the infectious strain. Neonatal infections have been reported to arise via contact with E. sakazakii in the birth canal or through post-birth environmental sources (Monroe and Tift, 1979, Steere et al., 1975 and Tift, 1977). Pathogenesis in neonates frequently involves bacteremia and/or sepsis, cerebrospinal fluid (CSF) infection and meningitis, brain
abscess and infarction, ventricle compartmentalization due to necrosis of brain tissue and liquefaction of white cerebral matter, cranial cystic changes, fluid collection and dilated

Table 1-4. Observations from case reports describing *E. sakazakii* infections

- In the University of Massachusetts Medical Center, 3.6% of patients with septicemia (from 1994 to 1996) were positive for *Enterobacter* species, 0.4% of patients with septicemia were positive for *E. sakazakii* in 1996, and five cases were diagnosed with nosocomially acquired *E. sakazakii* infections in 1995–1996.

- Thirty-one cases of infections in infants and children ranging from 3 days to 4 years linked to *E. sakazakii* were reported in the literature.

- 50% of children infected with *E. sakazakii* were < 1 week old.

- 75% of children infected with *E. sakazakii* were < 1 month old.

- 43% of those cases with meningitis experienced seizures.

- Case fatality rates of infants with non-meningital *E. sakazakii* infections were 33%, whereas meningitis patients had a higher fatality rate of 45%.

- After 1985, patients were treated with the third-generation cephalosporins in addition to combinations of ampicillin, gentamicin and chloramphenicol, which reduced the case fatality rate in meningital *E. sakazakii* patients from 62% to 14%.

- There were only four cases of *E. sakazakii* infections in adults reported in the literature and an additional four reported from the University of Massachusetts Medical Center.

- All adult *E. sakazakii* cases were in-patients ≥ 56 years of age, except for one 39-year-old male.

- Mean and median ages for the adult patients were 68 and 76 years, respectively.

- Case mortality rate of adult patients was 50%, with the University of Massachusetts Medical Center experiencing a 75% mortality rate.

- Most of the adult cases involved underlying complicating medical factors including 50% with malignancies.

- Sites of infection for the eight adult cases included bacteremia, osteomyelitis of the foot, pneumonia, and biliary sepsis.

\[a\]Summarized from Lai (2001).

ventricles and hemorrhagic and non-hemorrhagic intercerebral infarctions leading to cysticencephalomalacia (softening of the brain) and has been associated with necrotizing enterocolitis (Gallagher and Ball, 1991, Kleiman et al., 1981, Kline, 1988a, Ries et al., 1994,

Meningitis, an acute inflammation of the meninges surrounding the brain and the spinal chord, frequently results in mortality. Infection is most commonly caused by *Haemophilus*, meningococci and pneumococci in infants, as well as *E. coli*, *Enterobacter*, *Citrobacter diversus* and *Listeria* (Iversen and Forsythe, 2003, Kline, 1988a, Kline, 1988b and Nazarowec-White and Farber, 1997b). Meningitis is the most frequently reported condition in neonatal *E. sakazakii* infections, resulting in ca. 90% of the cases leading to brain abscesses (Burdette and Santos, 2000, Gallagher and Ball, 1991 and Kline, 1988a). These infections often increase inner cranial pressure, requiring aspiration of fluid and drainage of cerebral infarction, sometimes including insertion of a ventriculoperitoneal shunt to prevent cerebral damage (Muytjens et al., 1983).

Low birth weight (i.e., under 2.5 kg) has been identified as a contributor to higher risk of contracting illness (Muytjens et al., 1983). Neonatal meningitis caused by *Enterobacter* species is predictably high, with up to 92% mortality reported in cases involving *E. cloacae* (Rance et al., 1962). Meningital *E. sakazakii* infection has been reported as arising between the fourth and
fifth day after birth and can be fatal within a few hours to several days following the first clinical signs (Muytjens et al., 1983). Neonatal brain abscesses from Gram-negative rods are most frequently caused by *C. diversus* and *E. coli* (Foreman et al., 1984, Gallagher and Ball, 1991, Kline, 1988a and Kline, 1988b). Infant mortality for *E. sakazakii* meningitis is 40–80%, with death often occurring within hours of infection (Adamson and Rogers, 1981, Arseni et al., 1987, Joker et al., 1965, Kleiman et al., 1981, Muytjens et al., 1983, Nazarowec-White and Farber, 1997b, Urmenyi and Franklin, 1961 and Willis and Robinson, 1988). Seizure activity has been reported in about one-third of the cases of neonatal *E. sakazakii* meningitis, with physiological responses including grunting, bulging fontanelles, convulsions, twitching and an increase in cranial circumference (Muytjens et al., 1983 and Weir, 2002). These infections can cause hemorrhagic and non-hemorrhagic intercerebral infarctions, leading to cystic encephalomalacia (Ries et al., 1994). Up to 20% of newborns develop serious neurological complications following infection (Gebremariam, 1998).

The pathogenesis of neonatal *E. sakazakii* meningitis has not been fully defined. The process is believed to be by translocation of the bacterium through the chordus plexus and subsequent cellular invasion by means of pathogenic secretory factors (e.g., elastases, glycopeptides, endotoxins, collagenases and proteases) used to increase blood–brain barrier permeability, thus gaining access to the nutrient-rich cerebral matter (Iversen and Forsythe, 2003). Others have reported a similarity between the tropism of *E. sakazakii* and *C. diversus* for invasion of and infection in the central nervous system (Willis and Robinson, 1988, Kline, 1988a, Kline, 1988b and Burdette and Santos, 2000).

Bowen and Braden (2006) reviewed 46 cases of invasive infant *E. sakazakii* infections to assess risk factors. They reported that 40% of infected infants, were delivered via cesarean
section, eliminating the likelihood of *E. sakazakii* contamination via the birth canal. Ninety-two percent of the infants, for whom feeding information was available, consumed a reconstituted powdered infant formula and the pathogen was recovered from 68% of formulas in 22 cases investigated. Bowen and Braden (2006) also reported that 87% of *E. sakazakii* isolates recovered from these formulas were indistinguishable from clinical strains isolated from patients’ strains when characterized by biotype or genotype.

*E. sakazakii* can cause bacteremia and meningitis in neonatal infants by invading the intestinal epithelium. Increased intestinal permeability and subsequent invasion by gram-negative bacteria may be enhanced by heat stable toxins produced in the lipopolysaccharide membrane (Townsend et al., 2007). Seventy-five samples of powdered infant formula representing 31 brands from nine countries were found by Townsend et al. (2007) to contain endotoxins ranging from 40 to $5.5 \times 10^4$ endotoxin units (EU) per g.

*E. sakazakii* infections historically have been thought to originate from maternal vaginal contamination during passage of the infant through the birth canal; however, studies suggest that this hypothesis is implausible (Muytjens et al., 1983). For suspect cases of neonatal meningitis, many have recommended early detection by means of cranial ultrasonography, computed tomography scan (CT scan), or magnetic resonance imaging (MRI) (Bar-Oz et al., 2001, Burdette and Santos, 2000, Gallagher and Ball, 1991, Iversen and Forsythe, 2003 and Kline, 1988b). Monroe and Tift (1979) were the first to report a case of *E. sakazakii*-associated bacteremia in the absence of meningitis.

Many neonatal cases of *E. sakazakii* meningitis may have some relationship to necrotizing enterocolitis, which is associated with several bacterial pathogens and is the most common gastrointestinal disease in newborns (Muytjens et al., 1983 and Van Acker et al., 2001).
The illness affects ca. 2–5% of premature neonates, leads to death in 10–55% (Peter et al., 1999) and is characterized by ischaemia, bacterial colonization of the intestinal tract and increased levels of protein in the gastrointestinal lumen, the latter often attributable to the consumption of infant formula (Iversen and Forsythe, 2003). A positive correlation between necrotizing enterocolitis and oral formula feeding has been suggested (Kosloske, 1984, Iversen and Forsythe, 2003 and Van Acker et al., 2001). Babies fed only infant formula rather than breast milk are 10 times more likely to contract necrotizing enterocolitis (Lucas and Cole, 1990). Another study confirmed that in 125 infants, prior to the administration of antibiotics, *Enterobacter* species were the most prevalent bacteria, present in 29% of neonates (Chan et al., 1994).

The specific virulence factors of *E. sakazakii* remain elusive; however, pathogenicity of and production of exotoxins, aerobactin and hemagglutinin by *E. cloacae* have been documented (Keller et al., 1998). Until Pagotto et al. (2003) used the suckling mouse model, no animal model specifically addressing the minimum infectious dose, lethal dose, or virulence of *E. sakazakii* had been described. In these experiments, mice were challenged intraperitoneally and perorally to test for the production of enterotoxin by nine clinical isolates and eight food isolates of *E. sakazakii*, in addition to the type strain (ATCC 29544). The study also included negative and positive controls. Mice were challenged, after which they were euthenized and the intestines were examined for distension and fluid accumulation. Cell cultures (CHO, Vero, and Y-1 lines) were also tested for cytopathic effects caused by the bacterium. Four of eighteen strains (three clinical isolates and one food isolate) or 22% tested positive for production of enterotoxin. One strain of *E. sakazakii* was toxic to all three cell lines. Another strain was positive for enterotoxin production but negative for cytopathicity against cell cultures. Minimum lethal doses for the three *E. sakazakii* strains were the same, regardless of the method of challenge. All 18 strains...
were lethal to mice at oral doses of $10^8$ CFU/mouse; however, the type strain, ATCC 29544, tested negative for enterotoxin production. One clinical isolate and one food isolate were lethal via the peroral route at $10^7$ CFU, and one clinical isolate and one food isolate were lethal by intraperitoneal injection at populations as low as $10^5$ CFU. The authors concluded, by extrapolating from the suckling mouse model, that a minimum lethal dose in neonates would most likely require an unusually high number of viable cells such as might occur over time in temperature-abused infant formula. A recent study showed that lipopolysaccharide from *E. sakazakii* is toxic to N2a cells via the MTT test (Iversen et al., 2004e). Protease, phosphatase and lipase activities may contribute to host cell death.

In the more than 76 documented cases of neonatal and infant *E. sakazakii* infections, the infectious dose was not determined. Iversen and Forsythe (2003) speculated that a good first estimate for infection should be close to that postulated for *E. coli* O157:H7, *Listeria monocytogenes* 4b, or *Neisseria meningitidis*, i.e., ca. 1000 CFU. They noted that *Enterobacter* would not encounter extremely harsh pH conditions in the upper gastrointestinal tract of neonates and would pass rapidly into the small intestines. The conclusion by Iversen and Forsythe (2003) that outbreaks of *E. sakazakii* infections are due to gross temperature abuse or poor hygienic practices was questioned by Havelaar and Zwietering (2004), who provided another approach to assessing the risk of infections. Iversen and Forsythe (2004b) subsequently questioned the model, noting that it is based on assumptions and therefore needs to be verified.

Based on a level of contamination of ca. 0.36 – 66 CFU/100 g reported by Muytjens et al. (1988) and Nazarowec-White and Farber (1997c), and 18 g of powder reconstituted in a single feeding, at least 14 generations would be needed to produce 6000 CFU/feeding. This would require ca. 7 h at 37°C vs. 17.9 h at 21°C, 1.7 days at 18°C, 7.9 days at 10°C, and nearly 9 days
at 8°C (Iversen and Forsythe, 2003). However, these calculations are theoretical, as a fraction of a bacterial cell per bottle will not occur. The formula in the bottle will be either contaminated or not contaminated.

Adegbola and Old (1983) described fimbrial haemagglutinism in the genus Enterobacter. Two of four strains *E. sakazakii* were fimbriate mannose sensitive, haemagglutinin positive, produced thick fimbriae 7 – 8 nm in diameter and tested positive for a strong antibody coating. The other two strains were non-fimbriate and haemagglutinin negative. They observed that with increasing haemagglutinating power, the proportion of fimbriate bacteria increased.

**OUTBREAKS AND CASES OF INFECTIONS**

The number of documented outbreaks and cases of neonatal *E. sakazakii* infections are few. Summaries of some of the reports describing these infections are presented here.

**1958, St. Albans, England.** The first two documented cases of neonatal *E. sakazakii* meningitis occurred in 1958 (Urmenyi and Franklin, 1961). At that time, however, the bacterium was described as an unusual pigmented strain of the *cloacae* group. The two infants were born within a week of each other and died within 2 days of each other. The first infant was born to a mother experiencing mild toxemia. The child was readmitted to the hospital 12 h after discharge due to “left-sided fits” and jaundice. Additional complications included an increased pulse rate, enlarged liver and bulging anterior fontanelle from meningitis. The *E. sakazakii* isolate from the patient was sensitive to chloramphenicol and streptomycin; however, the infant died after 48 h treatment with oxytetracycline. The second infant was one of a pair of twins. Five days after birth, she abruptly collapsed with complications from jaundice, cerebral trauma and a hive-like rash and died shortly thereafter. Postmortem examinations revealed that white cranial matter in both infants had degenerated into a soft hemorrhagic mass. Brain, cerebrospinal fluid (CSF) and
bronchial swabs in the first infant, and brain, bronchial, liver and marrow swabs in the second infant tested positive for *E. sakazakii*. Environmental samples taken several days after infection, including swabs of the suspect incubator, were not positive for *E. sakazakii*. Nevertheless, it was surmised that the reason only one of the twins succumbed to the infection might be that the child was placed in the same incubator that had been used for an infected infant. The clinical isolate produced only slightly yellow pigmentation when cultured on nutrient agar at 37°C, yet produced a non-diffusible yellow-gold pigment when incubated at room temperature. This was the first confirmed case now recognized to have been caused by *E. sakazakii*. Septicemia due to a yellow-pigmented coliform, however, had been reported previously (Pangalos, 1929).

**1965, Denmark.** Joker et al. (1965) reported a case of neonatal meningitis caused by *E. sakazakii*, described as an atypical *Enterobacter*, forming yellow colonies on ordinary media. The patient was a 3.2-kg (6.9-lb) female delivered as a primigravida (first-time pregnancy) after 27 h of labor. Two days after birth, the child was suspected of having meningitis. *Enterobacter* was isolated from three specimens of cerebrospinal fluid but blood, feces and throat cultures tested negative. The child appeared to have recovered at 27 days and was discharged. At 2 months of age, she was re-admitted to the hospital with convulsions and an electroencephalogram showed severely abnormal brain waves. Following an aggressive antibiotic administration regimen of streptomycin, chloramphenicol, ampicillin, sulfadiazine, sulfadimidine, sulfamerazine and sulfacombin, the child recovered at about 4 months of age, yet experienced extreme mental impairment. Joker et al. (1965) did not state whether the child was fed breast milk or rehydrated powdered infant formula but attributed meningitis to the prolonged delivery period and cited a publication implicating extended labor as a causal factor in neonatal meningitis (Groover et al., 1961).
1979, Macon, GA, USA. Monroe and Tift (1979) reported a male term infant (birth weight 2.6 kg) as having *E. sakazakii* bacteremia at 7 days of age. Ampicillin administration for 10 days ameliorated the situation. Environmental samples of the nursery and/or samples from other patients and personnel were not taken. The physicians surmised that the source of infection was post-birth as evidenced from the 6-day delay in symptoms. This was the first reported case of non-meningital bacteremia caused by *E. sakazakii*.

1981, Indianapolis, IN, USA. Kleiman et al. (1981) reported a full-term, 5-week-old infant developing meningoencephalitis and cerebral ventricular compartmentation resulting in bulging fontanelles and grand mal seizures. Ampicillin and gentamicin treatments were administered and the child was discharged. Two months later, the circumference of the child's head increased and insertion of a ventriculoperitoneal shunt was performed. Tan 2–3-mm-diameter colonies of *E. sakazakii* formed on chocolate agar incubated under an atmosphere containing 10% carbon dioxide and 90% air and on CDC-anaerobe blood agar under a 10/5/85% mixture of hydrogen, carbon dioxide and nitrogen, respectively. Yellow-pigmented colonies developed when the culture was incubated aerobically for 48 h at 25°C. The child recovered from the acute illness after antibiotic therapy but was severely developmentally delayed.

1983, The Netherlands. Over a 6-year period, eight cases of neonatal meningitis involving *E. sakazakii* were reported in The Netherlands, with two of the eight patients also experiencing necrotizing enterocolitis (Muytjens et al., 1983). The bacterium was isolated from the blood and CSF of all eight patients, two of whom were delivered by Caesarean section. It was also confirmed that at least four of the eight infants were not colonized at birth (Muytjens, 1985). Twenty-three *Enterobacter* isolates were recovered from CSF of six patients, with eight of these testing positive for *E. sakazakii*. The remaining 15 were *E. cloacae, E. agglomerans*
and *E. aerogenes*. *Enterobacter* species but no *E. sakazakii* was isolated from the blood of patients. *E. sakazakii* isolates indistinguishable from those from CSF were recovered from prepared infant formula as well as from utensils used to prepare the formula. Muytjens and Kollee (1990) later reported that upon opting for sterile liquid formula in place of powdered formula, no further cases of *E. sakazakii* infections were reported in the following 8 years.

1985, Athens, Greece. Arseni et al. (1985) reported a case of neonatal septicemia caused by *E. sakazakii* co-mingled with *K. pneumoniae* in the neonatal intensive care unit of a children's hospital. The patient was born premature and sepsis was discovered 3 days after birth. Only *E. sakazakii* was isolated from the umbilical catheter as a pure culture, although both bacteria were isolated from blood. The two isolates were resistant to ampicillin, netilmicin, cefotaxime and amikacin. While the patient was being treated, an unspecified number of other infants was colonized, yet not infected by *E. sakazakii*, as revealed by rectal and throat swabs.

1986–1987, Reykjavik, Iceland. Three cases of *E. sakazakii* meningitis in full-term male neonates, two of whom were more than 38 weeks old, in Iceland were reported by Biering et al. (1989). One infant was treated with antibiotics but was severely mentally retarded and quadriplegic after recovery from infection. The second child was diagnosed with Down syndrome and died of complications from the *E. sakazakii* infection 5 days after birth. The third child was treated with antibiotics, after which he developed a seizure disorder and was moderately delayed in all developmental areas. All infants had been fed a rehydrated powdered infant formula administered within 2 h of preparation. *E. sakazakii* was not found on formula preparation utensils, in the preparation kitchen, or in environmental samples. An isolate was recovered from a time-indefinite bottle of refrigerated infant formula but not from freshly prepared formula. *E. sakazakii* was recovered in rehydrated samples from five packages of
rehydrated formula incubated for 4 h at 36°C. Formula from all lots tested was positive for the coliform. In addition, *E. cloacae* and *E. agglomerans* were recovered from the powdered formula. *E. sakazakii* was also recovered from urine, as well as from groin and anal swabs of a 3-day-old asymptomatic male child. Twenty-two of the twenty-three isolates from rehydrated formula were identical in biotype, antibiotic profile and plasmid profile to the four neonatal strains (Clark et al., 1990). It was concluded that infections must have originated from the rehydrated infant formula stemming from unknown contributing factors in the infected infants, as many neonates, including a twin brother of one infected patient, had received the same contaminated infant formula with no detectable pathogenicity. One of the causal factors may have been that formula bottles were occasionally left in heaters at 35–37°C for lengthy periods of time.

1987, Boston, MA, and New Orleans, LA, USA. Willis and Robinson (1988) reported that two infants, 4 weeks and 8 days of age, developed *E. sakazakii*-induced meningitis, necessitating ventricular shunts. Infections led to cerebral destruction, developmental damage and severe neurologic complications in both babies but subsided following treatment with moxalactam. No information was provided concerning possible sources of contamination; however, it was stated that the patients were never geographically proximate.

1988, Memphis, TN, USA. Simmons et al. (1989) reported an outbreak of neonatal *E. sakazakii*-induced septicemia and meningitis clearly linked to powdered infant formula which contained *E. sakazakii* and *E. cloacae* at populations of 8 CFU/100 g and 48 CFU/100 g, respectively. The outbreak involved four pre-term neonates that exhibited one or more symptoms of bacteremia, septicemia, urinary tract infection, abdominal distension and bloody diarrhea or stool. A blender was implicated as the possible source of contamination after testing
positive for *E. sakazakii* and *E. cloacae*. Other bacterial contaminants included *Pseudomonas fluorescens* and *Pseudomonas maltophilia*. The latter of the pseudomonads was isolated from CSF and stools of two of the patients. The blender was routinely rinsed with potable water and hand-cleaned on occasion with hexachlorophene and chlorhexidine. *E. sakazakii* infections of infants did not occur after use of the contaminated blender was discontinued and only sterilized blenders were used. It was determined that all isolates from the infant formula and three isolates from infants had the same plasmid and multilocus enzyme profile (Clark et al., 1990).

1990, Baltimore, MD, USA. Noriega et al. (1990) reported a case involving a 6-month-old female who had developed septicemia following small bowel complications, including an exploratory laparotomy and a gastrostomy tube. Blood cultures were positive for both *E. sakazakii* and *Leuconostoc mesenteroides*, while stool cultures were negative for both bacteria. The central venous catheter tip contained *E. sakazakii*. Following treatment with vancomycin and ampicillin, the infection resided. The authors reported that the blender used to rehydrate the powdered infant formula was heavily contaminated with *E. sakazakii* and *L. mesenteroides*. The hospital implemented a quality control protocol in which the blender was washed and autoclaved daily following each use. Rehydrated formula was also terminally pasteurized after receipt at the hospital and before administration.

1990, Cincinnati, OH, USA. Gallagher and Ball (1991) reported that an infant developed complications 2 days after birth. Ampicillin and cefotaxime were prescribed after a blood culture tested positive for the presence of *E. sakazakii*. Ultrasound and CT scans 4, 6, and 20 days after birth revealed a hemorrhage, abscess and brain infarction. After 28 days of antibiotic treatment, the problem was abated and the child was discharged. Twelve days later,
the infant developed meningitis and antibiotic therapy was commenced. CT scans revealed a large cranial cyst purulent with *S. aureus*.

**1995/1996, Boston, MA, USA.** Five cases of *E. sakazakii* infection were reported between January 1995 and December 1996 involving individuals 3, 39, 73, 76 and 82 years of age (Lai, 2001). All patients were experiencing complicative, potentially immunosuppressive illnesses and were being treated accordingly. Symptoms and treatments included carcinoma with radiation therapy, insertion of a gastronomy tube and emergent tracheostomy, jaundice and hepatic complications, aortic surgery with accompanying abdominal distress and fever and cecal volvulus. Only the two youngest patients survived following antibiotic treatment with gentamicin, cefotaxime, ceftazidime, cefuroxime axetil and clindamycin.

**1998, Belgium.** Van Acker et al. (2001) reported an outbreak of 12 cases of neonatal necrotizing enterocolitis in which four patients required operative treatment and male twins died within 3 weeks of each other in a Belgium neonatal intensive care unit (NICU). All 12 patients had been fed reconstituted infant formula prior to the illness, 10 of whom were fed with formula manufactured by a single manufacturer. Six of the twelve patients tested positive for *E. sakazakii* via blood culture, anal swabs and stomach aspirates. Two *E. sakazakii* strains with two differing yet unspecified morphologies were isolated from one patient. Eleven strains of *E. sakazakii* were isolated from the six *E. sakazakii*-positive patients. A survey of unopened cans of powdered infant formula yielded 14 *E. sakazakii* isolates. Molecular typing was carried out via arbitrarily primed PCR using the ERIC2 primer (enterobacterial repetitive intergenic consensus motif; 5’-AAGTAAGTGACTGGGGTGAGCG). Suspecting a link between the formula and the necrotizing enterocolitis, feeding of the formula was discontinued. The manufacturer's quality control records revealed that five samples were analyzed prior to distribution of the product.
One sample contained 20 coliforms/g, with < 1 coliform/g in the remaining four samples. The Codex Alimentarius standard requires a minimum of four of five control samples with < 3 coliforms/g, and a maximum of one of five samples with > 3 but ≤ 20 coliforms/g (FAO, 1994). Belgian law is more stringent, requiring < 1 coliform/g in all samples.

**2000, Winston Salem, NC, USA.** Burdette and Santos (2000) reported that a 3.3-kg (7.28-lb) female neonate born at week 35 of gestation developed symptoms typical of *E. sakazakii* infection, i.e., brain abscess 6 days after birth, including a high fever, irritability and seizure activity. A CT scan showed abnormal cerebritis-like indicators in the frontal lobe of the brain. Magnetic resonance imaging was used to identify infected cavities and lesions. The bacterium was isolated from both blood and CSF but not from urine. The acute condition cleared up after administration of ampicillin, cefotaxime and intravenous bactrim and draining purulent fluid via an emergency craniotomy. This fluid tested positive for *E. sakazakii*. The authors stated that this was the first reported case of *E. sakazakii* being isolated directly from a drained cranial abscess. Five weeks after admission, the child was discharged with no apparent neurological or developmental deficits.

**1993, 1995, 1997–2000, Israel.** Bar-Oz et al. (2001) reported two cases of neonatal meningitis that occurred in the NICU of a hospital in Israel in December 1999–January 2000. Both infants were underweight at birth, weighing 2.1 kg (4.75 lb) and 0.6 kg (1.37 lb), respectively. The first child was a full-term healthy female that had been fed rehydrated powdered infant formula and developed complications on the fourth day after birth. *E. sakazakii* was recovered from the CSF and blood. The child developed seizures and was confirmed by magnetic resonance imaging (MRI) and CT scans as having infarction, liquefaction and cavitation of the brain. The child required cefotaxime and a ventriculoperitoneal shunt to reduce
cranial pressure. The second child, born 9 weeks premature, was fed rehydrated infant formula by an enteral feeding tube. Nine days following birth, the child was diagnosed with an upper gastrointestinal hemorrhage and *E. sakazakii* was recovered from blood but not the CSF. The child recovered after a 10-day treatment with cefotaxime. As both children were born via Caesarean section, contamination attributable to passage through the birth canal was excluded. In addition to the two infected infants, three additional infants had *E. sakazakii*-positive stools for 3, 4 and 8 weeks, respectively, demonstrating colonization without infection. Antibiotic treatment did not eliminate *E. sakazakii* from these patients. Samples from the blender and rehydrated formula from the preparation kitchen were positive for *E. sakazakii*, yet samples of all other rehydrated formula, infant formula powder and preparation personnel were negative. The blender, noted to have a small crack in the base (Block et al., 2002), was no longer used for formula preparation but continued to test positive for *E. sakazakii* for 5 months.

Block et al. (2002) described four additional cases of *E. sakazakii* bacteremia, three of which occurred at hospitals in Israel. The first case involved a full-term formula-fed neonate in 1993. The second case occurred in 1995 in which *E. sakazakii* conjunctivitis developed in a female delivered by Caesarean section. The third case involved a 6-year-old boy in 1997 who had received a bone marrow transplant at the age of 3 days. *E. sakazakii* was isolated from a broviac catheter. A fourth case in 1998 involved a female 6 days after birth who was delivered vaginally and developed *E. sakazakii* meningitis.

**2001, Knoxville, TN, USA.** Himelright et al. (2002) and Weir (2002) described a 2001 outbreak of *E. sakazakii* infections in Tennessee in which 49 infants in a NICU were screened, with 10 infants testing positive for the bacterium and 1 dying of meningitis following 9 days of intravenous treatment with antibiotics. Seven of the ten *E. sakazakii*-positive neonates were
colonized but did not show symptoms of infection. The source of the bacterium was traced to a powdered infant formula specific for individuals with nutritional and malabsorption problems. Following this outbreak, the hang time, i.e., the length of time a given rehydrated infant formula is administered to an infant, was reduced from 8 h to 4 h (Himelright et al., 2002).

**ANTIBIOTIC RESISTANCE**

The frequency of clinical treatments of patients with broad spectrum antibiotics is thought to be a contributing cause of increased numbers of multi-antibiotic-resistant microorganisms, including *Enterobacter* species (Borderon et al., 1996, Burwen et al., 1994, Delphine et al., 2001, Ehrhardt and Sanders, 1993, Hervas et al., 2001, Huang et al., 2001, Landry et al., 1991, Pitout et al., 1997, Sakata and Maruyama, 1997, Sanders and Sanders, 1997, Snydman, 1991 and Wust et al., 1994). Pitout et al. (1997) cited numerous studies showing that as the number of patients in hospitals increases, so increases the number of isolates of *Enterobacter* resistant to quinolones, β-lactams and trimethoprim-sulfamethoxazole (Burwen et al., 1994, Landry et al., 1991, Pitout et al., 1997, Sanders and Sanders, 1997 and Wust et al., 1994). The amount of antibiotics utilized also increases as the size of the hospital increases, thus potentially exposing microorganisms to more as well as a wider array of antimicrobials. This theory is supported by Sakata and Maruyama (1997), who reported a steady emergence of multiple multi-antibiotic-resistant strains of *E. cloacae* from 1982 to 1995 corresponded with the type and volume of antibiotics administered. Effective means to treat antibiotic-resistant *Enterobacter* have been limited. Carbapenems or fluoroquinolones are commonly used to treat infected patients, although resistance to both antibiotics has been noted (Sanders and Sanders, 1997).
Because bacterial meningitis necessitates effective treatment, antibiotic susceptibility is of increased concern with neonatal *E. sakazakii* infections (Eng et al., 1987). *E. sakazakii* has been reported as being more sensitive than other *Enterobacter* species to some antibiotics, including typical susceptibility to aminoglycosides, ureidopenicillins, ampicillin and carboxypenicillins (Adamson and Rogers, 1981, Farmer et al., 1980, Hawkins et al., 1991, Jimenez and Gimenez, 1982, Muytjens and van der Ros-van De Repe, 1986, Monroe and Tift, 1979, Nazarowec-White and Farber, 1999 and Willis and Robinson, 1988). Farmer et al. (1980) tested 10 strains of *E. sakazakii* for antibiotic susceptibility and determined that the minimum inhibitory concentrations (MICs) to chloramphenicol and ampicillin were moderate, at 4–8 μg/ml and 2–4 μg/ml, respectively. Examination of 24 strains of *E. sakazakii* using the Kirby–Bauer disk method revealed that 96% were sensitive to nalidixic acid (30 μg), 100% to gentamicin (10 μg), 92% to streptomycin (10 μg), 100% to kanamycin (30 μg), 87% to tetracycline (30 μg), 100% to chloramphenicol (30 μg), 100% to ampicillin (10 μg) and 87% to carbenicillin (100 μg). The same strains were less susceptible to penicillin (0% at 10 U), cephalothin (13% at 30 μg), sulfadiazine (67% at 250 μg), and colistin (71% at 10 μg).

*E. sakazakii* infections have been traditionally treated with ampicillin in combination with gentamicin or chloramphenicol (Lai, 2001). The ampicillin–gentamicin treatment has been dubbed as the gold standard (Willis and Robinson, 1988). Unfortunately, *E. sakazakii* has developed resistance to these antibiotics by means of transposable elements and to β-lactams by the production of β-lactamase (Delphine et al., 2001, Lai, 2001, Muytjens et al., 1983 and Pitout et al., 1997). *Enterobacter* species are known to be prolific in their ability to inactivate broad spectrum penicillins and cephalosporins due to β-lactamase production, which appears to be increasing among strains of *E. sakazakii* (Chow et al., 1991, Emery and Weymouth, 1997 and
Lai, 2001). For this reason, a shift to carbapenems or the newer third-generation cephalosporins in combination with an aminoglycoside or trimethoprim–sulfamethoxazole has been suggested. Trimethoprim has also been suggested as a viable alternative (Lai, 2001 and Weir, 2002). Lai (2001) described cases of five *E. sakazakii* infections in which one or more of the isolates were resistant to cefazolin, ampicillin, cefotaxime, ceftazidime, piperacillin–tazobactam, gentamicin, ofloxacin and cefuroxime. One or more isolates were sensitive to cefotaxime, ceftazidime, piperacillin–tazobactam, gentamicin, ofloxacin, trimethoprim–sulfamethoxazole and imipenem–cilastatin. One strain from a 76-year-old patient was susceptible only to trimethoprim–sulfamethoxazole and aminoglycosides. A second strain from the same patient was susceptible to these antibiotics in addition to quinolones. Pitout et al. (1997) tested eight strains of *E. sakazakii* for the presence of β-lactamases. Antibiotic susceptibility testing was against ampicillin, ampicillin–sulbactam, amoxicillin–clavulanic acid, ticarcillin, ticarcillin–clavulanic acid, piperacillin, piperacillin–tazobactam, aztreonam, cephalothin, cefazolin, cefoxitin, cefotaxime, ceftriaxone, ceftazidime, cefepime and imipenem. Some of the eight strains were sensitive to the three β-lactams, ampicillin, cephalothin, and cefoxitin, while all wild-type *E. sakazakii* strains were susceptible to ampicillin, cefoxitin and cephalosporins. All eight strains tested for β-lactamases were positive for Bush group 1 β-lactamase (cephalosporinase). Block et al. (2002) examined *E. sakazakii* isolates from six neonatal and childhood infections and reported all as being β-lactamase positive, most likely representing Bush group 1 β-lactamase.

Nazarowec-White and Farber (1999) tested the antibiotic resistance of 17 strains of *E. sakazakii* and found four antibiotic susceptibility patterns (antibiograms). Five food isolates and all but one clinical isolate had the same antibiogram pattern as the type culture, viz., resistance to sulphisoxazole and cephalothin, yet susceptible to ampicillin, cefotaxime, chloramphenicol,
gentamicin, kanamycin, polymyxin-B, trimethoprim–sulphanmethoxazole, tetracycline and streptomycin. The authors concluded that antibiogram patterns were the least discriminatory of five analyses used to distinguish bacterial strains, i.e., biotyping, ribotyping, PFGE, RAPD and antibiotic susceptibility testing. Muytjens et al. (1983) reported that, although the *E. sakazakii* isolates in cases they investigated were susceptible in vitro to ampicillin, gentamicin, chloramphenicol and kanamycin, six of eight patients responded poorly and died.

Muytjens and van der Ros-van De Repe (1986) tested the antibiotic susceptibility of 195 *E. sakazakii* isolates, 157 of which were from confirmed clinical sources, against 29 antibiotics. Some of the sources (numbers of isolates in parenthesis) included the respiratory tract (35), digestive tract (31), utensils (21), CSF (17), superficial wounds (12), urine (9), upper respiratory tract (9) and blood (5). They reported using an agar dilution method with a base of Mueller–Hinton agar to determine MICs of antibiotics. *E. sakazakii* was the most susceptible of eight *Enterobacter* species evaluated for sensitivity to 29 antimicrobials. Concentrations of 24 of the 29 antibiotics necessary to inhibit at least 90% of the strains were \( \leq 8 \mu g/ml \). Antibiotics having MICs greater than 8 \( \mu g/ml \) were chloramphenical (16 \( \mu g/ml \)), cefaloridin (16 \( \mu g/ml \)), cefsulodin (32 \( \mu g/ml \)), cephalexin (\( > 128 \mu g/ml \)) and sulfamethoxazole (\( > 128 \mu g/ml \)). Concentrations necessary to inhibit 90% of the *E. sakazakii* strains were at least twofold lower than those required for *E. cloacae*. Kleiman et al. (1981) reported less, yet only moderate, resistance of an *E. sakazakii* strain isolated from a 5-week-old female with meningoencephalitis to cephalexin (MIC = 16 \( \mu g/ml \)).

Willis and Robinson (1988) detailed two cases of *E. sakazakii*-induced neonatal meningitis that, after being unresponsive to ampicillin–gentamicin therapy, resulted in abatement via treatment with moxalactam. Naqvi et al. (1985) eliminated *E. sakazakii* infection in one
patient by using cefotaxime. Block et al. (2002) concluded that general assumptions concerning antimicrobial therapy for *E. sakazakii* cannot be made, and treatment should be guided by clinical judgment and in vitro susceptibility testing.

**SOURCES OF CONTAMINATION**

**Clinical sources.** Farmer et al. (1980) reported that most *E. sakazakii* isolates from infected patients originate from CSF, blood, sputum, throat, nose, stool, gut, skin, wounds, bone marrow, eye, ear and breast abscess. Most isolates are rare if not sporadic. However, in a 7-month period, the bacterium was isolated from the respiratory tract of 29 patients in one hospital. Although *E. sakazakii* infections in newborns have been suspected as arising via passage through the mother's birth canal, this hypothesis seems untenable based on *E. sakazakii* infections in neonates born by Caesarean section (Bar-Oz et al., 2001, Muytjens and Kollee, 1990, Muytjens et al., 1983 and Urmenyi and Franklin, 1961). Analysis of plasmid DNA profiles has revealed that three or four of the five isolates from the same hospital were presumptive-positive for the same strain (Muytjens et al., 1983). Fecal, vaginal and cervical swabs of the mothers were negative for *E. sakazakii*. Samples from the anus, skin, nose, umbilical chord and outer ear of two infants on their day of birth were negative for the *Enterobacter*. Another infant who was tested for the presence of *E. sakazakii* in the outer ear, nose, umbilicus and gastric aspirate proved negative for the organism. These observations, coupled with infection of three infants delivered by Caesarean section, seem to also preclude contamination upon passage through the birth canal. The organism may persist in clinical settings over extended periods of time. For instance, three isolates in the same hospital over 11 years appeared to be the same strain based on ribotyping (Nazarowec-White and Farber, 1999).
**Environmental sources.** *Enterobacter* species are frequently isolated from soil, water, animals, sewage and human fecal samples (Sakazaki, 1974). Hallmann et al. (1997), in a review of endophytic bacteria in agricultural crops, noted that the genus *Enterobacter* is associated with the phytic flora. *Enterobacter* species have been isolated from corn roots and stems, cucumber roots, rough lemon roots and grapevine stems (Bell et al., 1995, Fisher et al., 1992, Gardner et al., 1982, Mahaffee and Kloeper, 1997 and McInroy and Kloeper, 1995).

It has been hypothesized that just as the primary reservoir for the coliform *E. coli* is feces, the reservoir for *E. sakazakii*, in addition to other coliforms, e.g., *Klebsiella oxytoca*, *K. pneumoniae*, *E. cloacae*, and *Citrobacter* species, may be primarily environmental and from plant materials (Mossel and Struijk, 1995 cited in Soriano et al., 2001). Iversen and Forsythe (2003) speculated that the principal environmental sources of *E. sakazakii* are water, soil and vegetables, and a secondary means of contamination may be vectors such as flies and rodents. However, Muytjens and Kollee (1990) did not recover *E. sakazakii* from bovine milk, cattle, domesticated animals, rodents, bird dung, grain, rotting wood, mud, soil, or surface water in The Netherlands. This is in contrast to our studies, including one industry study in the United States, in which *E. sakazakii* was recovered from 20 of 49 factory environmental sampling sites (data not published). Kandhai et al. (2004a) reported isolating *E. sakazakii* from 35 of 147 samples from a survey of 9 food factories and 16 households in The Netherlands. Factory samples were taken by sampling vacuum cleaner bags or by scraping or sweeping surfaces while household samples were taken exclusively from vacuum cleaner bags. Fourteen of the sixty-eight samples (21%) from a powdered milk factory were positive for *E. sakazakii*. In a chocolate factory, cereal factory, potato flour factory, pasta factory and spice factory, 2 of 8 (25%), 4 of 9 (44%), 4 of 15 (27%), 6 of 26 (23%) and 0 of 5 samples, respectively, were positive for the bacterium.
Five of sixteen household samples (31%) were positive for *E. sakazakii*. It was recommended that the widespread nature of *E. sakazakii* needs to be taken into consideration when designing preventive control measures (Kandhai et al., 2004a). Arts (2004) made two additional recommendations, viz., enhancement of the promotion of and support for breast feeding and inclusion of a warning on infant formulae and other breast-milk substitutes that the product might be contaminated with *E. sakazakii* and other microorganisms.

Kandhai et al. (2004b) evaluated a colorimetric screening assay to detect *E. sakazakii* in 152 dry samples of scrapings from dust, vacuum cleaner bags and spilled products from three milk powder plants. The bacterium was isolated from 18 of 152 (11.8%) samples. Cruz et al. (2004) isolated nine strains of *E. sakazakii* from 20 samples of dust; two strains were isolated from 48 samples of water in Mexico.

Kuzina et al. (2001) first reported isolating *E. sakazakii* from the Mexican fruit fly, *Anastrepha ludens*, and Hamilton et al. (2003) were the first to isolate *E. sakazakii* from the midgut of the stable fly larvae, *Stomoxys calcitrans*, suggesting that it may be a reservoir for the bacterium. Stable flies feed on blood of livestock and other domesticated animals, as well as humans and feral hosts, but are most commonly found in the vicinity of cattle. *S. calcitrans* can be found in all countries reporting *E. sakazakii* infections. Strains of *E. sakazakii*-producing mucoid or matt colonies have been isolated from the stable fly (Hamilton et al., 2003). Farm-isolated larvae were reared using established conditions (O’bratcha et al., 2000) and sterilized with 100% ethanol by immersion. Midguts were removed, plated on Luria-Bertani agar and incubated at 24°C overnight. Cells from colonies were identified by PCR and gene amplification of the 16s rRNA using the method of Hogg and Lehane (1999).
carotovora, Micrococcus luteus, Providencia stuartii and Serratia marcescens were isolated from farm-reared larvae.

_E. sakazakii_ has been isolated from a physician's stethoscope and an uninoculated bottle of bacterial culture medium (Farmer et al., 1980). Iversen and Forsythe (2003) suggested that the capsule produced by _E. sakazakii_ might increase its ability to attach to surfaces and form biofilms. Iversen et al. (2004d) studied biofilm formation by _E. sakazakii_ grown in an infant milk formula. The bacterium adhered to silicon, latex and polycarbonate in greater numbers than to stainless steel. A capsulated strain formed denser biofilms compared to a noncapsulated type strain. They recommended that bottles and utensils used to prepare infant formulae should be cleaned thoroughly as soon as possible after use to eliminate or minimize the formation of biofilms, which could be sources of infection. Zogaj et al. (2003) observed that an extracellular matrix, cell clumping, pellicle formation and biofilm formation by _E. sakazakii_ was associated with the expression of cellulose and curli fimbriae.

Kim et al. (2006a) conducted a study to determine growth characteristics of five strains of _E. sakazakii_ in a rich microbiological medium (tryptic soy broth), reconstituted infant formula, and lettuce juice as a produce model and to characterize subsequent attachment of stationary phase cells and biofilm formation on the surfaces of stainless steel and enteral feeding tubes at 12 and 25°C affected by temperature and nutrients provided by these media. Higher populations of cells attached at 25°C than at 12°C. Biofilm formation was determined by immersing enteral feeding tubes and stainless steel coupons at 4°C for 24 h in phosphate-buffered saline suspensions (7 log CFU/ml) to facilitate the attachment of 5.33 to 5.51 and 5.03 to 5.12 log CFU/cm², respectively, before they were immersed in tryptic soy broth (TSB), infant formula broth (IFB), or lettuce juice broth (LJB), followed by incubation at 12 or 25°C for up to 10 days.
Although no biofilms were produced at 12°C, when immersed in IFB at 25°C, the number of cells of test strains in biofilms increased by 1.42 to 1.67 log CFU/cm² and 1.16 to 1.31 log CFU/cm² on stainless steel and enteral feeding tubes, respectively. However, biofilms were not formed on TSB or LJB at 25°C.

Lee and Kim (2003) constructed a semi-pilot galvanized iron pipe model to assess the microbial quality of a public drinking water system. Municipally treated potable water was circulated in the iron pipe for 12 weeks and examined for the presence of bacteria. *E. sakazakii* was isolated from one influent sample and one effluent sample. Mosso et al. (1994) surveyed the bacterial quality of 26 thermal mineral water springs in Spain. Three of the springs were classified as hypothermal (< 30°C), ten were mesothermal (30–40°C), and thirteen were hyperthermal (> 40°C). Forty isolates of *Enterobacter* species, including *E. sakazakii*, *E. agglomerans* and *E. amnigenus*, and six unidentified isolates were recovered from 13 of the 26 springs surveyed. Ten of thirty-one *Enterobacter* isolates from hyperthermal springs were identified as *E. sakazakii*. Van Os et al. (1996) isolated *E. sakazakii* from grass silage in The Netherlands.

*E. sakazakii* has been isolated from floor drains, air, a vacuum canister, broom bristles, a room heater and electrical control box, transition socks, a clean-in-place (CIP) valve, a floor dryer, floor and condensate in a dry product processing environment in the United States (unpublished data). Iversen and Forsythe (2003), in reviewing relevant literature, reported a number of other environmental sources from which *E. sakazakii* has been isolated, including air in a hospital (Masaki et al., 2001), clinical materials (Janicka et al., 1999 and Tuncer and Ozsan, 1988), rats (Gakuya et al., 2001), soil (Neelam et al., 1987), rhizosphere (Emilani et al., 2001),
sediment and wetlands (Espeland and Wetzel, 2001), crude oil (Assadi and Mathur, 1991) and cutting fluids (Suliman et al., 1988).

**Foods as sources of *E. sakazakii***. Krieg and Holt (1984) stated that *E. sakazakii* was more prevalent in foods and the environment than in clinical settings. The bacterium has been isolated from a can of previously unopened non-fat dried milk (Farmer et al., 1980). Muytjens et al. (1988) cultured members of the family Enterobacteriaceae from 52.5% of 141 milk-substitute infant formulae obtained from 35 countries. Except for one sample from the former Soviet Union, populations did not exceed 1 CFU/g of any product. *E. sakazakii* was detected in 20 of 141 (14.2%) samples from 13 of the 35 countries. In a survey of infant formula products from 11 countries, Leuscher et al. (2004) isolated *E. sakazakii* from 8 of 58 (13.8%) samples. Iversen et al. (2004a) surveyed 82 samples of powdered infant formula milk and 404 other food products for the presence of *E. sakazakii*, *Salmonella* and other Enterobacteriaceae. The bacterium was isolated from 2 of 82 (2.4%) formulae, 5 of 49 (10.2%) dried infant foods, 3 of 72 (4.1%) milk powders, 2 of 62 (3.2%) cheese products and various dry food ingredients, including 40 of 122 (37.8%) herbs and spices. *Salmonella* was not isolated from the dry infant formula milk, dried infant foods, or milk samples. It was concluded that hygienic production of formula and milk powder as monitored by control of *Salmonella* and enumeration of Enterobacteriaceae did not control *E. sakazakii*.

Several reports have implicated rehydrated powdered infant formula as a source of *E. sakazakii* in neonatal infections (Biering et al., 1989, Block et al., 2002, Clark et al., 1990, Himelright et al., 2002, Muytjens et al., 1983, Muytjens et al., 1988, Noriega et al., 1990, Simmons et al., 1989, Smeets et al., 1998, Van Acker et al., 2001 and Weir, 2002). The first outbreak of *E. sakazakii* linked to powdered infant formula from a previously unopened can was
in 2001 (Centers for Disease Control, 2002, Himelright et al., 2002 and Weir, 2002). In another outbreak, powdered formula tested negative for *E. sakazakii*, yet the blender used to prepare the rehydrated formula was positive (Noriega et al., 1990). It was suggested that contamination could have arisen from a previous batch of powdered infant formula that was contaminated. The blender was washed in a dishwashing machine daily; however, it was suspected that the cleaning procedure was not sufficient to eliminate bacterial contamination. Caric (1993) indicated that the drying and filling areas of a food factory are potential sources of contamination. Nazarowec-White and Farber (1997a) stated that microbial pathogens can gain access to the powder from the environment or from the addition of ingredients at the powder stage. At least one strain of *E. sakazakii* (NCTC 8155) originated from dried milk (Farmer et al., 1980, Iversen and Forsythe, 2003 and Thornley, 1960).

In an outbreak of five cases of neonatal meningitis, *E. sakazakii* was isolated from a stirring spoon and a dish brush used to prepare infant formula and from prepared formula (Muytjens et al., 1983). Isolates were indistinguishable from clinical isolates by means of antibiograms, biogroups, pigment production and general morphology, with the exception of biochemical differences in one clinical isolate. Samples of water and powdered formula were negative for the coliform.

Gassem (1999) analyzed khamir, a fermented bread made from the Baydah and Hamra varieties of sorghum in southwest Saudi Arabia, for the presence of coliforms. They prepared the bread by combining the sorghum with water, onion, garlic, lemon juice and fenugreek and incubating the mixture for 24 h at 30°C. Products prepared from the two varieties had pH values of 3.92 and 3.85, respectively. Six *E. sakazakii* isolates were among the six coliforms detected in these breads. In another study (Gassem, 2002), 14 samples of sobia, a traditional fermented
beverage made in western and central provinces of Saudi Arabia, were examined for microbiological quality, titratable acidity and pH. Titratable acidity ranged from 0.04% to 0.30%, based on percent lactic acid, with the pH ranging from 3.37 to 5.53. *E. sakazakii* was most frequently isolated (1/3 of all samples) among 12 different bacteria, including lactics and coliforms recovered from the beverage.

Soriano et al. (2001) examined 370 food samples from restaurants in Spain to determine the incidence of pathogenic bacteria as well as the presence of spoilage microorganisms that might serve as food safety indicators. Lettuce, pork, beef, chicken and Spanish potato omelettes were analyzed. *E. sakazakii* was recovered from 1 of 40 samples of raw lettuce but not from ready-to-eat lettuce or the other food products. Cottyn et al. (2001) analyzed rice harvested from various sites in the Philippines for bacterial flora; of the 428 bacterial isolates examined, 184 were Gram-positive and 244 were Gram-negative. The most prevalent (25%) of the Gram-negative isolates were from the family Enterobacteriaceae, with the genus *Pantoea* and *Enterobacter* predominating. Four seed lots yielded 20 *E. sakazakii* isolates and five lots yielded 9 isolates of *E. cloacae*.

Because *E. sakazakii* has been isolated from rice, rice cereal is commonly fed to infants, and because *E. sakazakii* has proven to be pathogenic to neonates, Richards et al. (2005) conducted a series of experiments to determine survival and growth characteristics of *E. sakazakii* in infant cereal as affected by type of liquid used for reconstitution and storage temperature after reconstitution. Commercially manufactured dry infant rice cereal was reconstituted with water, apple juice, milk or liquid infant formula, inoculated with a 10-strain mixture of *E. sakazakii* at populations of 0.27, 0.93 and 9.3 CFU/ml and incubated at 4, 12, 21, or 30°C for up to 72 h. No growth occurred in cereal reconstituted with apple juice, regardless of
storage temperature, or in cereal reconstituted with water, milk or formula when these slurries with stored at 4°C. The lag time for growth in cereal reconstituted with water, milk, or formula was decreased as the incubation temperature (12, 21, or 30°C) was increased. Upon reaching maximum populations of 7-8 log CFU/ml, in some instances populations decreased to nondetectable levels during subsequent storage, commensurate to decreases in pH.

Other sources reported to harbor *E. sakazakii* include water, pipes and biofilm (Al-Hadithi and Al-Edani, 1995, Bartolucci et al., 1996 and Oliver, 1997), beer mugs (Schindler and Metz, 1990), sour tea (Tamura et al., 1995), cheese, minced beef, sausage meat and vegetables (Leclercq et al., 2002). Nazarowec-White and Farber (1997b) reported a personal communication from R. Foster who isolated the coliform from ground meat. In a Norwegian study, *E. sakazakii* was isolated from mung bean sprouts (Robertson et al., 2002). Nine strains of *E. sakazakii* were isolated from 50 samples of alfalfa sprouts in Mexico (Cruz et al., 2004). Kornacki (1998) isolated the organism from rice starch, rice flour and eggs.

Because *E. sakazakii* has been isolated from fresh produce, Kim and Beuchat (2005) performed a study to determine the survival and growth characteristics of *E. sakazakii* on fresh-cut apple, cantaloupe, strawberry, watermelon, cabbage, carrot, cucumber, lettuce, and tomato, and in unpasteurized juices prepared from these fruits and vegetables. Produce and juices were inoculated with 2 to 3 log CFU/g and 1 to 2 log CFU/ml, respectively, and stored at 4, 12, and 25°C. The authors also monitored populations of mesophilic aerobic bacteria (total counts), lactic acid bacteria, and molds and yeasts in juices inoculated with *E. sakazakii*. Populations of *E. sakazakii* did not change or gradually decreased in fresh-cut produce and juices stored at 4°C but grew at 12°C on fresh-cut apple, cantaloupe, watermelon, cucumber, and tomato and in all juices except apple, strawberry, cabbage, and tomato juices. All fresh-cut fruits and vegetables
except strawberry supported growth of *E. sakazakii* at 25°C. Growth occurred in all juices except apple, strawberry, and cabbage, prior to decreases in population to less than 1 CFU/ml after 2 to 3 days of storage, proportionate to the reduction in pH and increase in lactic acid bacterial populations. Counts of mesophilic aerobic bacteria increased in cucumber, cantaloupe, lettuce and carrot juices stored at 4°C. Mold and yeast populations increased in tomato and apple juices stored at 25°C but decreased to less than 1 CFU/ml in cucumber, cabbage and lettuce juices.

**INACTIVATION**

**Thermal resistance.** The atypically high number of *Enterobacter* species in powdered infant formulae has been explained by some as resulting from the relatively high thermal resistance of some species in the genus (Buchanan, 2003, Nazarowec-White and Farber, 1997a and Van Acker et al., 2001). Other studies suggest that persistence of *E. sakazakii* following pasteurization treatment is doubtful (Breeuwer et al., 2003, Buchanan and Edelson, 1999, Edelson-Mammel and Buchanan, 2004c, Nazarowec-White and Farber, 1997a and Nazarowec-White et al., 1999). One study revealed that the milk processing plant environment as well as ultra-high-temperature pasteurized milk cartons to be sources of *E. sakazakii* contamination (Skladal et al., 1993). This finding, however, does not conclusively prove that the organism is capable of surviving pasteurization.

Nazarowec-White and Farber (1997a) were the first to describe thermal inactivation and resistance characteristics of *E. sakazakii* in rehydrated powdered infant formula. The heating medium was an infant formula with the highest fat content (3.8 g/100 ml) of all formulae sold in Canada. Ten strains of *E. sakazakii* (five food isolates and five clinical isolates) at populations of 7 log<sub>10</sub> CFU/ml were heated at 52, 54, 56, 58, and 60°C. The resulting *D* values were
\[D_{52^\circ C} = 54.8\text{ min}, D_{54^\circ C} = 23.7\text{ min}, D_{56^\circ C} = 10.3\text{ min}, D_{58^\circ C} = 4.20\text{ min}, D_{60^\circ C} = 2.50\text{ min},\]

with a pooled \(z\) value of 5.82\(^{\circ}\)C. The authors calculated that a 6–7 \(\log_{10}\) kill would require heating at 60\(^{\circ}\)C for 15–17.5 min. Iversen et al. (2004d) determined \(D\) values for \(E. sakazakii\) in a rehydrated powdered milk formula. \(D\) values of 16.4, 5.1, 2.6, 1.1 and 0.3 min at 54, 56, 58, 60, and 62\(^{\circ}\)C, respectively, were reported for the type strain. \(D\) values for a capsulated strain were generally less but \(z\) values for type and capsulated strains were 5.8 and 5.7\(^{\circ}\)C, respectively.

High-temperature short-time (HTST) pasteurization (71.2\(^{\circ}\)C for 15 s) used to process dried infant formula would theoretically result in ca. a 21-\(D\) kill (Iversen et al., 2004d), which indicates that \(E. sakazakii\) cannot survive a commercial pasteurization process.

Nazarowec-White and Farber (1997) determined that \(D\)-values of \(E. sakazakii\) were among the highest species in the family Enterobacteriaceae. However, these data could not be extrapolated to continuous process pasteurization. Nazarowec-White et al. (1999) developed a linear model for heat inactivation of \(E. sakazakii\) during HTST pasteurization of bovine milk by taking into account physical stresses such as shear force (Fairchild et al., 1994; Mackey and Bratchell, 1989; Swartzel, 1984). Inoculated formulas were heated at 58 - 68\(^{\circ}\)C in 1\(^{\circ}\)C-increments at 3, 10, 16, 30, and 60 s (Nazarowec-White et al., 1999). \(D\) values were compared with those of \(L. monocytogenes\) calculated by Piyasena et al. (1998). The authors concluded that at 68\(^{\circ}\)C there was >1 log higher difference in thermal sensitivity of \(E. sakazakii\) over the more thermotolerant \(L. monocytogenes\).

Edelson-Mammel and Buchanan (2004c) examined the thermal resistance of 12 strains of \(E. sakazakii\) using a submerged vessel method reported in a previous study from the same laboratory (Buchanan and Edelson, 1999). Rehydrated infant formula was inoculated with \(E. sakazakii\) at ca. 8 \(\log_{10}\) CFU/ml and heated at 56, 58, 60, 65 and 70\(^{\circ}\)C. Isolates exhibited an
almost 20-fold divergence in thermal resistance, with strain ATCC 51329 being the least
thermally resistant. The most thermally resistant strain was a clinical isolate. The respective
mean $D_{58^\circ C}$ values for the two strains were 0.51 and 9.87 min. Approximately half of the strains
had $D_{58^\circ C}$ values of < 0.83 min, whereas half had $D$-values of > 5 min. It was concluded that the
two divergent thermally resistant groups could be divided into two distinct phenotypes. The $z$
value of the most thermally tolerant strain was calculated to be 5.6°C, very close to that reported
by Nazarowec-White and Farber (1997a). This strain was further examined by inoculating
powdered infant formula with a cell suspension, then rehydrating the powder in baby bottles with
deionized water at 50, 60, 70, 80, 90 and 100°C. Populations were reduced by 0.3 and 1.3 log$_{10}$
CFU/ml at 50 and 60°C, respectively, while treatment at all higher temperatures reduced
populations by > 4 log$_{10}$ CFU/ml, which was below the lowest detection limit of 100 CFU/ml. It
should be recognized that the behavior of cells after desiccation resulting from exposure to dry
powder may be different than that of cells not exposed to a dry environment.

Breeuwer et al. (2003) performed thermal inactivation studies using five strains of *E.
sakazakii* in the stationary phase. $D_{58^\circ C}$ values ranged from 0.39 to 0.60 min, with a mean of
0.48 min. The $z$ values of two strains were 3.1 and 3.6°C, respectively. These values are lower
than those reported in other studies (Edelson-Mammel and Buchanan, 2004c and Nazarowec-
White and Farber, 1997c). However, while the latter studies tested the thermal stability of *E.
sakazakii* in rehydrated infant formula, Breeuwer et al. (2003) used disodium hydrogen
phosphate/potassium dehydrogenate phosphate buffer with a neutral pH as a heating medium.
Differences in composition of heating media serve as a plausible explanation for the divergence
in observed $D$ values. The increased amount of fat, protein and carbohydrate in the infant
formula may protect *E. sakazakii* against thermal inactivation, thus resulting in higher $D$ values.
Spray or roller-dried milk, the primary ingredient in infant formula, casein, and whey proteins (β-lactoglobulins, α-lactalbumins, serum albumin and immunoglobulins) may also affect thermal inactivation rates.

Several studies have evaluated the effects of microwave heating on the destruction of microorganisms in milk. The mechanism by which microwaves cause the death of microbial cells is thought to involve thermal as well as non-thermal effects associated with electromagnetic radiation (Goldblith and Wang, 1967, Kindle et al., 1996, Lechowich et al., 1969, Najdovski et al., 1991, Rosenberg and Sinell, 1989 and Vela and Wu, 1979). Kindle et al. (1996) examined the effects of electromagnetic radiation on (2450 MHz) *E. sakazakii* strain ATCC 29544 (type culture) and two other strains. Cells were inoculated at a population of 5 log$_{10}$ CFU/ml into five rehydrated powdered infant formulae. Formulae were heated until the first signs of boiling, then cooled and analyzed for populations of surviving cells. Four of the five samples were negative for *E. sakazakii* following microwave treatment and one formula contained 20 CFU/ml. Differences in formula composition could account for different rates of inactivation of *E. sakazakii*. Thermal inactivation may also have been influenced by the concentration of solutes in various formulae. The bactericidal efficacy of microwave treatment of milk is the basis for recommending its use over more traditional methods to rewarm rehydrated powdered infant formula (Kindle et al., 1996). They reported that microwaving infant formula in baby bottles for 85–100 s to a temperature of 82–93°C can result in a > 4 log$_{10}$ CFU/ml destruction of *E. sakazakii*. However, Edelson-Mammel and Buchanan, 2004a, Edelson-Mammel and Buchanan, 2004b and Edelson-Mammel and Buchanan, 2004c suggested that due to the scalding hazard, a rehydration temperature of 70°C would be more appropriate and added that rehydration at this temperature would virtually assure that a serving would not contain *E. sakazakii*. 
Osmotic and desiccation resistance. *E. sakazakii* appears to have an unusual ability to survive when exposed to dry conditions. Survival of nine clinical and food strains of *E. sakazakii* in dry infant formula milk was studied by Caubilla-Barron et al. (2004). Initial reductions were 2–4 logs and reductions after 6 months were 4–7 logs. Rehydration with water at 60°C resulted in a 3-log decrease in viable cell number compared to rehydration at ambient temperature or 45°C. Iversen and Forsythe (2003) speculated that its survival under such conditions for periods of up to 2 years may be attributable to capsule formation. Breeuwer et al. (2003) described the resistance of *E. sakazakii* to high osmolarity and discussed mechanisms bacteria use to achieve this condition. Bacteria are known to prevent intercellular dehydration via accumulation of ions (e.g., K⁺) and compatible solutes (e.g., trehalose, proline, glycine betaine) that can increase the intracellular osmolarity and maintain a shell of water around macromolecules (Kempf and Bremer, 1998, Potts, 1994 and Leslie et al., 1995). An increase in the trehalose concentration in growth media has been shown to increase the resistance of stationary phase cells of *E. coli* to desiccation (Welsh and Herbert, 1999). Increasing the compatible solute concentrations in media used to culture *E. coli* and *Salmonella* has also proven effective in increasing osmotic resistance (Kempf and Bremer, 1998).

Breeuwer et al. (2003) tested the osmotic resistance of *E. sakazakii* at *a*ₜ 0.93 (in brain heart infusion broth supplemented with sorbitol) at 25°C and observed it to be more resistant than *E. agglomerans, E. coli, Salmonella senftenberg, Salmonella typhimurium* and *Salmonella enteritidis* to inactivation. The population of one strain of *E. sakazakii* decreased by ca. 90% during storage for 2 months. Populations of *Salmonella, E. coli, K. pneumoniae, Serratia rubidea* and *Citrobacter freundii* suspended in 75% sorbitol (a*ₜ* 0.81) decreased by 6 log₁₀ CFU/ml within 14 days. Populations of two strains of the *E. sakazakii* exposed to the same
conditions decreased only 3–4 log_{10} CFU in the same period of time, with an additional two strains being detectable after 4 weeks. The phase of growth played an important role in survival. For stationary phase cells suspended in a 75% sorbitol solution, the population of one strain of *E. sakazakii* decreased ca. 90% within 3 weeks, while cells in exponential growth phase decreased by 5 log_{10} within 1 week. Additional studies involved desiccating cells in phosphate buffer for 1 h at 20.7% relative humidity and holding pellets for 46 days before analyzing for survivors (Breeuwer et al., 2003). Exponential phase *E. sakazakii* decreased by ca. 7 log_{10} in 10 days, while stationary phase cells decreased by only 1–1.5 log_{10} during a 46-day trial. This is in contrast to stationary phase *E. coli* cells, which declined by > 4 log_{10} in 46 days. When trehalose was added to the *E. sakazakii* cell suspension prior to desiccation, viable exponential phase cells were reduced by ca. 2.5 log_{10} vs. ca. 7 log_{10} in the absence of the polyhydroxyl solute. Trehalose or glycine betaine, however, did not provide greater osmotic protection against sorbitol. The trehalose content in *E. sakazakii* was 500% higher in stationary phase cells than in exponential phase cells.

The genetic basis for survival of *E. sakazakii* when exposed to dry conditions \( a_w 0.23 \) was studied by Breeuwer et al. (2004). Desiccation results in an induction of seven genes from the heat shock regulon, four genes from the cyclic AMP receptor protein regulon, six genes involved in the stringent response and a number of genes involved in trehalose synthesis and cell wall functions such as lipid A and lipopolysaccharide biosynthesis. It was concluded that the response of *E. sakazakii* to dry stress involves a genome-wide expression of functionally different groups of genes.

**Antimicrobial activity of chitosan and its oligomers.** Chitosan, present in the shells of crustaceans such as shrimp, lobster and crab, is a non-toxic biopolymer (No et al., 2002). The
ability of chitosan and chitosan oligomers to inhibit tumors and lower LDL cholesterol levels, as well as their antimicrobial activities, has been described (Kendra and Hadwiger, 1984, Knorr, 1984, Muzzarelli, 1977, No et al., 2002, Sekiguchi et al., 1994, Sudarshan et al., 1992, Sugano et al., 1992 and Tokoro et al., 1988). It has been theorized that antimicrobial activity might be attributable to an interaction between negatively charged bacterial surface residues and positively charged chitosan molecules (Hadwiger et al., 1981, No et al., 2002, Sudarshan et al., 1992 and Young et al., 1982). Studies supporting the antimicrobial activity of chitosan are inconclusive, although it has been demonstrated that water-soluble chitosan preserves tofu against microbial spoilage (Chun et al., 1999). No et al. (2002) isolated seven different bacteria from 10 commercially produced South Korean brands of tofu, one of which was identified as \textit{E. sakazakii}. They studied the antibacterial effects of six chitosans and six chitosan oligomers in tofu immersion solutions to determine MICs. Five of six chitosans at a concentration of 0.1% exhibited strong inhibition and one exhibited weak inhibition, with an MIC of 400 μg/ml for all but the lowest molecular-weight chitosan.

**Antimicrobial action of commercial sanitizers.** Kim et al. (2006b) conducted a study with an objective of determining the effectiveness of chlorine, aqueous chlorine dioxide, and a peroxycetic acid-based sanitizer (Tsunami 200®) (applied for 1 and 5 min) in reducing populations of \textit{E. sakazakii} inoculated in an organic carrier (horse serum) onto the surface of apples, tomatoes, and lettuce at levels of 8.60 – 8.78 CFU/produce. Control fruits and vegetables underwent washing with water. When treated with Tsunami 200 at 40 μg/ml for 1 min, \textit{E. sakazakii} populations were reduced by ≥4.00 CFU/apple. Populations of \textit{E. sakazakii} on apples treated with 10 μg/ml chlorine dioxide for 1 and 5 min were reduced by 3.38 and 3.77 log CFU/apple, respectively, compared to controls. Tomatoes treated with 10 μg/ml chlorine or
chlorine dioxide or 40 µg/ml Tsunami 200 for 5 min resulted in reductions of $\geq 3.70$ CFU/fruit. Lettuce treated with chlorine at 10, 50, and 100 µg/ml for 1 min ranged from 1.61 to 2.50 CFU/sample, compared to controls. Lettuce treated with Tsunami 200 at 40 and 80 µg/ml for 5 min reduced *E. sakazakii* populations by $\geq 5.31$ log CFU/sample.

**Antimicrobial action of the lactoperoxidase system.** Lactoperoxidase (LPO) is a naturally occurring oxidoreductase that protects the neonatal gastrointestinal tract as well as the mammary glands against pathogenic microorganisms (Naidu, 2000). The enzyme has been detected in all mammalian milks tested (Seifu, 2005), although it is only present in human milk at 5% of the concentration in bovine milk (Watanabe, 2000). LPO is also present in human saliva, nasal fluid, luminal fluid, tears, and vaginal secretions (Ozer, 1999).

Lactoperoxidase is present at the second highest concentration of any enzyme in bovine milk, behind xanthine oxidase, and makes up about 1% of the whey protein (ca. 30 mg/ml) (De Wit and Van Hooydonk, 1996; Pruitt et al., 1991; Reiter, 1985). Gothefors and Marklund (1975) reported that bovine milk contains LPO at concentrations of 1.2 to 19.4 units/ml. Another LPOS constituent (thiocyanate or SCN⁻) is present at concentrations in bovine milk ranging from 1 to 15 µg/ml (Reiter and Harnulv, 1984), in contrast to 50 – 300 µg/ml SCN⁻ in human saliva and 40 – 50 µg/ml in human gastric juices (Bjorck et al., 1979, Korhonen, 1980). Mammals acquire SCN⁻ through dietary intake of glucosinolates and cyanogenic glucosides from vegetables such as cabbage, kale, brussel sprouts, cauliflower, turnips, rutabaga, cassava, potatoes, corn, millet, sugar cane, peas, and beans (Wolfson and Sumner, 1993; Reiter and Harnulv, 1984).

The LPO enzyme has been shown to protect somatic cells against the effects of peroxides. It is involved in the degradation of carcinogens (Tenovuo, 1985; Gothefors and Marklund, 1975; Stanislawski et al., 1989; Odajima et al., 1996), benign to mammalian cells, and serves as an
antioxidant against oxygen-derived species (Bjorck, 1990, Reiter and Harnulv, 1984). Doyle and Marth (1978) reported that in the presence of salt, the LPOS degrades aflatoxin.

The LPOS is composed of the three chemical components: the lactoperoxidase enzyme, thiocyanate (SCN\(^-\)), and H\(_2\)O\(_2\). In the presence of LPO, SCN\(^-\) is oxidized by H\(_2\)O\(_2\), which produces two primary antibacterial intermediate compounds, the hypothiocyanite ion (OSCN\(^-\)) and hypothiocyanous acid (HOSCN); however, data suggest that HOSCN displays the greater antibacterial activity of the two molecules (Seifu, 2005). Other short-lived intermediates produced by the LPOS that are less important in antimicrobial activity include thiocyanogen ((SCN)\(_2\)), cyanogen thiocyanate (NC-SCN), cyanosulfurous acid (HO2SCN), and cyanosulfuric acid (HO3SCN) (Pruitt and Kamau, 1991). Using the substrate ABTS (2,2'–azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]) as a reductant, LPO exhibits maximum activity in milk at pH 6.0 (Kumar and Bhatia, 1999; Pruitt et al., 1990). The antibacterial activity of the LPOS results from intermediate compounds oxidizing sulfhydryl groups on enzymes and proteins in the cytoplasmic membranes, inhibiting enzyme activity, creating leaky membranes, and preventing the uptake of critical compounds (Reiter and Harnulv, 1984). Thus, gram-negative bacteria may be more susceptible than gram-positive microorganisms to the LPOS. Various food systems and microbiological media have been used to test the efficacy of the LPOS (Table 1-5). The LPOS has been studied as a means to control pathogens in raw milk (Bjorck et al., 1979; FAO and WHO, 1991), pasteurized milk (Marks et al., 2001), skim milk (Vannini et al., 2004; Boussouel et al., 2000), UHT skim milk (Garcia-Graells et al., 2003; Zapico et al., 1998), reconstituted non-fat dry milk (Siragusa and Johnson, 1989), caprine milk (Seifu et al., 2004), infant formula (Banks and Board, 1985), fruit and vegetable juice (Van Opstal et al., 2006), ground beef
<table>
<thead>
<tr>
<th>Publication</th>
<th>Food system or medium</th>
<th>LPO</th>
<th>NaSCN</th>
<th>KSCN</th>
<th>SCN</th>
<th>Sodium percarbonate</th>
<th>H₂O₂</th>
<th>Glucose oxidase</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAO/WHO (1991)</td>
<td>Levels permitted in raw milk</td>
<td>na¹</td>
<td>14 µg/ml</td>
<td>na</td>
<td>na</td>
<td>30 mg/L</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Banks and Board (1985)</td>
<td>Infant formula</td>
<td>1.5 µ/ml</td>
<td>na</td>
<td>0.52 mM</td>
<td>na</td>
<td>na</td>
<td>0.1 µ/ml</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Kamau et al. (1990)</td>
<td>Measured as naturally occurring in milk</td>
<td>9.2 µg/ml</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Elliot et al. (2004)</td>
<td>Beef cubes</td>
<td>0.93 mg/ml</td>
<td>19.1 mg/ml</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>1 unit per 5 units LPO</td>
<td>18.2 mg /ml</td>
</tr>
<tr>
<td>Marks et al. (2001)</td>
<td>Pasteurized milk</td>
<td>na</td>
<td>10 µg/ml</td>
<td>na</td>
<td>na</td>
<td>10 µg/ml</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Vannini et al. (2004)</td>
<td>Skim milk</td>
<td>30 µg/ml (2.7 U/ml)</td>
<td>na</td>
<td>na</td>
<td>1.1 mg/ml</td>
<td>na</td>
<td>0.9 mg/ml</td>
<td>na</td>
<td></td>
</tr>
<tr>
<td>Boussouel et al. (2000)</td>
<td>Skim milk</td>
<td>35 µg /ml</td>
<td>na</td>
<td>25 µg /ml</td>
<td>na</td>
<td>na</td>
<td>1 µg /ml</td>
<td>200 µg /ml</td>
<td></td>
</tr>
<tr>
<td>Siragusa and Johnson (1989)</td>
<td>Tryptic soy broth with yeast extract or non-fat dry milk (reconstituted)</td>
<td>0.37 U/ml</td>
<td>na</td>
<td>0.3 mM</td>
<td>na</td>
<td>na</td>
<td>0.3 mM</td>
<td>na</td>
<td></td>
</tr>
<tr>
<td>McLay et al. (2002)</td>
<td>Todd Hewitt broth</td>
<td>187.5 or 213.75 µg /ml</td>
<td>na</td>
<td>486 µg /ml</td>
<td>na</td>
<td>na</td>
<td>0.1 or 1.0 units per 11.9 units LPO</td>
<td>3.96 or 11.9 g/L</td>
<td></td>
</tr>
<tr>
<td>Garcia-Graells et al. (2000)</td>
<td>UHT skim milk or potassium phosphate buffer</td>
<td>5 µg /ml</td>
<td>na</td>
<td>25 mM</td>
<td>na</td>
<td>na</td>
<td>25 mM</td>
<td>na</td>
<td></td>
</tr>
<tr>
<td>Elotmani and Assobhei (2003)</td>
<td>Agar diffusion method with saturated paper discs</td>
<td>3.5 µg /ml</td>
<td>na</td>
<td>50 µg /ml</td>
<td>na</td>
<td>na</td>
<td>2 µg /ml</td>
<td>1,300 µg /ml</td>
<td></td>
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<tr>
<td>Tan and Ockerman (2006)</td>
<td>Marinated broiler drumsticks</td>
<td>1 µg/ml</td>
<td>na</td>
<td>5.9 mM</td>
<td>na</td>
<td>na</td>
<td>2.5 mM</td>
<td>na</td>
<td></td>
</tr>
<tr>
<td>Nguyen et al. (2005)</td>
<td>Yeast extract broth or potato dextrose broth</td>
<td>25 µg /ml</td>
<td>100 µg /ml</td>
<td>na</td>
<td>na</td>
<td>50 mg/L</td>
<td>na</td>
<td>na</td>
<td></td>
</tr>
<tr>
<td>Seifu et al. (2004)</td>
<td>Caprine milk</td>
<td>na</td>
<td>14 µg /ml</td>
<td>na</td>
<td>na</td>
<td>30 mg/L</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Garcia-Graells et al. (2003)</td>
<td>Commercial sterilized skim milk</td>
<td>5 µg/ml</td>
<td>na</td>
<td>0.25 mM</td>
<td>na</td>
<td>na</td>
<td>0.25 mM</td>
<td>na</td>
<td></td>
</tr>
</tbody>
</table>

¹Not applied or not applicable
(Kennedy et al., 2000), beef cubes (Elliot et al., 2004), marinated broiler drum sticks (Tan and Ockerman, 2006), and fish (Elotamani and Assobhei, 2003).

Studies have documented the antimicrobial effect of the LPO system against *Bacillus cereus* in milk (Tenovuo et al., 1985; Zajac, 1981). Other studies have reported the bacteriostatic or bacteriocidal effects of the LPOS against *Escherichia coli*, *Salmonella*, *Campylobacter jejuni*, *Shigella* spp., *Pseudomonas* spp., *Staphylococcus aureus*, *Listeria monocytogenes*, *Brucella melitensis*, and *Streptococcus* spp., as well as against fungi and viruses (Seifu, 2005). The LPOS has also been used as an oral antimicrobial treatment in toothpaste products and has been studied as a means of human airway defense (Tenovuo, 2002, Gerson et al., 2000).

Banks and Board (1985) evaluated an LPOS for its effectiveness in reconstituted infant formula inoculated with several microorganisms isolated from lake water. The LPOS was established by the addition of LPO (1.5 µg/ml), glucose oxidase (0.1 µg/ml), and KSCN (0.52 mM). Enterobacteriaceae, initially at ca. 1.3 log CFU/ml increased to > 8.0 log CFU/g in untreated formulas, but were undetectable for up to 48 h in LPO-treated formulas (Banks and Board, 1985). Although glucose oxidase was used to generate H₂O₂ used in the LPOS, sodium percarbonate has proven to be a more economically feasible alternative (Banks and Board, 1985) to achieve the same ends (FAO and WHO, 1991), and has proven more efficient than direct addition of H₂O₂ in activating the LPOS (Ozer, 1999).

The LPOS has been accepted and recommended for use by the International Dairy Federation (IDF) as an antibacterial to preserve raw milk during transport to milk processing facilities in developing countries (IDF, 1988). Guidelines for preserving raw milk by use of the LPOS (FAO/WHO, 1991) call for the addition of 14 µg/ml of NaSCN, and 30 µg/ml of sodium percarbonate as an H₂O₂ generator. When NaSCN is added to milk at this level, concentrations
are much lower than those naturally occurring in human saliva and cruciferous vegetables such as cabbage and cauliflower. It was also reported that NaSCN will not interfere with iodine uptake by the thyroid gland. When the LPOS is activated according to these guidelines, raw milk can be preserved for 7–8 h at 30°C, 11–12 h at 25°C, 16–17 h at 20°C, or 24–26 h at 15°C (FAO/WHO, 1991). The document notes that when 14 µg/ml NaSCN is added, the milk should be mixed for 1 min followed by the addition of 30 µg/ml sodium percarbonate and mixing for an additional 2–3 min. It is essential, they state, that the ingredients be added in a prescribed order because the LPOS is initiated immediately upon generation of H₂O₂ by sodium percarbonate, and all H₂O₂ should be exhausted and reactions completed within 5 min.

Elliot et al. (2004) prepared separate solutions of LPO and NaSCN with deionized water and sterilized through an 0.2 µm filter. Beef cubes inoculated with L. monocytogenes, Staphylococcus aureus, Salmonella Typhimurium, Pseudomonas aeruginosa, and Yersinia enterocolitica, were treated with LPO at a concentration of 0.93 mg/ml. After 7 days at 12°C, S. Typhimurium and E. coli O157:H7 decreased from an initial population of ca. 4.3 log CFU/cm² to 3.7 and 3.5 log CFU/cm², respectively, compared to populations of 6.1 and 5.9 log CFU/cm² in control samples. After 24 h at 37°C, S. Typhimurium and E. coli O157:H7 increased from initial populations of 4.2 log CFU/cm² to 7.8 and 7.7 log CFU/cm², respectively.

Preparation and storage of chemical components used to create the LPOS vary among laboratories. Boussouel et al. (2000) created an LPOS in skim milk by adding 35 µg/ml LPO, inoculating with ca. 4 log CFU/ml Listeria monocytogenes and incubating at 25°C. No reductions in populations were reported. Min et al. (2006) prepared stock solutions of LPO and KSCN by dissolving in Butterfield’s buffer to study the inhibitory activity of LPOS in edible coatings applied to roasted turkey inoculated with Salmonella and E. coli. These pathogens, at
inoculum population of ca. 5.3 log CFU/ml, immediately declined by 2 - 3 log CFU/ml after applying coatings containing 0.036 – 0.063% LPO. Studies have suggested that activation of the LPOS in calf milk replacements lead to an increase in weight gain and to a reduction of incidence of diarrhea, and mortality in calves (Bjorck, 1990; Reiter et al., 1980, Reiter et al., 1981; van Leeuwen et al., 2000).

Neonatal illnesses could be further reduced by protecting premature and other at-risk infants in NICU’s against other pathogens known to cause neonatal infections, e.g., *Streptococcus agalactiae*, *E. coli*, *Haemophilus* spp., *Citrobacter diversus*, *Listeria* spp., and other *Enterobacter* spp. (*meningititis, cloacae, aerogenes, agglomerans, hormechie, and gergovial*) (Willis and Robinson, 1988; Nazarowec-White and Farber, 1997a.; Weir, 2002, Muytjens et al., 1983; Iversen and Forsythe, 2003; Kline, 1988a, Kline, 1988b). The LPOS could also control of growth of other microorganisms that have been isolated from powdered infant formulas (viz., *Enterobacter cloacae* (Simmons et al., 1989; Muytjens et al., 1988; Casewell et al., 1981), *Enterobacter agglomerans, amnigenus, and intermedium, Klebsiella pneumoniae* and oxytoca, *Serratia plymuthica, Citrobacter freundii* and diversus, *Hafnia alvei, Yersinia intermedia and frederiksenii, Escherichia vulneris, hermanii, and adecarboxylata, *Buttiauxella agrestis, Cedecea, Rahnella aquatilis, (Muytjens et al., 1988), Salmonella* spp. (Rowe et al; 1987; Collins et al., 1968; FAO/WHO, 2006;) *Bacillus* (Noriega et al., 1990; Van Acker et al., 2001); *Staphylococcus*, and *Acinetobacter* (Van Acker et al., 2001), may also be achievable using the LPOS.

Activation of the LPOS in reconstituted formulas could also protect against other microorganisms known to be inhibited by the LPOS, e.g., *Campylobacter jejuni* (Beumer et al.,
1985; Borch et al., 1989; Beumer et al., 1985), Bacillus cereus (Zajac et al., 1981), Shigella flexneri and sonnei (Van Opstal et al., 2006), Helicobacter pylori (Shin et al., 2002), Staphylococcus aureus (Elliot et al., 2004; Vannini et al., 2004; Garcia-Graells et al., 2003; Kennedy et al., 2000; Bosch et al., 2000), L. monocytogenes (Elliot et al., 2004; Vannini et al., 2004; Kennedy et al., 2000), E. coli (Min et al., 2006; Sermon et al., 2005; Vannini et al., 2004; Garcia-Graells et al., 2003; Kennedy et al., 2000; Bosch et al., 2000) Bacillus subtilis, Pseudomonas putida, Salmonella Typhimurium and Enteritidis, Proteus vulgaris (Vannini et al, 2004), Yersinia enterocolitica (Elliot et al., 2004), Pseudomonas aeruginosa (Elliot et al., 2004 and Bosch et al., 2000), Pseudomonas fluorescens (Garcia-Graells et al., 2003), Salmonella enterica serotypes Agona, Gaminara, Michigan, and Montevideo (Min et al., 2006), Salmonella Enteritidis (Touch et al., 2004; Min et al., 2006), Salmonella Typhimurium (Elliot et al., 2004), Brucella melitensis (Seifu et al., 2004), fungi (Popper and Knorr, 1997; Jacob et al., 2000), and viruses (Belding et al., 1970; Yamaguchi et al, 1993; Pourtois et al, 1990).

Vannini et al. (2004) used lactoperoxidase that had a manufacturer’s reported activity of 80 – 100 U/mg. The enzyme was added at 30 µg/ml to create the LPOS in skim milk and inoculated with ca. 4.2 log CFU/ml of E. coli strain 555. After incubating at 37°C for 3 h, the population decreased to ca. 2.2 log CFU/ml and between 6 and 24 h was undetectable (detection limit was 0.5 CFU/ml).

Garcia-Graells et al. (2003) created a stock solution of 10 mg/ml LPO by combining LPO in 50% glycerol and 50% phosphate buffered saline and storing at 4°C. Aqueous stock solutions of KSCN and H₂O₂ were prepared and sterilized by passage through 0.22-µm filters. The LPOS was created in skim milk with the addition 5 µg/ml of LPO. Milk was then inoculated with E. coli wild-type MG1655 at ca. 5.8 log CFU/ml, treated with hydrostatic pressure at 300 MPa, and
incubated at 20°C. After high-pressure treatment, populations fell by only ca. 0.1 log CFU/ml. However, at 6 h, populations decreased to below the detection limit (20 CFU/ml) and remained at that level up to 24 h. In another study, Garcia-Graells et al. (2000) reported that the LPOS was activated in skim milk with 5 µg/ml of LPO. Each sample was inoculated with one of four strains of *E. coli*, and incubated at 20°C. In this case, populations of one strain of *E. coli* (wild-type MG1655), initially at ca. 4.2 log CFU/ml, decreased by less than 0.5 log CFU/ml within 24 h. The three other strains behaved in similar fashion and in the case of one strain (ATCC 11775), populations increased after 6 h of incubation.

Lactoperoxidase is typically purchased in a concentrated form and diluted to create a stock solution, which is added. Opstal et al. (2006) prepared a stock solution of LPO and soybean peroxidase thiocyanate. The chemicals were added to a solution made up of 50% (w/v) glycerol in phosphate-buffered saline, and stored at 4°C. Zapico et al. (1998) prepared LPO in sterile deionized water, filtered it through an 0.22 µm pore-size filter, and stored it at –40°C. NaSCN was prepared in an aqueous solution; no storage temperature was specified. Sermon et al. (2005) prepared a stock solution of LPO in 50% glycerol in phosphate-buffered saline and stored at –18°C. Kennedy et al. (2000) dissolved LPO in water to a concentration of 10 µg/ml and stored it at –20°C. McClay et al. (2002) prepared NaSCN and LPO stock solutions with deionized water, followed by filtration (0.2 µm porosity) using a 32-mm diameter syringe filter.

Studies have suggested that the activation of the LPOS in calf milk replacements leads to an increase in weight gain and to a reduction of diarrhea and mortality in calves (Bjorck, 1990; Reiter et al., 1980, Reiter et al., 1981; van Leeuwen et al., 2000). Future research might address activation of the LPOS by addition of naturally occurring food and animal extracts.
Enterobacter sakazakii poses a threat, especially to neonates fed reconstituted infant formula in hospitals and neonatal intensive care wards. The pathogen has been implicated in neonatal infections and traced to contaminated preparation equipment such as spoons and blenders. Activation of LPOS in infant formula in these facilities could serve a number of purposes, including prevention of growth of the pathogen in unintentionally temperature-abused infant formulas, and inactivation of the pathogen on the surface of formula preparation equipment as well as in permanently affixed enteral feeding tubes. It should be noted, however, that the use of this system would not replace acceptable hygienic practices.

**PRESENCE AND BEHAVIOR IN INFANT FORMULA**

Dried cow (bovine) milk and milk products are potential sources of bacteria pathogenic to humans. In one study, coliforms were detected in 3 of 124 samples of spray dried milk, 6 of 54 samples of roller dried milk and 13 of 38 samples of infant formula from 10 factories at populations of > 1 CFU/g (Ghodeker et al., 1980). Populations higher than 90 CFU/g were found in 25, 10 and 5 samples, respectively, and > 1 CFU/g was detected in 3, 6 and 13 samples, respectively. Multiple bacterial pathogens known to occasionally be found in powdered infant formulae include species of *Klebsiella, Citrobacter, Enterobacter, Yersinia, Staphylococcus* and *Streptococcus* (Anderson et al., 1984, Baldwin et al., 1984, Casewell et al., 1981, Fagerman, 1986, Gill and Gill, 1981, Muytjens et al., 1988, Schroeder et al., 1983 and Simmons et al., 1989). The FDA has published bulletins (U.S. Food and Drug Administration, 1988 and U.S. Food and Drug Administration, 2002b) highlighting the dangers of bacterial contamination of enteral formula products, most of which contain powdered milk as the major ingredient. In one study, 28% of 208 enteral formulae for nosocomial patients was reported to contain bacteria (Navajas et al., 1992).
Powdered infant formula has an $a_w$ of ca. 0.2 and is formulated so as to mimic the nutritional profile of human milk rather than cow milk (Breeuwer et al., 2003). Nazarowec-White and Farber (1997a) noted ways that cow milk is modified so as to achieve this goal, e.g., reducing protein and mineral content, increasing the amount of whey protein, increasing the carbohydrate content, increasing the Ca/P ratio, modifying the fat and adding vitamins. Production of powdered infant formula is achieved by either “wet” or “dry” processing (Caric, 1993). The wet process involves combining all essential ingredients with liquid skimmed milk and fat components and heating the mixture at ca. 81°C for 20 s. All components are then added to the mixture and heated to 107–110°C for 60 s, followed by concentrating in a falling film evaporator. The mixture is finally heated at 80°C prior to spray drying (Caric, 1993 and Nazarowec-White and Farber, 1997a). In the dry process, pasteurized evaporated skim milk is dry blended with the balance of essential ingredients (essential fatty acids, vitamins, whey, stabilizers and emulsifiers), pasteurized for 60 s at 110°C and spray dried. It has been noted that problems associated with this method include a higher probability of post-processing contamination, ingredient mixing difficulties and ingredient separation, including lactose segregation (Iversen and Forsythe, 2003, Lambert-Legace, 1982, Nazarowec-White and Farber, 1997a and Nazarowec-White and Farber, 1997b). It is often the case that dry and wet processing procedures are combined by adding the more soluble ingredients during the liquid phase and the less soluble ones into the spray-dried powder matrix (Caric, 1993, Nazarowec-White and Farber, 1997a and Nazarowec-White and Farber, 1997b). A definitive statement concerning which process is inherently more or less likely to result in contamination of products with *E. sakazakii* cannot be made. This is because in-factory contamination is most likely to occur at some point between the spray drying and packaging steps. Critical to preventing product contamination is
how the enteric population in the drying and post-drying pre-packaging factory environments is controlled. Factories will differ in many ways (e.g., age, building materials, design and cleanability) that will influence the level of efficiency in controlling the microbial ecology therein. Hence, risk of formula contamination can be expected to be a function of the particular factory environment rather than solely manufacturing processes.

The 1980 Infant Formula Act, revised in 1986, regulates the production and distribution of infant formula in a unique fashion by setting minimum standards for 29 nutrients (Baker, 2002 and U.S. Food and Drug Administration, 1985). The act mandates adherence to good manufacturing practices (GMPs) and clear labeling, although there is no requirement for sterility. Baker (2002) reported that the FDA limit for bacterial aerobic plate counts in infant formula powder is $4 \log_{10} \text{CFU/g}$. Zink (2003) noted that additional restrictions imposed on powdered infant formula by the FDA are $\leq 3.05 \text{MPN/g}$ for coliforms and $S. \text{aureus}$, $\leq 100 \text{CFU/g}$ for $Bacillus \text{cereus}$ and zero tolerance for both listeriae and salmonellae.

$E. \text{sakazakii}$ has been isolated at varying frequencies from infant formulae examined in several studies and surveys (Block et al., 2002, Muytjens et al., 1988, Nazarowec-White and Farber, 1997b, Postupa and Aldová, 1984 and Van Acker et al., 2001). One of the most notable surveys was conducted by Muytjens et al. (1988) in which the organism was detected in 14.9% of 141 samples of powdered infant formulae originating from 35 countries. Positive samples originated from 13 countries. $E. \text{sakazakii}$ was the third most commonly isolated bacterium, just behind $E. \text{agglomerans}$ and $E. \text{cloacae}$; however, only one of the Enterobacteriaceae exceeded 1 CFU/g. Simmons et al. (1989) reported that $E. \text{sakazakii}$ could out-compete $E. \text{cloacae}$, the second most common $Enterobacter$, despite $E. \text{sakazakii}$ being present in products at lower populations than other bacteria. Muytjens et al. (1988) suggested that contamination of
powdered infant formulae with Enterobacteriaceae must occur post-processing and following the final thermal treatment.

In a survey in the former Czechoslovakia, four strains of *E. sakazakii* were recovered from powdered milk and two strains were recovered from powdered infant formula (Postupa and Aldová, 1984). Other reports have confirmed or implicated powdered infant formula as a source of bacteria responsible for meningitis and associated with neonatal necrotizing enterocolitis (Bar-Oz et al., 2001, Biering et al., 1989, Clark et al., 1990, Muytjens and Kollee, 1990, Noriega et al., 1990, Simmons et al., 1989 and Van Acker et al., 2001). Some in the pediatric community, however, question the ability of *E. sakazakii* to induce necrotizing enterocolitis. The CDC (2002) reported an outbreak of neonatal *E. sakazakii* infections linked to infant formula. Studies have also confirmed the link between powdered infant formula and neonatal infection (Muytjens et al., 1983, Postupa and Aldová, 1984, Muytjens et al., 1988, Biering et al., 1989 and Noriega et al., 1990). The CDC has reported a definitive link between the presence of *E. sakazakii* in powdered infant formula in an unopened can and an outbreak of *E. sakazakii* infection (Centers for Disease Control, 2002 and Baker, 2002).

Nazarowec-White and Farber (1997c) examined powdered infant formula manufactured by five companies (48 cans/company) and isolated *E. sakazakii* at an average population of 0.36 CFU/100 g from eight cans, i.e., 3.3% of the cans analyzed. They also determined the minimum growth temperature of 10 food and clinical isolates by inoculating rehydrated infant formula with 3.0 log_{10} CFU of *E. sakazakii*/ml. Minimum temperatures were between 5.5 and 8.0°C (7°C for the ATCC 29544 type strain). Iversen et al. (2004d) reported that six clinical and food strains grew between 6 and 45°C, with an optimum of 37–43°C, and Kandhai et al. (2004c) reported that the bacterium grew in reconstituted infant formula at 8–47°C. Studies have indicated that up
to 20% of household refrigerator temperatures are kept at $\geq 10^\circ$C (Daniels, 1991, Harris, 1989, Rhodehamel, 1992 and Van Garde and Woodburn, 1987), thus providing temperatures at which \textit{E. sakazakii} will grow. Nazarowec-White and Farber (1997c) reported the lag times and generation times for the 10 \textit{E. sakazakii} strains in infant formula held at 4, 10 and 23$^\circ$C. No differences in behavior of these strains were noted among test formulae and growth did not occur at 4$^\circ$C. The lag times at 23 and 10$^\circ$C were 2–3 h and 19–47 h, respectively, and mean generation times were 0.67 and 4.64 h, respectively. \textit{E. sakazakii} grew more rapidly than \textit{Salmonella} or \textit{E. coli}. Iversen et al. (2004d) reported that 70 strains grew to ca. $10^9$ CFU/ml overnight in TSB at 37$^\circ$C and 44$^\circ$C but none grew within 24 h at 47$^\circ$C. The growth phase of \textit{E. sakazakii} inoculated into powdered infant formula had no significant effect on lag time or growth rate in reconstituted formula (Kandhai et al., 2004c).

Lihono et al. (2004) examined the use of probiotic cultures to control the growth of \textit{E. sakazakii} in rehydrated infant formula at 30 and 35$^\circ$C. \textit{Enterococcus faecium} was more inhibitory than \textit{Lactobacillus acidophilus} or \textit{Pediococcus acidilacticii}, largely as a result of decreased pH caused by the production of acids.

The rate of inactivation of \textit{E. sakazakii} in powdered infant formula held at room temperature has been studied (Edelson-Mammel and Buchanan, 2004b). The population decreased by approximately 2.5 logs (6.0 log CFU/ml to 3.5 log CFU/ml of rehydrated formula) during the initial 5 months. Over the course of the subsequent year, the population decreased an additional 0.5 log, indicating that \textit{E. sakazakii} can survive for an extended period of time in powdered infant formula.

An outbreak of \textit{E. sakazakii} infections in Tennessee was the first reported in the U.S. resulting in a voluntary recall of product by a producer (Himelright et al., 2002; Weir, 2002). An
epidemiologic investigation of microbial cultures from water and the hospital environment failed to isolate *E. sakazakii*, while PFGE patterns of isolates from opened and unopened cans of infant formula were identical to the neonatal patient isolates. In a survey of 16 NICUs, 25% reported using powdered infant formula as the sole feeding source while 31% reported using powdered formula in addition to other formulas (Himelright et al., 2002). A more detailed summary of this outbreak including FDA guidelines for the preparation of powdered infant formula in NICUs was published by the CDC (Himelright et al., 2002). A voluntary recall of ca. 1.5 million cans of another brand of dehydrated infant formula containing *E. sakazakii* occurred in 2002 (FDA, 2002a).

In 2002, the FDA heightened awareness to *E. sakazakii* testing in powdered infant formula. At least one producer of powdered formula responded to this increased emphasis on the microorganism (Wallingford, 2003). Writing the CFSAN, Wallingford (2003) argued that since the infectious does of *E. sakazakii* for term infants is greater than the zero tolerance level, a practicle approach would be the use of MPN testing for *E. sakazakii* in finished product. This approach, however, fails to address the probability of consumer/hospital personnel abuse in formula preparation that may result in contamination with *E. sakazakii* and subsequent growth. Based on a premise that *E. sakazakii* is an opportunistic pathogen affecting only the immunocompromised, it should not be considered an adulterant as are other more virulent bacteria, Wallingford stated that, “*We believe the true risk to public health needs to be factored into the tolerance set for E. sakazakii. If the objective is to eliminate not only organisms pathogenic in normal hosts but all potential opportunistic pathogens, then we have moved to a point where all powdered products need to be sterile.*” The potential negative consequences of feeding sterile infant formula were expressed and it was suggested that there is a direct
correlation between exposure to microorganisms and the development of neonatal immunity, citing Kalliomaki and Isolauri (2002). With regard to demanding a sterile powdered infant formula, Wallingford (2003) went on to say that “Besides the impracticality of achieving this objective with current technology, there is a potential risk to long-term health, especially allergy, of too little exposure to ordinary environmental microbes.”

Lots of powdered infant formula implicated in the 2001 Tennessee outbreak were voluntarily recalled (Himelright, 2002; Weir, 2002) after the death of a premature infant who had been fed the formula (FDA, March 29, 2002). The formula linked to the death is a special product typically marketed only to health care professionals for the treatment of individuals of all ages with illnesses that prevent the absorption of fats in their normal diet (Himelright et al., 2002; Weir, 2002; CDC, 2002). A voluntary recall of another brand of dehydrated infant formula containing *E. sakazakii* has also been made (FDA, November, 2002).

HAZARD ANALYSIS AND RISK MANAGEMENT

It has been demonstrated that powdered infant formula is not commercially sterile and may harbor *E. sakazakii*. However, studies have not established the minimum number of cells needed to cause clinical symptoms, barring poor preparation, temperature abuse, refrigeration and hygienic practices that have been frequently implicated as contributing factors to infections (Block et al., 2002, Clark et al., 1990, Iversen and Forsythe, 2003 and Smeets et al., 1998). In an attempt to lower the risk of infant formula causing neonatal *E. sakazakii* infections, interim guidelines were issued in the U.S. (Himelright et al., 2002), drawing from recommendations issued by the American Dietetic Association (1991) for proper procedures for preparing, feeding and storing powdered infant formula in health care facilities (Table 1-6). It should be noted that these guidelines are for health care facilities and do not apply to home settings (Baker, 2002). In
Table 1-6. Guidelines for preparation and handling infant formula

- Formula products should be selected based on nutritional needs; alternatives to powdered forms should be chosen when possible.
- Trained personnel should prepare powdered formula using aseptic techniques in a designated preparation room.
- Manufacturer's instructions should be followed; product should be refrigerated if not fed immediately and discarded if not used within 24 h after preparation.
- The administration or “hang” time for continuous enteral feeding should not exceed 4 h.
- Written hospital guidelines should be available in the event of a product recall, including notification of health-care providers, a system for reporting, follow-up of specific formula products used, and retention of recall records.

*aSummarized from Himelright et al. (2002).

A letter to health professionals, the FDA (2002c) delineated further recommendations that were later revised to eliminate the recommendation of rehydrating formula with boiling water. Justification for the revision to remove the boiling water step was based on problems associated with the practice, including a potential for loss of heat-sensitive nutrients, changes in physical characteristics of some formulae, the inability to ensure destruction of *E. sakazakii* and possible injury to formula preparation personnel and the infant as a result of scalding. The FDA (2002b) noted the possibility of *E. sakazakii* infections in hospitalized neonates, indicating that the most likely contributing factor is the use of milk-based powdered formula. It was stressed that the likelihood of contracting infections is greater in premature or other immunocompromised infants; however, the warning did not apply to liquid infant formula, which is sold as a commercially sterile product, or to healthy full-term infants at home. Guidelines for Preparation of Formula and Breastmilk in Health Care Facilities, published by the American Dietetic Association (1991), provides 132 guidelines in eight chapters focused on physical facilities, equipment, utensils and supplies, personnel, formula preparation and handling, expressed human milk, delivery and bedside management of infant feedings, microbiology and infection control.
and quality assurance. The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) jointly convened a workshop on *E. sakazakii* in early 2004 (FAO/WHO, 2004) in response to a request for scientific advice from the Codex Committee on Food Hygiene to provide input for the revision of the Recommended International Code of Hygienic Practice for Goods and Infants and Children. An extensive list of recommendations to FAO, WHO, Codex, their member countries, and Non-Governmental Organizations (NGOs) was issued (FAO/WHO, 2004) (Table 1-7). The extent to which these recommendations have been accepted and implemented by the infant formula industry and in settings where infant formula is reconstituted and fed to infants is unknown.

Thermal treatment of rehydrated infant formula may be a practical way of minimizing neonatal risk to *E. sakazakii* infection (Edelson-Mammel and Buchanan, 2004c, Jaspar et al., 1990, Kindle et al., 1996, Muytjens and Kollee, 1990 and Nazarowec-White et al., 2003). Simmons et al. (1989) recommended using refrigeration and limiting hang time to prevent or retard the growth of *E. sakazakii*. Jaspar et al. (1990) recommended powdered infant formula preparation interventions as a means of preventing infections caused by *E. sakazakii*. Suggestions included disinfecting blenders as well as boiling spoons, bottles and nipples prior to formula preparation. They also recommended storing rehydrated formula at refrigeration temperatures as well as heating the formula in a microwave oven just prior to feeding. Others have echoed the last two suggestions and have cautioned that bottle warmers may pose a risk of prolonged exposure to temperatures at which *E. sakazakii* can rapidly grow (Muytjens and Kollee, 1990).

Iversen and Forsythe (2003) made recommendations focused on reducing the probability of neonatal and infant infections caused by infant formulae. These include controlling the initial
Table 1-7. Joint FAO/WHO recommendations to the powdered infant formula industry and infant caregivers concerning processing, preparing and handling powdered and reconstituted products

- In situations where infants are not breast-fed, caregivers, particularly of infants at high risk, should be regularly alerted that powdered infant formula is not a sterile product and can be contaminated with pathogens that can cause serious illness and provided with information that can reduce the risk.

- In situations where infants are not breast-fed, caregivers of high-risk infants should be encouraged to use, whenever possible and feasible, commercially sterile liquid formula or formula which has undergone an effective point of use decontamination procedure (e.g., use of boiling water to reconstitute or by heating reconstituted formula).

- Guidelines should be developed for the preparation, use and handling of infant formula to minimize risk.

- The infant food industry should be encouraged to develop a greater range of commercially sterile alternative formula products for high-risk groups.

- The infant food industry should be encouraged to reduce the concentration of prevalence of *E. sakazakii* in both the manufacturing environment and powdered infant formula. To this end, the infant food industry should consider implementing an effective environmental monitoring program and the use of Enterobacteriaceae rather than coliform testing as an indicator of hygienic control in factory production lines.

- In revising its Code of Practice, Codex should better address the microbiological risks of powdered infants formula and, if deemed necessary, include the establishment of appropriate microbiological specifications for *E. sakazakii* in powdered infant formula.

- FAO/WHO should address the particular needs of some developing countries in establishing effective measures to minimize risk in situations where breast-milk substitutes may be used in exceptionally difficult circumstances, e.g., feeding infants of HIV-positive mothers or low-birth-weight infants.

- The use of internationally validated detected and molecular typing methods for *E. sakazakii* and other relevant microorganisms should be promoted.

- Investigation and reporting of sources and vehicles, including powdered infant formulae, of infection by *E. sakazakii* and other Enterobacteriaceae should be encouraged. This could include the establishment of a laboratory-based network.

- Research should be promoted to gain a better understanding of the ecology, taxonomy, virulence and other characteristics of *E. sakazakii* and on ways to reduce its levels in reconstituted powdered infant formula.

\(^{a}\)Summarized from FAO/WHO (2004).
populations of *E. sakazakii* in raw materials on receipt, reducing populations during heat treatment of raw milk and related ingredients, preventing an increase in population of *E. sakazakii* by avoiding post-processing contamination, applying microbiological criteria and providing appropriate information and preparation instructions, e.g., labeling and consumer education.

**RESEARCH NEEDS**

Studies involving *E. sakazakii* have focused on methods to eliminate the coliform from powdered infant formula, thermal resistance, environmental reservoirs, pathogenicity, antibiotic resistance, exopolysaccharide production, development of rapid methods detection, enumeration and identification, subtyping and predictive modeling, but additional research in these and other areas is needed. The urgency for more information in some areas is greater than in others. One study using a suckling mouse model to determine virulence mechanisms and minimum infectious dose has suggested the possibility of enterotoxin production by *E. sakazakii* (Pagotto et al., 2003) but other virulence factors associated with the bacterium remain unknown. Potential correlations between pathogenicity and pigmentation, shape and texture of colonies, DNase production and the use of other animal models and cell cultures as enterotoxin assay systems need to be investigated (Pagotto et al., 2003).

Lai (2001), recognizing the propensity of *E. sakazakii* to infect certain individuals or groups of individuals in particular ways, commented that tropism for the central nervous system in neonates and infants remains a mystery. Pathogenicity studies are underway and virulence factors of the bacterium are slowly being characterized; however, the ability of *E. sakazakii* to affect some full-term healthy neonates but not others in the same setting is enigmatic. The 1986 Reykjavik, Iceland, outbreak involving one of two twin boys is a case in point (Biering et al.,
Research has not fully explored traditional or technologically advanced treatments for their efficacy in eliminating the pathogen from the powdered milk or powdered infant formula. Baker (2002) recommended researching irradiation of powdered infant formula as an approach to control *E. sakazakii*. It was also suggested that research into protecting neonates from pathogens by using pro- or pre-biotics should be conducted.

Studies to determine conditions that influence survival and growth or cause death of *E. sakazakii* in dry and reconstituted infant formulae are needed, given the likelihood that post-process contamination is the principle route of contamination. Other areas in need of research attention include studies of conditions affecting biofilm formation by *E. sakazakii* in processing plants and hospital settings (e.g., in tubes used for enteral feeding), competitive exclusion to control or prevent growth, efficacy of sanitizers, methods to recover and resuscitate injured cells and evaluation of practices associated with preparing and feeding infant formulae in hospitals and in the home. Surveys of neonatal wards, NICUs and food processing environments for the presence of *E. sakazakii* and an evaluation of hygienic practices in hospitals and the home that may contribute to neonatal infections would also provide information of value when developing intervention strategies to eliminate *E. sakazakii* infections.

Iversen and Forsythe (2003) recommended further work to define the role of capsule production as it relates to desiccation resistance and thermal destruction, as well as characterization of virulence factors. Further investigation should also be done in the areas of phage typing, serotyping, virulence factors, tolerance to desiccation, heat and pH, lag times across a range of temperatures and in an array of food matrices, biofilm formation and the use of bacteriocins, organic acids, disinfectants and other chemicals to control the growth of the *E. sakazakii*. 
REFERENCES


http://www.eatright.org/Public/NutritionInformation/104_17242.cfm


Taxonomic and nomenclature changes in Enterobacteriaceae. Center for Disease Control
and Prevention, Atlanta, GA.

toxicants subcommittee meeting: Enterobacter sakazakii contamination in powdered
infant formula. U.S. Food and Drug Administration, Food Advisory Committee Mtg.,


Burdette, J.H. and Santos, C. 2000. Enterobacter sakazakii brain abscess in the neonate: The

System. 1994. Ceftazidime resistance among selected nosocomial gram negative bacilli

Molecular characterization of Vibrio cholerae O1 strains by pulsed-field gel


Casewell, M.W., J.E. Cooper, and M. Webster. 1981. Enteral feeds contaminated with


New Orleans, Louisiana.

Biochemical Techniques. American Society for Microbiology 104th General Meeting.
New Orleans, Louisiana.


96


containing probiotic cultures American Society for Microbiology 91st Annual Meeting.
Phoenix, AZ.

Relationship of *Enterobacter sakazakii* and related organisms. American Society for
Microbiology 104th General Meeting. New Orleans, Louisiana.

336:1519–1523.


Mahaffee WF, and J.W. Kloepper. 1997. Temporal changes in the bacterial communities of soil,
rhizosphere, and endorhiza associated with field-grown cucumber (*Cucumis sativus* L.)

Marks, N.E., A.S. Grandison, and M.J. Lewis. 2001. Challenge testing of the lactoperoxidase

Watanabe, K. Oishi, and T. Nagatake. 2001. Detection of gram-negative bacteria in
patients and hospital environments at a room in geriatric wards under the infections


Min, S., L.J. Harris, and J. Krochta. 2006. Inhibition of *Salmonella enterica* and *Escherichia coli* O157:H7 on roasted turkey by edible whey protein coatings incorporating the lactoperoxidase system. *J. Food Prot.* 69:784-793.


Quantitative, standardized assays for determining the concentrations of bovine 
191:278-286.


of septicemia with meningitis and hemorrhagic encephalitis in premature infants. *J. 

7:147-150.

Reiter, B. 1985. The biological significance of the non-immunological protective proteins in 

evaluation of the growth-promoting effect of the lactoperoxidase system in newborn 

Reiter, B., and G. Harnulv. 1984. Lactoperoxidase antibacterial system: Natural occurrence, 

Reiter, B., V.M. Marshall, and S.M. Philips. 1980. The antibiotic activity of the lactoperoxidase-


U.S. Food and Drug Administration. 1985. Guidelines concerning notification and testing of infant formula. Accessed on 11/21/03 at:
http://vm.cfsan.fda.gov/~dms/inf-guid.html


U.S. Food and Drug Administration. 2002a. FDA Alerts public regarding recall of powdered infant formula. Accessed on 11/13/03 at:
http://www.fda.gov/bbs/topics/NEWS/2002/NEW00849.html

U.S. Food and Drug Administration Talk Paper. April 12, 2002b. FDA warns about possible *Enterobackter sakazakii* infections in hospitalized newborns fed powdered infant formulas. Accessed on 11/13/03 at:
http://www.cfsan.fda.gov/~dms/inf-ltr3.html

U.S. Food and Drug Administration. 2002d. Isolation and enumeration of Enterobacter sakazakii from dehydrated powdered infant formula. Accessed on 11/05/03 at:  
http://www.cfsan.fda.gov/~comm/mmesakaz.html

Accessed on 11/13/03 at: http://www.fda.gov/oc/po/firmrecalls/meadjohnson03_02.html

http://www.cfsan.fda.gov/~comm/mmesakqa.html


fda.gov/ohrms/dockets/dailys/03/Sept03/090503/95N-0309-emc-000008-03.doc


CHAPTER 2

PERFORMANCE OF MEDIA FOR RECOVERING STRESSED CELLS OF

ENTEROBACTER SAKAZAKII AS DETERMINED USING SPIRAL PLATING AND

ECOMETRIC TECHNIQUES

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ABSTRACT

The method used by the U.S. Food and Drug Administration to analyze powdered infant formula for the presence of *Enterobacter sakazakii* involves the rehydration of samples in sterile distilled water followed by enrichment in Enterobacteriaceae enrichment broth (EE broth), streaking enriched cultures on violet red bile glucose (VRBG) agar, and subculturing presumptive positive colonies on tryptic soy agar (TSA). Yellow-pigmented colonies formed on TSA are then subjected to biochemical confirmation tests. Several media have been recently developed to differentiate and select for *E. sakazakii*. However, the ability of *E. sakazakii* cells that have become injured as a result of exposure to various environmental stresses, or stresses inherent in EE broth, to resuscitate and grow in or on these media has not been described. We undertook a study to determine the performance of differential, selective media for supporting resuscitation and colony development by cells of *E. sakazakii* that had been exposed to stress environments. Cells of four strains of *E. sakazakii* isolated from powdered infant formula were exposed to five stress conditions: heat (55°C for 5 min), freezing (-20°C for 24 h, thawed, frozen again at -20°C for 2 h, thawed), acidic pH (3.55), alkaline pH (11.25), and desiccation in powdered infant formula (aw 0.25, 25°C for 30 days). Control and stressed cells were spiral plated on TSA supplemented with 0.1% pyruvate (TSAP) (control medium), two new fluorogenic agars developed by Leuschner, Baird, Donald, and Cox (LBDC) and Oh and Kang (OK), fecal coliform agar (FCA), Druggan-Forsythe-Iversen medium (DFI), VRBG agar, and Enterobacteriaceae enrichment agar (EE). With the exception of desiccation-stressed cells, suspensions were also plated on these media and on R & F *Enterobacter sakazakii* chromogenic plating medium (RF) using the ecometric technique. The general order of performance of media for recovering control and heat-, freeze-, acid-, and alkaline-stressed cells by spiral plating was
TSAP > LBDC > FCA > OK > VRBG > DFI > EE; the general order for recovering desiccated cells was TSAP > LBDC > FCA > OK > DFI > VRBG > EE. Using the ecometric technique, the general order was TSAP > LBDC > FCA > RF > VRBG > OK > EE > DFI for growth indices of stressed cells. Results indicate that differential, selective media vary greatly in their ability to support resuscitation and colony formation by stressed cells of *E. sakazakii*. The general order of performance of media was similar using spiral plating and ecometric techniques although results from spiral plating should be considered more conclusive.

**INTRODUCTION**

*Enterobacter sakazakii* is recognized as an emerging pathogen, causing neonatal septicemia and meningitis (Simmons, 1989; Himelright et al. 2001; Weir, 2002). The bacterium also has been associated with necrotizing enterocolitis in neonates, as well as infections in other immunocompromised individuals. The method used by the U.S. Food and Drug Administration (FDA) to recover and identify *E. sakazakii* in powdered infant formula requires rehydration in sterile distilled water overnight at 36°C followed by enrichment in Enterobacteriaceae enrichment broth (EE broth) overnight at 36°C, streaking on violet red bile glucose (VRBG) agar and incubating overnight at 36°C, subculturing presumptive positive colonies from VRBG on tryptic soy agar (TSA), and incubating plates for 48 - 72 h at 25°C (USFDA, 2002). Yellow-pigmented presumptive positive *E. sakazakii* colonies are then subjected to confirmation tests using the API 20E biochemical identification system, which requires incubation for an additional 18 - 24 h. EE broth and VRBG agar contain selective and differential ingredients (oxgall and brilliant green in EE broth, and bile salts #3 and crystal violet in VRBG) that may prevent resuscitation of injured *E sakazakii*, precluding its detection in powdered infant formula. The FDA method is also quite protracted, requiring 6 – 7 days to complete.
Numerous media have been developed for recovering *E. sakazakii* from powdered infant formula. Oh and Kang (2004) described a fluorogenic selective and differential medium (OK) for the isolation of *E. sakazakii*. A fluorogen, 4-methylumbelliferyl α-D-glucoside, was added to the medium as an indicator of the presence of α-glucosidase, which is produced by *E. sakazakii*. Bile salts #3 was added to select for enteric organisms and ferric citrate and sodium thiosulfate were added to differentiate H₂S-producing Enterobacteriaceae (e.g., *Citrobacter*, *Salmonella*, *Edwardsiella*, and *Proteus*). This fluorogen was also added to a medium (LBDC) developed by Leuschner et al. (2004) for the presumptive detection of *E. sakazakii* in infant formula. No biochemically selective agents were added to the medium. Iversen et al. (2004a) developed a differential, selective medium (Druggan-Forsythe-Iversen medium) (DFI) for the recovery of *E. sakazakii*. The chromogen 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside (XαGlc) was used as a differential agent. This moiety is cleaved by α-glucosidase creating blue-green *E. sakazakii* colonies. A selective agent, sodium desoxycholate, along with H₂S markers (sodium thiosulfate and ferric ammonium citrate) are also present in the medium. Hsing-Chen and Wu (1992) developed fecal coliform agar (FCA) to recover stressed fecal coliforms. Bromcresol purple was used as an indicator of pH change caused by the utilization of lactose. Calcium lactate, which precipitates around fecal coliform colonies after reacting with carbon dioxide, and bile salts #3 were added as differential and selective agents, respectively. R & F Laboratories (West Chicago, IL) developed R & F *Enterobacter sakazakii* chromogenic plating medium. This medium contains an unidentified chromogen that causes *E. sakazakii* colonies to appear blue-black in color. It also contains unidentified dyes, an inducer, and bile salts as differential and selective inhibitory agents.
The objective of this research was to determine and compare the ability of eight agar media to recover and support colony development of healthy and heat-, freeze-, acid-, alkaline-, and desiccation-stressed cells of *E. sakazakii*.

**MATERIALS AND METHODS**

**Bacterial strains.** Four strains of *E. sakazakii* were examined for their response to stress and recovery on non-selective and differential, selective media. Strain 4921, isolated from powdered infant formula in a 1988 Memphis, Tennessee outbreak of *E. sakazakii* infections, had the same plasmid and multilocus enzyme profile as clinical isolates from infected infants (Simmons, et al., 1989). Strain Frm-TN, acquired from the Division of Healthcare Quality Promotion at the Centers for Disease Control and Prevention, Atlanta, Georgia, was isolated from powdered infant formula implicated in a 2001 case of *E. sakazakii* neonatal infection (Himelright et al., 2002; Weir, 2002). The PFGE pattern of this strain was identical to that of clinical isolates from the infected infant. Strains ES132 and 111389 were isolated from powdered infant formula produced in two separate commercial processing facilities in August and September, 2003.

**Preparation of cells for exposure to stress environments.** Stock cultures of all strains were stored in glycerol (15%) solution at -20°C prior to use in experiments. Cultures were streaked onto tryptic soy agar (TSA; Difco, Becton Dickinson, Sparks, Maryland) slants and incubated at 37°C for 24 h, then stored at 4°C. Cells were grown in brain heart infusion broth (BHI; Difco, Becton Dickinson) (10 ml) at 37°C for 24 h, with loop transfers (ca. 10 µl) at 24-h intervals immediately prior to exposing to stress conditions.
Procedure for stressing cells. Unstressed (control) cells and cells exposed to heated, freezing, acid, alkaline and desiccated environments were enumerated on eight agar media using spiral plating and ecometric techniques.

**Heat stress.** Nine milliliters of sterile potassium phosphate buffer (0.1 M, pH 6.8) in 16 x 125 mm screw-cap test tubes were adjusted at 55°C by immersing tubes in an Isotemp 228 water bath (Fisher Scientific, Pittsburgh, Pennsylvania). One milliliter of a 24-h BHI broth culture of *E. sakazakii* was deposited in the tube and held for 5 min. Heated suspensions were cooled by placing under running tap water for 1 min, serially diluted in a sterile 0.1% peptone solution, and surface plated on test media.

**Freeze stress.** Cells were freeze-stressed by adding 1 ml of a 24-h BHI broth culture to 9 ml of potassium phosphate buffer (0.1M, pH 6.8) in 16 x 125 mm screw-capped test tubes and freezing at -20°C for 24 h. Suspensions were thawed at 21°C, frozen again at -20°C for 2 h, thawed at 21°C, serially diluted in 0.1% peptone solution, and surface plated on test media.

**Acid stress.** Cells were acid stressed by adding 1 ml of a 24-h BHI broth culture to 9 ml of potassium phosphate buffer (0.1 M) adjusted to pH 3.49 with 85% lactic acid and held at 21°C for 30 min. Cell suspensions were neutralized by depositing 1 ml into 9 ml of potassium phosphate buffer (0.1 M, 6.8 pH). Suspensions were serially diluted in 0.1% peptone solution and surface plated on test media. The pH of the 24-h BHI broth culture, the acidified potassium phosphate buffer after adding 1 ml of culture, and the buffer after adding 1 ml of the treated suspension was measured.

**Alkaline stress.** Alkaline stress of *E. sakazakii* cells was achieved by adding 1 ml of a 24-h BHI broth culture to 9 ml of phosphate buffer (0.1M) adjusted to pH 11.65 with sodium hydroxide (2 M) and holding at 21°C for 5 min. The pH of alkaline suspensions of cells was
adjusted to pH 6.88 by adding 1 ml to 9 ml of potassium phosphate buffer (0.1 M, pH 6.8). Suspensions were serially diluted in 0.1% peptone solution and surface plated on test media. The pH of the 24-h BHI broth culture, the alkaline potassium phosphate buffer after adding 1 ml of culture, and the buffer after adding 1 ml of treated suspension was measured.

**Desiccation stress.** Cells were desiccation-stressed by spraying 24-h BHI cultures of *E. sakazakii* onto the surface of a commercially manufactured powdered infant formula (Enfamil with Iron, Infant Formula, Milk-Based Powder, Mead-Johnson Nutritionals, Evansville, Indiana). Powdered infant formula (100 g) was distributed evenly on the bottom of a sterile 30-cm diameter stainless steel mixing bowl. Spray-inoculation of powdered infant formula was done using a chromatography reagent sprayer (Model 422530-0050, Kontes Glass Company, Vineland, New Jersey). The sprayer was held 35 cm above the powdered infant formula and sprayed at ca. 2 psi with nitrogen gas as a carrier. The powdered infant formula was mixed with a sterile spoon between applications of each of four inocula (0.025 ml, 0.75 sec) to achieve a final inoculum of ca. 0.1 ml of culture per 100 g of powdered infant formula. Inoculated powdered infant formula was aseptically deposited in a sterile 500-ml screw-cap bottle, hermetically sealed, shaken for 2 min, analyzed for population of *E. sakazakii* (0 day analysis), and held at 21°C for 31 days before again analyzing for the number of surviving *E. sakazakii*. Control (uninoculated) or inoculated powdered infant formula (10 g) were combined with 90 ml sterile 0.1% peptone solution and 250 µl were spiral plated on TSA on day 0; 100 µl samples were plated on TSAP on day 31. Plates were incubated for 24-h at 37°C before examining for presumptive colonies of *E. sakazakii*. The water activity (aw) of powdered infant formula was measured before and after inoculation on day 0 and again after storage for 31 days at 21°C using an AquaLab Model CX2 Water Activity Measurement Device, Pullman, Washington.
Recovery of stressed cells. Suspensions of control (unstressed) and stressed *E. sakazakii* cells were surface-plated on one non-selective medium and on seven differential, selective agar media. Media were prepared in an A-S-10 Agar Sterilizer (New Bruswick Scientific Co., Edison, New Jersey) and dispensed (14±1 ml) in Petri dishes (90 mm diameter) using a PourMatic Media Dispenser (Model MP-320, New Bruswick Scientific).

Tryptic soy agar supplemented with 0.1% sodium pyruvate (TSAP). TSAP served as a non-selective control medium to recover healthy cells and cells in various states of physiological and structural debilitation caused by exposure to stress environments. Pyruvate was added to TSA for the purpose of enhancing resuscitation of injured cells.

Leuschner, Baird, Donald, and Cox (LBDC) agar (2004). Nutrient agar (Oxoid Inc., Basingstoke, Hampshire, United Kingdom) was prepared according to the manufacturer’s instructions, cooled to 50°C, and supplemented with the fluorogen, 4-methylumbelliferyl α-D-glucoside (50 mg/L) (Sigma Chemical Co., St. Louis, Missouri) prior to dispensing into Petri dishes.

Oh and Kang agar (OK) (2004). This medium was prepared by combining 1 L of deionized water with tryptone (20 g) (Difco, Becton Dickinson), bile salts #3 (1.5 g) (Difco, Becton Dickinson), agar (15 g) (Difco, Becton Dickinson), sodium thiosulfate (1 g) (Sigma Chemical Co.), and ferric citrate (1 g) (MP Biomedicals; LLC, Aurora, Ohio). Ingredients were added to water, dissolved by heating, and autoclaved at 121°C for 15 min. After cooling to 50°C, the medium was supplemented with 4-methylumbelliferyl α-D-glucoside (50 mg/L) before dispensing into Petri dishes.

Fecal coliform agar (FCA). Fecal coliform agar was developed by Hsing-Chen and Wu (1992). Leclercq et al. (2002) reported that FCA recovered higher numbers of fecal coliforms.
than did violet red bile lactose (VRBL) agar, and reported the formation of typical colonies of *E. sakazakii* on FCA and VRBL. Two solutions were separately prepared. Solution A contained tryptone (20 g), bile salts #3 (1.5 g), lactose (10 g), yeast extract (5 g), sodium chloride (5 g), and 850 ml of deionized water. All ingredients were dissolved in water at 50±1°C. Solution B contained calcium lactate (14 g), β-glycerophosphate (1 g) (glycerophosphate disodium salt pentahydrate; MP Biomedicals, LLC, Aurora, Ohio), and 150 ml of deionized water. All ingredients were dissolved in water at 50±1°C. Solution B was slowly poured into Solution A and heated while mixing until the combined solutions became cloudy. The pH of the mixture was adjusted to 7.0±0.2 with a 1 N NaOH solution. Bromcresol purple (3 ml of a solution consisting of 1 g of bromcresol purple [Acros, Fisher Scientific] in 100 ml of 20% ethanol) and 15 g of agar were added to the solution. The mixture was boiled for 2 min, cooled to 50±1°C, and dispensed in Petri dishes.

**Druggan-Forsythe-Iversen (DFI).** This medium was developed by Iversen et al. (2004a) and is manufactured by Oxoid Inc. as Chromogenic *Enterobacter sakazakii* agar (DFI formulation). The medium was prepared according to the manufacturer’s instructions.

**Violet red bile glucose (VRBG) agar.** VRBG agar (Oxoid) was prepared according to the manufacturer’s instructions.

**Enterobacteriaceae Enrichment (EE) agar.** This medium was made by adding agar (15 g/ L) to the Mossel formulation of Enterobacteriaceae Enrichment Broth Mossel (Difco, Becton Dickinson).

**R & F Enterobacter sakazakii chromogenic plating medium (RF).** This medium was prepared and provided by R & F Laboratories, West Chicago, Illinois.
**Spiral plating.** Suspensions of control and stressed cells of *E. sakazakii* were spiral plated in duplicate on all media except for RF medium. Spiral plating was done using an Autoplate 4000 Automated Spiral Plater (Spiral Biotech, Norwood, Massachusetts); 50 µl of suspension was plated using the exponential deposition mode. Plates were incubated for 24 h at 37°C before the number of *E. sakazakii* colonies were counted.

**Ecometric evaluation.** With the exception of desiccation-stressed *E. sakazakii*, suspensions of control cells and stressed cells were streaked on TSAP and differential, selective media using a modification of the ecometric technique (Mossel et al., 1980). The external surface of the bottom of each 90-mm diameter Petri dish was marked with a permanent felt pen into quadrants and numbered 1 through 4. A sterile disposable 10-µl plastic inoculating loop (VWR International, Bridgeport, New Jersey) was immersed in a suspension of control or stressed cells and the suspension filling the loop was deposited on the surface of the agar in the first quadrant. Sequentially, and without re-immersing the loop in suspension, five parallel lines were sequentially streaked on the surface of agar in each of the four quadrants, with one additional streak transversing the middle of the plate at the cross-section of the quadrants, creating a total of 21 streaks. Plates were incubated at 37°C and examined for growth after incubating for 24 h and again after 48 h. Scoring of plates consisted of assigning a growth index (GI) value of 0 to 4.2 to each plate. Growth indices were calculated by counting the number of streaks (out of 21) on each plate that yielded at least one colony (1 CFU) after incubating for 24 and 48 h and multiplying that number by 0.2

**Statistical analysis.** Three independent replicate trials were conducted using spiral plating and ecometric plating techniques. Data from spiral plating were analyzed to determine significant differences ($P \leq 0.05$) in the number of control cells of *E. sakazakii* recovered on
various media, excluding RF, which was not evaluated. Significant differences in the number of control cells and cells exposed to a given stress treatment and plated on various test media were also determined. The same analyses were done to determine significant differences in GI calculated for control and stressed cells in suspensions streaked on media using the ecometric technique. Mean separation of values were determined by the least significant difference by the Student’s t test using general linear models on SAS software version 8.0 (Statistical Analysis Systems Institute, Cary, North Carolina).

RESULTS AND DISCUSSION

Recovery of control (unstressed) cells by spiral plating. The number of control (unstressed) cells of strains 4921 and 111389 recovered on TSAP was significantly higher than on selective media (Table 1). LBDC was equivalent to TSAP in recovering control cells of strains ES132 and Frm-TN. Enterobacteriaceae enrichment agar (EE) recovered significantly fewer control cells of strains ES132 and 111389 than all other media.

Recovery of heat-stressed cells by spiral plating. Significantly higher numbers of control cells of all test strains, compared to number of heat-stressed cells, were recovered on TSAP and all selective media (Table 2-1). Thermally-stressed cells of all strains were recovered in significantly higher numbers on TSAP than on selective media. Overall, LBDC recovered higher numbers of control and heat-stressed cells than the differential, selective media across all strains. The other five differential, selective media performed similarly in recovering heat-injured cells of all test strains except 4921, which was recovered in higher numbers on FCA than on the other selective media.

Data for the populations of control cells of all test strains and for heat-stressed cells of all test strains recovered on each test medium were separately composited and analyzed for
TABLE 2-1. Populations of control and heat-stressed cells of *Enterobacter sakazakii* recovered on TSAP and differential selective media.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Recovery medium</th>
<th>Control Cells</th>
<th>Heat-stressed cells</th>
<th>R²</th>
<th>R³</th>
<th>R⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES132</td>
<td>TSAP</td>
<td>a 9.21 a</td>
<td>a 8.78 b</td>
<td>0.43</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>LBDC</td>
<td>a 9.15 a</td>
<td>b 8.49 b</td>
<td>0.29</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FCA</td>
<td>b 9.06 a</td>
<td>c 7.91 b</td>
<td>0.87</td>
<td>1.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DFI</td>
<td>b 9.02 a</td>
<td>c 7.59 b</td>
<td>1.19</td>
<td>1.43</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OK</td>
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<td>c 8.08 b</td>
<td>0.70</td>
<td>0.97</td>
<td></td>
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<tr>
<td></td>
<td>VRGB</td>
<td>b 9.06 a</td>
<td>c 7.72 b</td>
<td>1.06</td>
<td>1.34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EE</td>
<td>c 8.74 a</td>
<td>c 7.97 b</td>
<td>0.81</td>
<td>0.77</td>
<td></td>
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<td>4921</td>
<td>TSAP</td>
<td>a 9.40 a</td>
<td>a 8.85 b</td>
<td>0.55</td>
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<td></td>
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<td></td>
<td>LBDC</td>
<td>b 8.99 a</td>
<td>b 8.69 b</td>
<td>0.16</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FCA</td>
<td>b 8.94 a</td>
<td>c 8.52 b</td>
<td>0.33</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DFI</td>
<td>b 8.81 a</td>
<td>c 8.16 b</td>
<td>0.69</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OK</td>
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<td>c 8.25 b</td>
<td>0.60</td>
<td>0.62</td>
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<td></td>
<td>VRGB</td>
<td>b 8.37 a</td>
<td>c 7.88 b</td>
<td>0.97</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EE</td>
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<td>c 5.29 b</td>
<td>3.56</td>
<td>1.05</td>
<td></td>
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<td>111389</td>
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<td>1.24</td>
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<td></td>
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<td>b 6.99 b</td>
<td>0.90</td>
<td>1.98</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>b 5.38 b</td>
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<tr>
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</tr>
<tr>
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<td>4.65</td>
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<tr>
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<td>b 5.54 b</td>
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<tr>
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<tr>
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<td>a 7.21 b</td>
<td>1.89</td>
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<tr>
<td></td>
<td>LBDC</td>
<td>a 9.05 a</td>
<td>b 6.93 b</td>
<td>0.28</td>
<td>2.12</td>
<td></td>
</tr>
<tr>
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<td>FCA</td>
<td>b 8.87 a</td>
<td>c 5.23 b</td>
<td>1.98</td>
<td>3.64</td>
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<tr>
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<td>bc 8.73 a</td>
<td>c 5.42 b</td>
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<tr>
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<td>2.87</td>
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<td>EE</td>
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<td>c 5.23 b</td>
<td>1.98</td>
<td>2.25</td>
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</tr>
</tbody>
</table>

1 Mean values in the same row that are not followed by the same letter are significantly different (P ≤ 0.05). Within strain, mean values in the same column that are not preceded by the same letter are significantly different.
2 Within strain, reduction in the number of control cells recovered on differential selective media compared to the number of control cells recovered on TSAP.
3 Within strain, reduction in the number of heat-stressed cells recovered on differential, selective media compared to the number of heat-stressed cells recovered on TSAP.
4 Within strain, and recovery medium, reduction in the number of heat-stressed cells recovered compared to the number of control cells recovered.
significant differences. TSAP performed better than all other media for recovering control and
heat-stressed cells (Table 2-2). LBDC outperformed all selective media except FCA, which was
not different than DFI, OK, VRBG, and EE in recovering heat-stressed cells. The general order
of performance of media for recovering heat-stressed E. sakazakii by spiral plating was TSAP >
LBDC > FCA > OK > DFI > VRBG > EE.

The theoretical $D_{55^\circ C}$ value for the composite of all four strains, which was reduced by an
average of 0.69 log CFU within 5 min, was calculated to be 7.25 min. Reductions were 0.43,
0.55, 1.24, and 1.89 log CFU/ml for strains ES132, 4921, 111389, and FRM-TN, respectively.
Corresponding $D_{55^\circ C}$ values were 11.63, 9.09, 4.03, and 2.65 min (Table 2-3). These $D_{55^\circ C}$
values should be considered only as theoretical, however, because reductions in populations
resulting from heat treatment were less than 1.9 log CFU/ml and cells were subjected to only one
heating time.

Results show that there is more than a four-fold difference in heat resistance among the
four strains, which is not atypical of the disparity in $D$ values for E. sakazakii reported by others.
Edelson-Mammel and Buchanan (2004a) examined the thermal resistance of twelve strains of E.
sakazakii in rehydrated powdered infant formula using a submerged coil method apparatus
(Buchanan and Edelson, 1999). A 19-fold difference in $D_{58^\circ C}$ was reported (Table 2-3). $D_{56^\circ C}$
vvalues ranged from 18.52 to 23.81 min, which were considerably greater than the four-strain
average $D_{55^\circ C}$ value of 7.25 min calculated in our study. Nazarowec-White and Farber (1997)
were the first to describe thermal inactivation and the heat resistance characteristics of E.
sakazakii in rehydrated powdered infant formula. Pooled $D$ values for five food isolates and five
clinical isolates at 52, 54, 56, 58, and 60$^\circ C$ are listed in Table 2-3.
TABLE 2-2. Populations of control, heat-stressed, freeze-stressed, acid-stressed, and alkaline-stressed cells of a composite of four strains of *Enterobacter sakazakii* recovered on TSAP and differential, selective media

<table>
<thead>
<tr>
<th></th>
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<th></th>
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<th></th>
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</thead>
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<td>8.08 a</td>
<td>8.45 a</td>
<td>8.75 a</td>
<td>8.51 a</td>
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<td>9.04 b</td>
<td>8.31 b</td>
<td>8.02 a</td>
<td>8.21 b</td>
<td>8.70 a</td>
<td>8.39 b</td>
</tr>
<tr>
<td>FCA</td>
<td>8.96 bc</td>
<td>8.02 bc</td>
<td>7.48 b</td>
<td>7.22 c</td>
<td>8.33 b</td>
<td>7.96 c</td>
</tr>
<tr>
<td>DFI</td>
<td>8.88 bc</td>
<td>7.66 c</td>
<td>7.03 b</td>
<td>6.73 c</td>
<td>8.25 b</td>
<td>7.78 d</td>
</tr>
<tr>
<td>OK</td>
<td>8.85 c</td>
<td>7.87 c</td>
<td>7.29 b</td>
<td>6.89 c</td>
<td>8.26 b</td>
<td>7.85 cd</td>
</tr>
<tr>
<td>VRGB</td>
<td>8.79 cd</td>
<td>7.51 c</td>
<td>7.24 b</td>
<td>6.86 c</td>
<td>8.29 b</td>
<td>7.80 cd</td>
</tr>
<tr>
<td>EE</td>
<td>8.42 d</td>
<td>7.37 c</td>
<td>6.66 b</td>
<td>6.56 c</td>
<td>7.82 c</td>
<td>7.39 e</td>
</tr>
</tbody>
</table>

1. Within each column, mean values that are not followed by the same letter are significantly different (P ≤ 0.05).
2. Within control or stress condition, reduction in the number of cells recovered on differential, selective media compared to the number of cells recovered on TSAP.
3. Within a composite of all stress conditions, reduction in the number of cells recovered on differential, selective media compared to the number recovered on TSAP.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Medium</th>
<th>Bacterial Strain</th>
<th>D-value (min) at various temperatures (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>This study</td>
<td>Phosphate buffer</td>
<td>ES132</td>
<td>11.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4921</td>
<td>9.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>111389</td>
<td>4.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Frm-TN</td>
<td>2.65</td>
</tr>
<tr>
<td>Breeuwer et al. (2003)</td>
<td>Infant formula milk</td>
<td>1387-2</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Phosphate buffer</td>
<td>1387-2</td>
<td>20.20 7.10 2.40 0.48</td>
</tr>
<tr>
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<td></td>
<td>16</td>
<td>8.30 6.40 1.10 0.40</td>
</tr>
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</tr>
<tr>
<td></td>
<td></td>
<td>145</td>
<td>0.27</td>
</tr>
<tr>
<td>Iversen et al. (2004b)</td>
<td>Tryptic soy broth</td>
<td>11467 (type strain)</td>
<td>14.90 2.70 1.30 0.90 0.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>823</td>
<td>10.20 1.20 1.70 0.20 0.20</td>
</tr>
<tr>
<td></td>
<td>Infant formula milk</td>
<td>11467</td>
<td>16.40 5.10 2.60 1.10 0.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>823 (capsulated)</td>
<td>11.70 3.90 3.80 1.80 0.20</td>
</tr>
<tr>
<td>Nazarowec-White and Farber (1997)</td>
<td>Infant formula milk</td>
<td>5 Pif-isolated strains</td>
<td>54.82 18.57 9.75 3.44 2.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 Clinical strains</td>
<td>54.76 36.72 10.91 5.45 3.06</td>
</tr>
<tr>
<td>Edelson-Mammel and Buchanan (2004a)</td>
<td>Infant formula milk</td>
<td>51329</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NQ2-Environ</td>
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<tr>
<td></td>
<td></td>
<td>NQ3-Environ</td>
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<td>LCDC 674</td>
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<td>CDC A3(10)</td>
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<tr>
<td></td>
<td></td>
<td>NQ1-Environ</td>
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<td>29544</td>
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<td></td>
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<td>SK 90</td>
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<td></td>
<td></td>
<td>LCDC 648</td>
<td>9.02</td>
</tr>
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<td>4.01C</td>
<td>9.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>607</td>
<td>21.05 9.87 4.41 0.59 0.07</td>
</tr>
</tbody>
</table>
Breeuwer et al. (2003) performed thermal inactivation studies using *Escherichia sakazakii* isolated from powdered infant formula manufacturing plants. Cells in the stationary growth phase had *D* values considerably lower than those reported by Nazarowec-White and Farber (1997). Five strains heated at 58°C were determined to have *D* values of 0.27 - 0.50 min (Table 2-3). Two strains had *D*\(_{53^\circ C}\) values of 8.3 and 20.2 min, *D*\(_{54^\circ C}\) values of 6.4 and 7.1 min, and *D*\(_{56^\circ C}\) values of 1.1 and 2.4 min. These values are closer to the pooled *D*\(_{55^\circ C}\) value of 7.25 min observed in our study.

Iversen et al. (2004b) determined *D* values for the type strain of *Escherichia sakazakii* (NCTC 11467) as well as a capsulated strain (823) (Table 2-3). Cells were heated in infant formula milk and in TSB. *D* values of 16.4, 5.1, 2.6, 1.1, and 0.3 min at 54, 56, 58, 60, and 62°C, respectively, were reported when the type strain was heated in infant formula milk. Studies in various laboratories show disparities of 19-fold for *D*\(_{56^\circ C}\) values, 22-fold for *D*\(_{60^\circ C}\) values, and over 36-fold for *D*\(_{58^\circ C}\) values.

The lower *D* values reported by Breeuwer et al. (2003), compared to those reported by others (Edelson-Mammel and Buhannan, 2004a; Nazarowec-White and Farber, 1997; Iversen et al., 2004b) could be due in part to variations in strain, composition of heating media, and methodology. We heated *Escherichia sakazakii* cells in potassium phosphate buffer (pH 6.8), which is similar to the neutral pH disodium hydrogen phosphate/potassium dihydrogen phosphate buffer used by Breeuwer et al. (2003). In other studies, infant formula milk or TSB were used to suspend cells during heating. Rehydrated powdered infant formula and UHT infant formula milk contain fat, protein, and carbohydrate, which are not present in phosphate buffers. These components may protect cells against thermal inactivation. Other research supporting this hypothesis has demonstrated that the rate of thermal inactivation of *Escherichia coli* in milk increases with
an increase in fat content (Jay, 2000; Carpenter, 1967). Batish et al. (1980) suggested that
tolerance of enterococci is enhanced in infant formula milk, which contains elevated levels of fat
and dissolved solids.

**Recovery of freeze-stressed cells by spiral plating.** A significantly higher number of
control cells compared to freeze-stressed cells of all test strains were recovered on all media
(Table 2-4). TSAP performed significantly better than all differential, selective media in
recovering control cells of strains 4921 and 111389 but not strains ES132 and Frm-Tn. LBDC
outperformed all other selective media in recovering freeze-stressed cells. Freeze-stressed cells
of strain 111389 and Frm-TN were recovered in equal numbers on TSAP and LBDC.

When data were analyzed by combining values for all four strains of freeze-stressed cells
as a composite, TSAP and LBDC were shown to perform better than all other media (Table 2-2).
The general order of performance of media used to recover freeze-stressed cells by spiral plating
was TSAP, LBDC > FCA, OK, VRBG, DFI, EE.

**Recovery of acid-stressed cells by spiral plating.** A significantly higher number of
control cells, compared to acid-stressed cells, of all strains of *E. sakazakii* were recovered on
TSAP and all selective media (Table 2-5). Recovery of control cells of strains 4921 and 111389,
but not strains ES132 and Frm-TN, was significantly lower on LBDC compared to TSAP. TSAP
performed significantly better than all selective media in recovering acid-stressed cells. There
were no significant differences in performance of FCA, DFI, OK, VRBG, and EE in recovering
acid-stressed cells of the four test strains.

Comparison of media for their performance in recovering a composite of the four acid-
stressed strains revealed that media fell into three distinct categories (Table 2-2). The order of
performance was TSAP > LBDC > FCA, OK, VRBG, DFI, EE. This order differs only slightly
TABLE 2-4. Populations of control and freeze-stressed cells of *Enterobacter sakazakii* recovered on TSAP and differential, selective media.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Recovery medium</th>
<th>Control cells</th>
<th>Freeze-stressed cells</th>
<th>( R^2 )</th>
<th>( R^3 )</th>
<th>( R^4 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES132</td>
<td>TSAP</td>
<td>a 9.21 a</td>
<td>a 7.92 b</td>
<td></td>
<td></td>
<td>1.29</td>
</tr>
<tr>
<td></td>
<td>LBDC</td>
<td>a 9.15 a</td>
<td>b 7.83 b</td>
<td>0.06</td>
<td>0.09</td>
<td>1.32</td>
</tr>
<tr>
<td></td>
<td>FCA</td>
<td>b 9.06 a</td>
<td>cd 7.05 b</td>
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<td>0.87</td>
<td>1.21</td>
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<tr>
<td></td>
<td>DFI</td>
<td>b 9.02 a</td>
<td>d 6.80 b</td>
<td>0.19</td>
<td>1.12</td>
<td>2.22</td>
</tr>
<tr>
<td></td>
<td>OK</td>
<td>b 9.05 a</td>
<td>ed 7.07 b</td>
<td>0.16</td>
<td>0.85</td>
<td>1.98</td>
</tr>
<tr>
<td></td>
<td>VRGB</td>
<td>b 9.06 a</td>
<td>c 7.25 b</td>
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<td>0.67</td>
<td>1.81</td>
</tr>
<tr>
<td></td>
<td>EE</td>
<td>c 8.74 a</td>
<td>d 6.67 b</td>
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<td>1.25</td>
<td>2.07</td>
</tr>
<tr>
<td>4921</td>
<td>TSAP</td>
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<td>a 8.03 b</td>
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<td>1.37</td>
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<td>b 7.96 b</td>
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<td>0.07</td>
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<td>c 7.30 b</td>
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<td>0.73</td>
<td>1.64</td>
</tr>
<tr>
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<td>DFI</td>
<td>b 8.81 a</td>
<td>d 6.63 b</td>
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<td>c 7.21 b</td>
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<td>0.82</td>
<td>1.66</td>
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<tr>
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<td>VRGB</td>
<td>b 8.37 a</td>
<td>d 6.63 b</td>
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<td>1.40</td>
<td>1.74</td>
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<td>EE</td>
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<td>d 3.94 b</td>
<td>3.06</td>
<td>4.09</td>
<td>2.40</td>
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<tr>
<td>111389</td>
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<td>a 8.11 b</td>
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</tr>
<tr>
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<td>a 8.08 b</td>
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<td>0.03</td>
<td>0.89</td>
</tr>
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<td>0.24</td>
<td>1.09</td>
</tr>
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<td>d 7.42 b</td>
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<td>c 7.64 b</td>
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<td>0.47</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td>VRGB</td>
<td>c 8.85 a</td>
<td>c 7.59 b</td>
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<td>0.52</td>
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<td>e 6.89 b</td>
<td>0.46</td>
<td>1.22</td>
<td>1.78</td>
</tr>
<tr>
<td>Frm-TN</td>
<td>TSAP</td>
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<td>ab 8.01 b</td>
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<td></td>
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<td></td>
<td>LBDC</td>
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<td>a 8.31 b</td>
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<td>-0.30</td>
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</tr>
<tr>
<td></td>
<td>FCA</td>
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<td>b 7.23 b</td>
<td>0.23</td>
<td>0.78</td>
<td>1.64</td>
</tr>
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<td>0.37</td>
<td>1.26</td>
<td>1.98</td>
</tr>
<tr>
<td></td>
<td>OK</td>
<td>de 8.26 a</td>
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<td>1.21</td>
<td>1.46</td>
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<tr>
<td></td>
<td>VRGB</td>
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<td>b 6.91 b</td>
<td>0.53</td>
<td>1.10</td>
<td>1.66</td>
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<td></td>
<td>EE</td>
<td>e 7.48 a</td>
<td>b 6.61 b</td>
<td>1.62</td>
<td>1.40</td>
<td>0.87</td>
</tr>
</tbody>
</table>

1 Mean values in the same row that are not followed by the same letter are significantly different (\( P \leq 0.05 \)). Within strain, mean values in the same column that are not preceded by the same letter are significantly different.

2 Within strain, reduction in the number of control cells recovered on differential, selective media compared to the number of control cells recovered on TSAP.

3 Within strain, reduction in the number of freeze-stressed cells recovered on differential, selective media compared to the number of freeze-stressed cells recovered on TSAP.

4 Within strain and recovery medium, reduction in the number of freeze-stressed cells compared to the number of control cells recovered.
TABLE 2-5. Populations of control and acid-stressed cells of *Enterobacter sakazakii* recovered on TSAP and differential selective media.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Recovery medium</th>
<th>Control cells</th>
<th>Acid-stressed cells</th>
<th>R²</th>
<th>R³</th>
<th>R⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES132</td>
<td>TSAP</td>
<td>a 9.21 a</td>
<td>a 8.36 b</td>
<td>0.85</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LBDC</td>
<td>a 9.15 a</td>
<td>b 8.16 b</td>
<td>0.20</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
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<td>c 4.67 b</td>
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<td>e 7.48 a</td>
<td>c 5.97 b</td>
<td>1.67</td>
<td>1.51</td>
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</tbody>
</table>

1 Mean values in the same row that are not followed by the same letter are significantly different \( (P \leq 0.05) \). Within strain, mean values in the same column that are not preceded by the same letter are significantly different.

2 Within strain, reduction in the number of control cells recovered on differential, selective media compared to the number of control cells recovered on TSAP.

3 Within strain, reduction in the number of acid-stressed cells recovered on differential, selective media compared to the number of acid-stressed cells recovered on TSAP.

4 Within strain and recovery medium, reduction in the number of acid-stressed cells recovered compared to the number of control cells recovered.
from that for freeze-stressed cells in that TSAP performed better than LBDC for acid-stressed cells. Mean pH values of BHI culture and buffered suspensions during acid stressing of cells are shown in Table 2-6. The mean pH values of BHI cultures after 24 h of incubation, after acidification, and after neutralization for a composite of the four strains were 6.14, 3.54, and 6.41, respectively. Edelson-Mammel and Buchanan (2004c) examined twelve strains of *E. sakazakii* for resistance to low pH stress. Cells were inoculated into BHI and grown to stationary phase by incubating at 36°C for 18 h. Cultures were then transferred to TSB adjusted to either pH 3.0 or pH 3.5 with hydrochloric acid and held at 36°C up to 5 h. There were no reductions of viable numbers of *E. sakazakii* cells in TSB at pH 3.5 during the first 2 h of incubation. However, after 5 h, the population of the type strain (ATCC 29544) was reduced by approximately 1 log CFU/ml. The population of strain ATCC 51329 decreased by 2, 3, and 4 logs at 3, 4, and 5 h, respectively. The remaining ten strains decreased by < log 0.5 CFU/ml at the 5-h incubation time. Three strains (ATCC 51239, EWFAKRC11NNV1493, and LCDC 648) were the most sensitive at pH 3, decreasing by 4, 4, and 5 logs, respectively, within 1 h. The two most acid-resistant strains (CDC A3[10], and SK 90) decreased by 3.5 and 4 logs, respectively, after incubating for 3 h. In our study, stressing *E. sakazakii* in phosphate buffer at pH 3.5 for 30 min at 21°C resulted in log CFU/ml reductions of (strain in parenthesis) 0.85 (ES132), 0.64 (4921), 0.68 (111389), and 1.46 (Frm-TN) (Table 2-5). This lower tolerance to acid stress, compared to tolerance of strains studied by Edelson-Mammel and Buchanan (2004a), may have been influenced by factors such as age of the cells, composition of and time of exposure to the stress medium, and temperature during exposure of cells to acidic environment.

**Recovery of alkaline-stressed cells by spiral plating.** Significantly higher numbers of control cells of all test strains, compared to alkaline-stressed cells, were recovered on all media (Table 2-7). TSAP and LBDC performed significantly better than other media in recovering
TABLE 2-6. pH values of *E. sakazakii* suspensions before and after acid stress treatment

<table>
<thead>
<tr>
<th>Strain</th>
<th>pH values</th>
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<th></th>
</tr>
</thead>
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<td>24-h culture</td>
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<td>Neutralized suspension plus acid</td>
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<td>6.00</td>
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<td>111389</td>
<td>6.20</td>
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<td>6.40</td>
</tr>
<tr>
<td>Frn-TN</td>
<td>6.09</td>
<td>3.56</td>
<td>6.43</td>
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</table>
TABLE 2-7. Populations of control and alkaline-stressed cells of *Enterobacter sakazakii* recovered on TSAP and differential selective media.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Recovery medium</th>
<th>Control cells</th>
<th>Alkaline-stressed cells</th>
<th>( R^2 )</th>
<th>( R^3 )</th>
<th>( R^4 )</th>
</tr>
</thead>
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<tr>
<td>ES132</td>
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<tr>
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<td>0.04</td>
<td>0.37</td>
</tr>
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<td>0.70</td>
</tr>
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<td>bc 8.35 b</td>
<td>0.16</td>
<td>0.47</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>VRBG</td>
<td>b 9.06 a</td>
<td>b 8.48 b</td>
<td>0.15</td>
<td>0.34</td>
<td>0.58</td>
</tr>
<tr>
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<td>EE</td>
<td>c 8.74 a</td>
<td>c 8.15 b</td>
<td>0.47</td>
<td>0.67</td>
<td>0.59</td>
</tr>
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<td>a 8.84 b</td>
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<td>0.02</td>
<td>0.17</td>
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<td>a 8.82 b</td>
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<td>0.34</td>
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<td>0.47</td>
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<td>0.33</td>
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<tr>
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<td>b 7.16 b</td>
<td>1.62</td>
<td>1.22</td>
<td>0.32</td>
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</tbody>
</table>

1 Mean values in the same row that are not followed by the same letter are significantly different \( (P \leq 0.05) \). Within strain, mean values in the same column that are not preceded by the same letter are significantly different.

2 Within strain, reduction in the number of control cells recovered on differential, selective media compared to the number of control cells recovered on TSAP.

3 Within strain, reduction in the number of alkaline-stressed cells recovered on differential, selective media compared to the number of alkaline-stressed cells recovered on TSAP.

4 Within strain and recovery medium, reduction in the number of alkaline-stressed cells recovered compared to the number of control cells recovered.
alkaline-stressed cells among all strains. Fewer control and alkaline-stressed cells were recovered on EE than on other media, although differences were not always statistically significant. The number of alkaline-stressed cells of strain FRM-TN recovered was not significantly different on selective, differential media except LBDC.

Mean pH values of BHI cultures and alkaline-buffered suspensions at various points during stressing cells are listed in Table 2-8. Average mean pH values of a composite of four test strains of _E. sakazakii_ in BHI after 24 h of incubation, after alkalization, and after neutralization were 6.36, 11.26, and 6.90, respectively.

Data for alkaline-stressed cells of a composite of all four strains were analyzed to determine the overall rankings of the performance of media (Table 2-2). The order of performance for media used to recover alkaline-stressed cells by spiral plating was TSAP, LBDC > FCA, VRGB, OK, DFI > EE. This order of performance is in general agreement with the order observed for recovery of heat-, freeze-, and acid-stressed cells.

**Comparison of media for recovering cells exposed to a composite of stress conditions by spiral plating.** Composite values from spiral plating experiments using heat-, freeze-, acid-, and alkaline-stressed cells were analyzed to determine significant differences in number of control and stressed _E. sakazakii_ recovered on each test medium (Table 2-2). A significantly higher number of stressed cells were recovered on TSAP than on differential, selective media. LBDC recovered a higher number of stressed cells than other differential selective media. The general order of performance in recovering stressed cells was TSAP > LBDC > FCA, OK, VRGB, DFI > EE. These results demonstrate the inferiority of differential, selective media in recovering injured _E. sakazakii_, regardless of the stress condition causing the injury. LBDC was superior to all other differential, selective media, however, making it an attractive candidate for
<table>
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<th>Suspension plus alkali</th>
<th>Neutralized suspension plus alkali</th>
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<td>6.89</td>
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</tbody>
</table>
use in direct plating of samples that may contain stressed or injured cells of *E. sakazakii* in the presence of other bacteria. The high performance of LBDC in recovering stressed *E. sakazakii* is attributed in part to the fact that it contains only one differential ingredient, 4-methylumbelliferyl α-D-glucoside, whereas all other media except TSAP contain numerous ingredients that provide differential, selective characteristics. Oxoid nutrient agar (ONA), used as a basal medium in LBDC agar, differs from TSA in that ONA contains Lab-Lemco powder (a meat extract) and an unspecified peptone as nitrogen sources, whereas TSA contains an enzymatic digest of soybean meal and a pancreatic digest of casein as nitrogen sources. ONA also contains yeast extract. EE and VRBG agar are both recommended by the FDA method, respectively, for enriching and detecting *E. sakazakii* in dehydrated powdered infant formula (USFDA, 2002). Results from our study indicate that agar made from EE broth and VRBG agar inhibit the recovery of injured cells.

One of the first sites of debilitation caused by thermal assault is the destruction of cellular enzymes that protect against ultraviolet radiation and destructive oxygen species (Czechowicz et al., 1996). It is therefore important for recovery media to contain oxygen scavengers that protect against free radicals harmful to injured cells. Examples of oxygen scavengers added to growth media include pyruvate, blood, and catalase (Flowers et al., 1977) ascorbic acid (Dave and Shah, 1997), L-cysteine (Nebra et al., 2002), sodium thioglycolate, which works by blocking H₂O₂ formation (Nebra et al., 2002), ferrous sulfate (Uyttendaele and Debevere, 1996), sodium metabisulfite (Uyttendaele and Debevere, 1996), and Oxyrase® (Oxyrase Inc., Mansfield, Ohio), which works by reducing O₂ into H₂O₂, thereby lowering the oxygen tension (Fung, 1992). We supplemented TSA with 0.1% pyruvate to enhance the resuscitation and growth of cells that may have been injured as a result of exposure to stress environments. Baird-Parker and Davenport (1965) demonstrated that pyruvate enhanced the repair of damaged bacterial cells that were
otherwise inhibited in their ability to resist toxic oxidizing substances that were present as a result of the destruction of catalase within the cell. They concluded that pyruvate creates a beneficial environment by decomposing the oxidizer, hydrogen peroxide. Brewer et al. (1977) later confirmed this hypothesis. Leyer and Johnson (1992) reported that the recovery of Salmonella cells after treatment with organic acid was increased by 1,000-fold using a recovery medium containing 0.1% sodium pyruvate. Czechowicz et al. (1996) reported that populations of unheated and heat-stressed (57°C for 50 min and 57°C for 60 min) E. coli O157:H7 cells were recovered in higher numbers in media containing sodium pyruvate than in media without pyruvate. Their data showed that TSAP recovered 10-fold more of the injured bacterial cells than were recovered on TSA. Plate count agar (PCA) supplemented with pyruvate resulted in a 4-log higher recovery of heat-injured cells than on TSA. The higher recovery on PCA was attributed not only to the addition of sodium pyruvate but also to the additional sources of energy and growth factors (glucose and yeast extract, respectively) that are not contained in TSA. They concluded that pyruvate enhanced cellular repair following heat damage. Glucose also has been demonstrated to be effective in promoting the recovery of injured bacterial cells (Draughon and Nelson, 1981).

Other studies have also shown that pyruvate increases the recovery of heated and unheated bacterial cells, e.g., Clostridium perfringens (Hood et al., 1990), Staphylococcus aureus (Hurst et al., 1976), Salmonella senftenberg (Rayman et al., 1978), Shigella flexneri (Smith and Dell, 1990), and in the recovery of fungi from foods (Koburger, 1986). Pyruvate has also been shown to enhance the recovery of freeze- and heat-stressed E. coli (McDonald et al., 1983) and E. coli O157:H7 from ground beef (Line et al., 1991).
We hypothesize that supplementation of LBDC with pyruvate would enhance the recovery of injured *E. sakazakii*, resulting in a performance level similar to that of TSAP. Across all stress conditions and test strains examined in our study, there was only a 0.12-log CFU/ml reduction in the number of cells recovered on LBDC versus TSAP (Table 2-2). Compared to TSAP, recovery of control, heat-, freeze-, acid-, and alkaline-stressed cells of *E. sakazakii* on LBDC was reduced by 0.19, 0.22, 0.06, 0.24, and 0.05 logs, respectively. This placed LBDC in a statistically lower grouping than that of TSAP, but the overall performance of LBDC for recovering cells was superior to the other five differential, selective media. The addition of sodium pyruvate, or perhaps other oxygen scavengers to the selective media evaluated in our study may also prove beneficial in resuscitating injured *E. sakazakii* cells. The high performance of LBDC in recovering *E. sakazakii* would argue for its use as an alterative to traditional selective recovery media, particularly for products containing low populations of background microflora. An added benefit of LBDC over some of the other test media is the ease of detecting fluorescent colonies of *E. sakazakii*.

The Oh and Kang (OK) agar also contained 4-methylumbelliferyl α-D-glucoside as an indicator of the presence of α-glucosidase-producing bacteria such as *E. sakazakii*. When viewed under a 6-watt, dual-bulb, UV illumination lamp (Model VLCLC, Vilber Lourmat, Marne la Valee, France), *E. sakazakii* colonies fluoresce a light blue color. Fluorescence is more pronounced when colonies are viewed under a wavelength of 365 nm than under 245 nm. Although viewing the surface of agar on individual Petri dishes with a UV lamp for detection of presumptive positive identification of *E. sakazakii* is more time consuming than detecting presumptive positive colonies using a chromogenic agar such as DFI, the significantly higher recovery of injured cells on LBDC agar makes this medium more attractive.
The behavior of *E. sakazakii* exposed to the four stress conditions was further analyzed by comparing recovery of a composite of all test strains on TSAP and on differential, selective media (Table 2-9). Significantly higher numbers of control and stressed cells were recovered on TSAP than on differential, selective media. Control cells, when spiral plated on TSAP or differential, selective media, were recovered in significantly higher populations (9.23 versus 8.86 log CFU/ml, respectively) than stressed cells that had been exposed to heat, freezing, acid, or alkaline conditions. When stressed cells were plated on differential, selective media, the order of ability of stressed cells to recover was alkaline-stressed cells > thermally-stressed cells > acid-stressed cells = freeze-stressed cells.

**Recovery of desiccation-stressed cells by spiral plating.** The $a_w$ of powdered infant formula before spray-inoculation on day 0 was 0.234. Post-inoculation $a_w$ values of powdered infant formula subsequently inoculated with strains ES132, 4921, 111389, and Frm-TN were 0.253, 0.261, 0.258, and 0.226, respectively on day 0. After storage of inoculated powdered infant formula for 31 days at 21°C, $a_w$ values were 0.244, 0.243, 0.258, and 0.225, respectively.

Reductions of *E. sakazakii* populations in powdered infant formula during the 31-day storage period at 21°C are shown in Table 2-10. Decreases in populations ranged from log 0.37 to 0.85 CFU/g of powdered infant formula. Significantly fewer desiccation-stressed cells of strain 111389 were recovered on EE than on all other differential, selective media from inoculated powdered infant formula stored at 21°C for 31 days (Table 2-11). Significantly fewer desiccation-stressed cells of strain 4921 were recovered on EE and VRBG than on TSAP and other differential, selective media. No cells of strain 4921 were recovered from powdered infant formula plated on EE (minimum detection limit of 50 CFU/g of powdered infant formula).
TABLE 2-9. Populations of composites of four strains of control and heat-, freeze-, acid-, and alkaline-stressed cells of *E. sakazakii* recovered on TSAP and composites of differential, selective media

<table>
<thead>
<tr>
<th>Stress condition</th>
<th>Population (log CFU/ml) recovered&lt;sup&gt;1&lt;/sup&gt;</th>
<th>TSAP</th>
<th>Differential, selective media</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean values in the same column that are not followed by the same letter are significantly different (P ≤ 0.05). Within stress condition, mean values in the same row that are not preceded by the same letter are significantly different.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>a 9.23 a</td>
<td>b 8.86 a</td>
<td>0.37</td>
</tr>
<tr>
<td>Thermal</td>
<td>a 8.54 bc</td>
<td>b 7.91 c</td>
<td>0.95</td>
</tr>
<tr>
<td>Freeze</td>
<td>a 8.02 c</td>
<td>b 7.53 d</td>
<td>1.33</td>
</tr>
<tr>
<td>Acid</td>
<td>a 8.45 bc</td>
<td>b 7.53 d</td>
<td>1.33</td>
</tr>
<tr>
<td>Alkaline</td>
<td>a 8.75 b</td>
<td>b 8.35 b</td>
<td>0.51</td>
</tr>
</tbody>
</table>

<sup>1</sup> Within the same row, reduction in the number of control or stressed cells recovered on differential, selective media compared to the number recovered on TSAP.
TABLE 2-10. Populations of desiccation-stressed cells of *Enterobacter sakazakii* recovered from powdered infant formula.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Day 0(^2)</th>
<th>Day 31(^3)</th>
<th>R(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES132</td>
<td>a 4.63 a</td>
<td>a 4.17 b</td>
<td>0.46</td>
</tr>
<tr>
<td>4921</td>
<td>b 4.55 a</td>
<td>b 3.99 b</td>
<td>0.56</td>
</tr>
<tr>
<td>111389</td>
<td>c 4.33 a</td>
<td>c 3.48 b</td>
<td>0.85</td>
</tr>
<tr>
<td>Frm-TN</td>
<td>d 4.05 a</td>
<td>c 3.68 b</td>
<td>0.37</td>
</tr>
</tbody>
</table>

\(^1\)Reduction in the number of *E. sakazakii* recovered from reconstituted powdered infant formula 2 h after inoculation (day 0) and after storing at 21ºC for 31 days. Mean values in the same row that are not followed by the same letter are significantly different (P ≤ 0.05). Mean values in the same column that are not preceded by the same letter are significantly different.

\(^2\)Recovery on TSA.

\(^3\)Recovery on TSAP.
TABLE 2-11. Populations of desiccation-stressed cells of *Enterobacter sakazakii* recovered from powdered infant formula stored at 21°C for 31 days on TSAP and differential, selective media.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Recovery Medium</th>
<th>Population (log CFU/g) recovered</th>
<th>Desiccation stressed</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES132</td>
<td>TSAP</td>
<td>4.17 ab</td>
<td>-0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LBDC</td>
<td>4.18 ab</td>
<td>-0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FCA</td>
<td>4.36 a</td>
<td>-0.19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DFI</td>
<td>3.96 b</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OK</td>
<td>4.10 ab</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VRBG</td>
<td>3.99 b</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EE</td>
<td>3.92 b</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>4921</td>
<td>TSAP</td>
<td>3.99 a</td>
<td>-0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LBDC</td>
<td>4.02 a</td>
<td>-0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FCA</td>
<td>3.90 b</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DFI</td>
<td>3.54 c</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OK</td>
<td>4.04 a</td>
<td>-0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VRBG</td>
<td>3.07 d</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EE</td>
<td>0.00(^3)</td>
<td>3.99</td>
<td></td>
</tr>
<tr>
<td>111389</td>
<td>TSAP</td>
<td>3.48 a</td>
<td>-0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LBDC</td>
<td>3.50 a</td>
<td>-0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FCA</td>
<td>3.47 a</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DFI</td>
<td>3.29 bc</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OK</td>
<td>3.45 ab</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VRBG</td>
<td>3.28 c</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EE</td>
<td>2.94 d</td>
<td>0.54</td>
<td></td>
</tr>
<tr>
<td>Frm-TN</td>
<td>TSAP</td>
<td>3.68 a</td>
<td>-0.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LBDC</td>
<td>3.61 ab</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FCA</td>
<td>3.44 cd</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DFI</td>
<td>3.40 cd</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OK</td>
<td>3.51 bc</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VRBG</td>
<td>3.30 d</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EE</td>
<td>3.27 d</td>
<td>0.41</td>
<td></td>
</tr>
</tbody>
</table>

1. Within strain, mean values in the same column that are not followed by the same letter are significantly different (\( P \leq 0.05 \)).

2. Within strain, reduction in the number of desiccation-stressed cells recovered on differential, selective media compared to the number of cells recovered on TSAP.

3. Minimum detection limit was 50 CFU/g of Pif.
Composite populations of all four strains of desiccation-stressed cells recovered were analyzed to determine the performance of TSAP and differential, selective recovery media in resuscitating cells and supporting colony formation. The order of performance of media was TSAP, LBDC, FCA, OK > DFI, VRBG, EE (Table 2-12). This order is similar to that observed for *E. sakazakii* that had been exposed to other stress conditions, i.e., that TSAP and LBDC performed better than other differential, selective media. Results further support the use of LBDC as a direct plating medium for detecting injured *E. sakazakii*.

Other researchers have investigated the response of *E. sakazakii* to desiccation stress. Edelson-Mammel and Buchanan (2004a; 2004b) inoculated *E. sakazakii* into powdered infant formula in a dropwise manner, followed by thorough mixing. Data indicated that after storage for ca. 100 days populations were reduced by ca. 1 log CFU/g and then subsequently by 2.5 (day 150), 3.0 (day 550), and ca. 3.3 (day 700) log CFU/g. Breeuwer et al. (2003) desiccated *E. sakazakii* cells by transferring 50 µl of exponential or stationary phase BHI cultures to 12-well culture plates, followed by drying at 25°C for 1 h at 20.7% relative humidity. Desiccated cultures were periodically analyzed throughout a 46-day storage period at 25°C. Extrapolating from the survival curve for stationary phase cells, destruction of desiccated cells on day 30 was between log 0 and ca. 1.3 log CFU/ml, for the four test strains. Analysis on day 46 revealed the death of 1-1.5 log CFU/ml for cells in the stationary phase before destruction. Cells of one strain (1387-2) of exponential phase cells of *E. sakazakii* were less resistant to desiccation stress and decreased by ca. 7.0 log CFU/ml over a 46-day period. When a compatible solute (trehalose) was added to the medium in which cells were desiccated, recovery of cells was increased by 2 logs.
TABLE 2-12. Populations of desiccation-stressed cells of a composite of four strains of *Enterobacter sakazakii* recovered on TSAP and differential, selective media

<table>
<thead>
<tr>
<th>Recovery medium</th>
<th>Population (log CFU/g) recovered&lt;sup&gt;1&lt;/sup&gt;</th>
<th>R&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSAP</td>
<td>3.91 a</td>
<td></td>
</tr>
<tr>
<td>LBDC</td>
<td>3.92 a</td>
<td>-0.01</td>
</tr>
<tr>
<td>FCA</td>
<td>3.96 a</td>
<td>-0.05</td>
</tr>
<tr>
<td>DFI</td>
<td>3.63 b</td>
<td>0.28</td>
</tr>
<tr>
<td>OK</td>
<td>3.87 a</td>
<td>0.04</td>
</tr>
<tr>
<td>VRGB</td>
<td>3.57 b</td>
<td>0.34</td>
</tr>
<tr>
<td>EE</td>
<td>3.44 b</td>
<td>0.47</td>
</tr>
</tbody>
</table>

<sup>1</sup> Mean values that are not followed by the same letter are significantly different (P ≤ 0.05).

<sup>2</sup> Reduction in the number of desiccation-stressed cells recovered on differential, selective media compared to the number recovered on TSAP.
Desiccation of test strains used in this study and those examined by Edelson-Mammel and Buchanan (2004b) in powdered infant formula caused death of fewer cells than reported in BHI cultures by Breeuwer et al. (2003). Ingredients in the powdered infant formula used in this study include lactose, taurine, L-carnitine, potassium chloride, potassium citrate, potassium hydroxide, magnesium phosphate, calcium phosphate, ferrous sulfate, manganese sulfate, cupric sulfate, zinc sulfate, sodium selenite, the nucleotides adenosine 5’-monophosphate, cytidine 5’-monophosphate, disodium guanosine 5’-monophosphate, and disodium uridine 5’-monophosphate, and constituents of nonfat milk and whey protein concentrate. Many of these compounds may protect *E. sakazakii* from low aw stress. Ingredients in the BHI broth used by Breeuwer et al. (2003) that might serve as compatible solutes include glucose, disodium phosphate, sodium chloride, and constituents in proteose peptone, calf brain infusion solids, and beef heart infusion solids. Caubilla-Barron et al. (2004) studied the survival of nine strains of *E. sakazakii* following desiccation in freeze-dried and air-dried infant milk formula. Initial populations decreased by 2 logs immediately following desiccation, and further decreased by 4 logs after storage for 6 months at an unspecified temperature. The type strain (ATCC 11467) decreased by 4 logs initially and 7 logs within 6 months of storage.

**Recovery of control cells by the ecometric technique.** Suspensions of control and stressed cells of *E. sakazakii* were streaked on TSAP and seven differential, selective media using the ecometric technique. Growth indices are shown in Table 2-13. A lower growth index (GI) was obtained for strains 4921 and 111389 on non-selective TSAP than on differential, selective media. The GI of strain 4921 plated on EE (1.73) was significantly lower than GI values obtained on all other media.
TABLE 2-13. Growth indices of heat-stressed, freeze-stressed, acid-stressed, and alkaline-stressed cells of *Enterobacter sakazakii* recovered on TSAP and differential, selective media using the ecometric technique

<table>
<thead>
<tr>
<th>Strain</th>
<th>Recovery medium</th>
<th>Control cells</th>
<th>Heat-stressed cells</th>
<th>Freeze-stressed cells</th>
<th>Acid-stressed cells</th>
<th>Alkaline-stressed cells</th>
<th>Growth index¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>R²</td>
<td>R²</td>
<td>R²</td>
<td>R²</td>
<td>R²</td>
<td></td>
</tr>
<tr>
<td>ES132</td>
<td>TSAP</td>
<td>3.40 ab</td>
<td>1.98 abc</td>
<td>3.27 a</td>
<td>1.49 a</td>
<td>2.18 a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LBDC</td>
<td>3.04 b</td>
<td>0.36</td>
<td>2.36 ab</td>
<td>-0.38</td>
<td>2.80 ab</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>FCA</td>
<td>3.78 a</td>
<td>0.38</td>
<td>1.76 bc</td>
<td>0.22</td>
<td>2.62 abc</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>DFI</td>
<td>3.31 ab</td>
<td>0.09</td>
<td>1.53 c</td>
<td>0.45</td>
<td>1.69 ed</td>
<td>1.58</td>
</tr>
<tr>
<td></td>
<td>OK</td>
<td>3.49 ab</td>
<td>-0.09</td>
<td>2.49 a</td>
<td>-0.51</td>
<td>1.33 c</td>
<td>1.94</td>
</tr>
<tr>
<td></td>
<td>VRBG</td>
<td>3.42 ab</td>
<td>-0.02</td>
<td>2.04 abc</td>
<td>-0.06</td>
<td>2.20 bed</td>
<td>1.07</td>
</tr>
<tr>
<td></td>
<td>EE</td>
<td>3.44 ab</td>
<td>-0.04</td>
<td>1.76 bc</td>
<td>0.22</td>
<td>1.76 ed</td>
<td>1.51</td>
</tr>
<tr>
<td>4921</td>
<td>TSAP</td>
<td>2.82 d</td>
<td>2.58 ab</td>
<td>2.73 a</td>
<td>1.36 ab</td>
<td>1.53 bc</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LBDC</td>
<td>3.60 abc</td>
<td>-0.78</td>
<td>2.56 ab</td>
<td>0.02</td>
<td>2.22 a</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>FCA</td>
<td>3.71 ab</td>
<td>-0.89</td>
<td>2.93 a</td>
<td>-0.35</td>
<td>2.56 a</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>DFI</td>
<td>3.00 cd</td>
<td>-0.18</td>
<td>1.51 cd</td>
<td>1.07</td>
<td>0.73 c</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>OK</td>
<td>3.87 a</td>
<td>-1.05</td>
<td>2.27 abc</td>
<td>0.31</td>
<td>0.84 bc</td>
<td>1.89</td>
</tr>
<tr>
<td></td>
<td>VRBG</td>
<td>3.23 bcd</td>
<td>-0.41</td>
<td>1.87 bcd</td>
<td>0.71</td>
<td>1.16 bc</td>
<td>1.57</td>
</tr>
<tr>
<td></td>
<td>EE</td>
<td>1.73 c</td>
<td>1.09</td>
<td>1.27 d</td>
<td>1.31</td>
<td>0.07 d</td>
<td>2.66</td>
</tr>
<tr>
<td></td>
<td>RF</td>
<td>3.11 bcd</td>
<td>-0.29</td>
<td>2.80 a</td>
<td>-0.22</td>
<td>1.47 b</td>
<td>1.26</td>
</tr>
<tr>
<td>111389</td>
<td>TSAP</td>
<td>2.69 d</td>
<td>-0.53</td>
<td>2.07 a</td>
<td>1.71 c</td>
<td>0.87 a</td>
<td>1.71 ab</td>
</tr>
<tr>
<td></td>
<td>LBDC</td>
<td>3.22 bc</td>
<td>-0.53</td>
<td>2.04 a</td>
<td>0.03</td>
<td>2.38 ab</td>
<td>-0.67</td>
</tr>
<tr>
<td></td>
<td>FCA</td>
<td>3.98 a</td>
<td>-1.29</td>
<td>0.42 bc</td>
<td>1.65</td>
<td>2.96 a</td>
<td>-1.25</td>
</tr>
<tr>
<td></td>
<td>DFI</td>
<td>3.13 ed</td>
<td>-0.44</td>
<td>0.18 c</td>
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</tr>
<tr>
<td></td>
<td>OK</td>
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<td>-1.27</td>
<td>0.11 c</td>
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<td>1.22 cd</td>
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</tr>
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<td>-1.24</td>
<td>0.24 c</td>
<td>1.83</td>
<td>1.78 bc</td>
<td>-0.07</td>
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<td>3.24 bc</td>
<td>-0.55</td>
<td>0.64 b</td>
<td>1.43</td>
<td>1.36 cd</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>RF</td>
<td>3.62 ab</td>
<td>-0.93</td>
<td>0.40 bc</td>
<td>1.67</td>
<td>1.36 cd</td>
<td>0.35</td>
</tr>
<tr>
<td>Frm-TN</td>
<td>TSAP</td>
<td>3.27 bc</td>
<td>1.78 a</td>
<td>2.09 ab</td>
<td>0.71 a</td>
<td>1.16 ab</td>
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<td></td>
<td>LBDC</td>
<td>3.27 bc</td>
<td>0.00</td>
<td>1.60 a</td>
<td>0.18</td>
<td>1.87 ab</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>FCA</td>
<td>3.82 a</td>
<td>-0.55</td>
<td>0.62 b</td>
<td>1.16</td>
<td>2.58 a</td>
<td>-0.49</td>
</tr>
<tr>
<td></td>
<td>DFI</td>
<td>2.76 c</td>
<td>0.51</td>
<td>0.44 b</td>
<td>1.34</td>
<td>1.24 b</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>OK</td>
<td>3.24 bc</td>
<td>0.03</td>
<td>0.49 b</td>
<td>1.29</td>
<td>1.69 ab</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>VRBG</td>
<td>3.42 ab</td>
<td>-0.15</td>
<td>0.51 b</td>
<td>1.27</td>
<td>1.27 b</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>EE</td>
<td>3.69 ab</td>
<td>-0.42</td>
<td>0.44 b</td>
<td>1.34</td>
<td>1.42 b</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>RF</td>
<td>3.42 ab</td>
<td>-0.15</td>
<td>0.60 b</td>
<td>1.18</td>
<td>1.80 ab</td>
<td>0.29</td>
</tr>
</tbody>
</table>

¹ Within strain, mean values in the same column that are not followed by the same letter are significantly different (P ≤ 0.05).

² Within strain and control or stress condition, difference between the growth index obtained on differential, selective media compared to the index obtained on TSAP.
Analysis of a composite of all four strains of control cells revealed that *E. sakazakii* had a significantly higher GI (3.82) on FCA than all other media except OK (3.60) (Table 2-14). Growth indices for EE (3.03), DFI (3.05), and TSAP (3.04) were statistically equivalent to that of LBDC (3.28), but significantly lower than the four other media. The general order of performance of media in recovering control cells as determined by ecometric evaluation was FCA > OK > VRBG, RF > LBDC, DFI, TSAP, EE; no significant differences in GI were noted in the performance of VRGB and RF, or in the performance of LBDC, DFI, TSAP, and EE.

**Recovery of heat-stressed cells by ecometric evaluation.** Growth indices for heat-stress cells on TSAP and LBDC were equivalent or significantly higher than those obtained on other media for all four test strains (Table 2-13). Composite values for all four strains of heat-stressed cells were analyzed (Table 2-14). GI scores on TSAP (2.10) and LBDC (2.14) were significantly higher than scores for all other media. The general order of performance in supporting recovery of heat-stressed cells was TSAP, LBDC > FCA, RF, OK, VRBG, EE, DFI. This order differs slightly from that obtained by spiral plating (TSAP > LBDC, FCA > OK, VRBG, DFI, EE) (Table 2-2).

**Recovery of freeze-stressed cells by the ecometric technique.** Growth indices for freeze-stressed cells varied greatly among strains and recovery media (Table 2-13). All strains had consistently low GI scores on EE. Indices for all four strains of freeze-stressed cells were analyzed as a composite (Table 2-14) to determine the performance of each recovery medium. Growth indices for TSAP, LBDC, and FCA were not significantly different but were higher than indices obtained for other media. Growth indices of DFI and EE were significantly lower than those on all other media except OK. The general order of media performance was TSAP, LBDC,
TABLE 2-14. Growth indices (GI) of a composite of four strains of control or heat-stressed, freeze-stressed, acid-stressed, and alkaline-stressed cells of Enterobacter sakazakii recovered on TSAP and differential, selective media using the ecometric technique

<table>
<thead>
<tr>
<th>Recovery medium</th>
<th>Control cells R^2</th>
<th>Heat-stressed cells R^2</th>
<th>Freeze-stressed cells R^2</th>
<th>Acid-stressed cells R^2</th>
<th>Alkaline-stressed cells R^2</th>
<th>All stress conditions R^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSAP</td>
<td>3.04 d</td>
<td>2.10 a</td>
<td>2.45 a</td>
<td>1.11 a</td>
<td>1.64 a</td>
<td>1.83 a</td>
</tr>
<tr>
<td>LBDC</td>
<td>3.28 cd</td>
<td>-0.24</td>
<td>2.14 a</td>
<td>-0.04</td>
<td>2.32 a</td>
<td>-0.24</td>
</tr>
<tr>
<td>FCA</td>
<td>3.82 a</td>
<td>-0.78</td>
<td>1.43 b</td>
<td>0.67</td>
<td>2.68 a</td>
<td>0.64 b</td>
</tr>
<tr>
<td>DFI</td>
<td>3.05 d</td>
<td>-0.01</td>
<td>0.91 c</td>
<td>1.19</td>
<td>1.13 c</td>
<td>0.38 cd</td>
</tr>
<tr>
<td>OK</td>
<td>3.60 ab</td>
<td>-0.60</td>
<td>1.33 bc</td>
<td>0.77</td>
<td>1.27 bc</td>
<td>0.42 bc</td>
</tr>
<tr>
<td>VRBG</td>
<td>3.50 bc</td>
<td>-0.46</td>
<td>1.16 bc</td>
<td>0.94</td>
<td>1.60 b</td>
<td>0.38 cd</td>
</tr>
<tr>
<td>EE</td>
<td>3.03 d</td>
<td>0.01</td>
<td>1.04 bc</td>
<td>1.06</td>
<td>1.15 c</td>
<td>0.24 d</td>
</tr>
<tr>
<td>RF</td>
<td>3.41 bc</td>
<td>-0.37</td>
<td>1.37 bc</td>
<td>0.73</td>
<td>1.67 b</td>
<td>0.51 bc</td>
</tr>
</tbody>
</table>

1 Mean values in the same column that are not followed by the same letter are significantly different (P ≤ 0.05).
2 Within control or stress condition, difference between the growth index obtained on differential, selective media compared to the growth index obtained on TSAP.
FCA > VRBG, RF, OK, DFI, EE. There were no significant differences, however, in the performance of OK, RF, and VRBG and in the performance of OK, DFI, and EE. The ecometric technique appeared to be more sensitive that spiral plating in discerning media performance.

**Recovery of acid-stressed cells by the ecometric technique.** Growth indices for TSAP and LBDC in recovering acid-stressed cells were consistently highest for all strains (Table 2-13). Strain 4921 had a GI of 0 on EE. This low score is in agreement with the dismal recovery of strain 4921 on EE using the spiral plating technique. Growth indices for EE were consistently lowest or second to the lowest among test media.

Data for all four strains of acid-stressed cells were analyzed as a composite (Table 2-14) to determine the overall performance of each medium. Growth indices for TSAP and LBDC were not significantly different but were significantly higher than all other media. The general order of performance was TSAP, LBDC > FCA, RF, OK > DFI, VRBG > EE. There are similarities between this order and that obtained using the spiral plating technique, which gave a general order of performance of TSAP > LBDC > FCA, OK, VRBG, DFI, EE.

**Recovery of alkaline-stressed cells by the ecometric technique.** The GI for alkaline-stressed cells of strain 4921 on EE was significantly lower than on all other media (Table 2-13). Overall, the GI scores for TSAP, LBDC, and FCA was highest.

Analysis of composite data for all four strains of alkaline stressed cells revealed that GI scores were in the order of TSAP, LBDC, FCA > DFI, OK, VRBG, EE, RF (Table 2-14). This order of performance is similar to that observed for spiral plated alkaline-stressed cells, but spiral plating was somewhat more discerning, revealing that TSAP, LBDC > FCA and that EE was inferior to all other media.
Comparison of media for recovering cells exposed to a composite of all stress conditions by the ecometric technique. Data from ecometric evaluations using heat-, freeze-, acid-, and alkaline stressed cells were combined and analyzed for significant differences in GI on each test medium (Table 2-14). The order of performance was TSAP, LBDC > FCA > RF, VRGB, OK > DFI, EE. While this order is similar to that observed for spiral plating, the performance of specific media using the two methods was not always the same. Examples are shown in Table 2-15.

Data from ecometric evaluation were analyzed by combining GI scores for the four strains of *E. sakazakii* as a composite (Table 2-16). When plated on differential, selective media, the order of recovery according to stress conditions was control > freeze > thermal, alkaline > acid. This differs from the order obtained from spiral plating (control > alkaline > thermal > acid, freeze) (Table 2-9). A side-by-side comparison of spiral plating and the ecometric technique reveals several differences in performance of media for recovering stressed *E. sakazakii* cells on differential, selective media (Table 2-17).

When ecometric evaluation was done using TSAP alone, the order of recovery was control > freeze > thermal > alkaline > acid (Table 2-16). Data from spiral plating, on the other hand, showed that the general order of cells as affected by stress condition was control > alkaline > thermal > acid > freeze, although there were no significant differences among the number of thermal-, acid-, and alkaline-stressed cells recovered or among the number of thermal-, freeze-, and acid-stressed cells recovered (Table 2-9). A side-by-side comparison of the two techniques for recovering stressed cells reveals differences in recovery of stressed *E. sakazakii* cells on TSAP (Table 2-18).

<table>
<thead>
<tr>
<th>Spiral plating</th>
<th>Ecometric technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSAP &gt; LBDC</td>
<td>TSAP = LBDC</td>
</tr>
<tr>
<td>DFI &gt; EE</td>
<td>DFI = EE</td>
</tr>
<tr>
<td>OK = FCA</td>
<td>OK &lt; FCA</td>
</tr>
<tr>
<td>OK = DFI</td>
<td>OK &gt; DFI</td>
</tr>
<tr>
<td>VRGB = FCA</td>
<td>VRGB &lt; FCA</td>
</tr>
<tr>
<td>VRGB = DFI</td>
<td>VRGB &gt; DFI</td>
</tr>
</tbody>
</table>
TABLE 2-16. Growth indices of a composite of four strains of heat-stressed, freeze-stressed, acid-stressed, and alkaline-stressed cells of *Enterobacter sakazakii* recovered on TSAP and a composite of differential, selective media determined using the ecometric technique

<table>
<thead>
<tr>
<th>Stress Condition</th>
<th>TSAP</th>
<th>R²</th>
<th>Differential, selective media</th>
<th>R²</th>
<th>R³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>a 3.04 a</td>
<td>b 3.39 a</td>
<td>-0.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Freeze</td>
<td>a 2.45 b</td>
<td>0.59</td>
<td>b 1.69 b</td>
<td>1.70</td>
<td>0.76</td>
</tr>
<tr>
<td>Thermal</td>
<td>a 2.09 c</td>
<td>0.95</td>
<td>b 1.34 c</td>
<td>2.05</td>
<td>0.75</td>
</tr>
<tr>
<td>Alkaline</td>
<td>a 1.64 d</td>
<td>1.40</td>
<td>b 1.30 c</td>
<td>2.09</td>
<td>0.34</td>
</tr>
<tr>
<td>Acid</td>
<td>a 1.11 e</td>
<td>1.93</td>
<td>b 0.51 d</td>
<td>2.88</td>
<td>0.60</td>
</tr>
</tbody>
</table>

1 Mean values in the same column that are not followed by the same letter are significantly different (P ≤ 0.05). Mean values in the same row that are not preceded by the same letter are significantly different.

2 Within TSAP or a composite of differential, selective media, difference in growth index obtained for stressed cells and the growth index obtained for control cells.

3 Difference between the growth index obtained on differential, selective media compared to the growth index obtained on TSAP.
TABLE 2-17. Differences in spiral plating and ecometric evaluation in the recovery of *E. sakazakii* plated on differential, selective media.

<table>
<thead>
<tr>
<th>Spiral plating</th>
<th>Ecometric technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freeze &lt; thermal</td>
<td>Freeze &gt; thermal</td>
</tr>
<tr>
<td>Freeze &gt; acid</td>
<td>Freeze = acid</td>
</tr>
<tr>
<td>Freeze &gt; alkaline</td>
<td>Freeze &lt; alkaline</td>
</tr>
<tr>
<td>Thermal = alkaline</td>
<td>Thermal &lt; alkaline</td>
</tr>
</tbody>
</table>
TABLE 2-18. Differences in spiral plating and ecometric evaluation in the recovery of *E. sakazakii* plated on TSAP

<table>
<thead>
<tr>
<th>Spiral Plating</th>
<th>Ecometric Plating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freeze = Thermal</td>
<td>Freeze &gt; Thermal</td>
</tr>
<tr>
<td>Freeze &lt; Alkaline</td>
<td>Freeze &gt; Alkaline</td>
</tr>
<tr>
<td>Freeze = Acid</td>
<td>Freeze &gt; Acid</td>
</tr>
<tr>
<td>Thermal = Alkaline</td>
<td>Thermal &gt; Alkaline</td>
</tr>
<tr>
<td>Thermal = Acid</td>
<td>Thermal &gt; Acid</td>
</tr>
<tr>
<td>Alkaline = Acid</td>
<td>Alkaline &gt; Acid</td>
</tr>
</tbody>
</table>
Ecometric evaluation and subsequent modifications of the technique have been used as a consistent and efficient means of testing the efficiency of media (Mossel et al., 1979, 1980, 1983; Urbanova, 1999; Johnson and Murano, 1999; Kociubinski et al., 1999; Corry et al., 1986; Corry and Atabay, 1997, Weenk, 1992; Uyttendaele, et al., 2001; Kornacki et al., 2003). However, our study revealed that, overall, direct (spiral) plating is superior to ecometric evaluation to determine the proficiency of media for recovering healthy and injured cells of *E. sakazakii*.

**Appearance of colonies on test media.** Colonies formed by the four strains of *E. sakazakii* on TSAP and differential, selective media incubated at 37°C for 24 h were examined for color, size, and overall appearance (Tables 2-19 – 2-22). Strain ES132 formed consistently larger colonies than all other strains on all media. Strain 111389 was the only strain that produced a matte colony rather than mucoid colonies on TSAP, VRBG, and EE. All strains produced yellow pigmentation, to varying degrees, on TSAP. Only strain 4921 lightened the color of FCA. Growth of strain 4921 on EE was very weak forming punctiform colonies. Only strain 111389 produced colonies with a rubbery consistency on TSAP, OK, LBDC, and EE. Colonies of strains 11389 and 4921 had rubbery consistencies on VRBG, DFI, FCA, and RF.

Because data from direct plating are generally considered the standard by which ecometric evaluation is measured, spiral plating, in this study, should be considered more definitive, and data should receive greater weight than GI scores obtained by ecometric evaluation. However, even without data for RF media obtained by spiral plating, some conclusions can still be drawn concerning its performance in comparison to the seven other media based on observations from ecometric evaluation. Because ecometric evaluation showed that RF = VRBG, OK > DFI, EE, it is concluded that RF medium performs equivalent to, if not
<table>
<thead>
<tr>
<th>Observation</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ave. colony diameter (mm)</td>
<td>TSAP</td>
</tr>
<tr>
<td></td>
<td>3.32</td>
</tr>
<tr>
<td></td>
<td>VRBG</td>
</tr>
<tr>
<td></td>
<td>4.61</td>
</tr>
<tr>
<td></td>
<td>OK</td>
</tr>
<tr>
<td></td>
<td>2.78</td>
</tr>
<tr>
<td></td>
<td>LDBC</td>
</tr>
<tr>
<td></td>
<td>1.87</td>
</tr>
<tr>
<td></td>
<td>EE</td>
</tr>
<tr>
<td></td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>DFI</td>
</tr>
<tr>
<td></td>
<td>2.85</td>
</tr>
<tr>
<td></td>
<td>FCA</td>
</tr>
<tr>
<td></td>
<td>3.50</td>
</tr>
<tr>
<td></td>
<td>RF</td>
</tr>
<tr>
<td></td>
<td>2.90</td>
</tr>
<tr>
<td>Colony color</td>
<td></td>
</tr>
<tr>
<td>Mucoid light creamy yellow</td>
<td></td>
</tr>
<tr>
<td>Mucoidal. Tri-colored. Center: deep</td>
<td></td>
</tr>
<tr>
<td>yellow. Inner ring: darker yellow.</td>
<td></td>
</tr>
<tr>
<td>Outer edge: purple</td>
<td></td>
</tr>
<tr>
<td>Shiny light creamy yellow</td>
<td></td>
</tr>
<tr>
<td>Mucoid. Yellow creamy trans-luscent</td>
<td></td>
</tr>
<tr>
<td>Creamy yellow trans-luscent</td>
<td></td>
</tr>
<tr>
<td>Blue center surrounded by cream</td>
<td></td>
</tr>
<tr>
<td>colored outer ring</td>
<td></td>
</tr>
<tr>
<td>Slight sheen. Yellow colonies</td>
<td></td>
</tr>
<tr>
<td>with lightening of media around</td>
<td></td>
</tr>
<tr>
<td>colonies</td>
<td></td>
</tr>
<tr>
<td>Black with sheen</td>
<td></td>
</tr>
<tr>
<td>Form</td>
<td>Circular</td>
</tr>
<tr>
<td>Circular</td>
<td>Circular</td>
</tr>
<tr>
<td>Circular</td>
<td>Circular</td>
</tr>
<tr>
<td>Circular</td>
<td>Circular</td>
</tr>
<tr>
<td>Circular, yet filamentous within</td>
<td></td>
</tr>
<tr>
<td>colony</td>
<td></td>
</tr>
<tr>
<td>Circular</td>
<td></td>
</tr>
<tr>
<td>Elevation</td>
<td>Convex</td>
</tr>
<tr>
<td>Convex</td>
<td></td>
</tr>
<tr>
<td>Slightly raised</td>
<td></td>
</tr>
<tr>
<td>Convex</td>
<td></td>
</tr>
<tr>
<td>Raised</td>
<td></td>
</tr>
<tr>
<td>Raised</td>
<td></td>
</tr>
<tr>
<td>Raised</td>
<td></td>
</tr>
<tr>
<td>Umbonate</td>
<td></td>
</tr>
<tr>
<td>Flat</td>
<td></td>
</tr>
<tr>
<td>Margin</td>
<td>Entire</td>
</tr>
<tr>
<td>Entire</td>
<td></td>
</tr>
<tr>
<td>Entire</td>
<td></td>
</tr>
<tr>
<td>Entire</td>
<td></td>
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<td>Entire</td>
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<td>Entire</td>
<td></td>
</tr>
<tr>
<td>Entire</td>
<td></td>
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<tr>
<td>Entire</td>
<td></td>
</tr>
<tr>
<td>Entire</td>
<td></td>
</tr>
<tr>
<td>Observations when touched with</td>
<td></td>
</tr>
<tr>
<td>inoculating loop</td>
<td></td>
</tr>
<tr>
<td>Thin creamy consistency</td>
<td></td>
</tr>
<tr>
<td>Soft sticky creamy consistency</td>
<td></td>
</tr>
<tr>
<td>Soft, wet consistency</td>
<td></td>
</tr>
<tr>
<td>Soft sticky</td>
<td></td>
</tr>
<tr>
<td>Soft creamy</td>
<td></td>
</tr>
<tr>
<td>Soft creamy</td>
<td></td>
</tr>
<tr>
<td>Soft creamy</td>
<td></td>
</tr>
<tr>
<td>Soft creamy</td>
<td></td>
</tr>
<tr>
<td>Soft creamy</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 2-19.** Morphological characterization of *Enterobacter sakazakii* strain ES132 on TSAP and differential, selective media.
<table>
<thead>
<tr>
<th>Observation</th>
<th>TSAP</th>
<th>VRBG</th>
<th>OKA</th>
<th>LDBC</th>
<th>EE</th>
<th>DFI</th>
<th>FCA</th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ave. colony diameter (mm)</td>
<td>2.22</td>
<td>2.32</td>
<td>1.39</td>
<td>1.30</td>
<td>0.50</td>
<td>1.50</td>
<td>2.10</td>
<td>1.20</td>
</tr>
<tr>
<td>Colony color</td>
<td>Shiny, with deep pronounced yellow pigmentation</td>
<td>Dark yellow center with purple edge</td>
<td>Shiny yellow translucent</td>
<td>Yellow translucent</td>
<td>Mucoid slightly yellow translucent</td>
<td>Dark blue center surrounded by cream colored outer ring</td>
<td>Cream colored. No lightening of the media</td>
<td>Dark gray.</td>
</tr>
<tr>
<td>Form</td>
<td>Circular</td>
<td>Circular</td>
<td>Circular</td>
<td>Circular</td>
<td>Punctiform</td>
<td>Circular</td>
<td>Circular, yet filamentous within colony</td>
<td>Circular</td>
</tr>
<tr>
<td>Elevation</td>
<td>Umbonate</td>
<td>Pulvinate with concaved center</td>
<td>Raised</td>
<td>Convex</td>
<td>Pulvinate</td>
<td>Umbonate</td>
<td>Umbonate</td>
<td>Convex</td>
</tr>
<tr>
<td>Margin</td>
<td>Entire to undulate</td>
<td>Undulate</td>
<td>Entire</td>
<td>Entire</td>
<td>Entire</td>
<td>Entire</td>
<td>Entire</td>
<td>Entire</td>
</tr>
<tr>
<td>Observations when touched with inoculating loop.</td>
<td>Thick pasty consistency</td>
<td>Rubbery consistency. Entire colony adheres to loop when touched or either snaps back</td>
<td>Soft, smooth consistency</td>
<td>Soft, pasty consistency</td>
<td>Soft, sticky consistency. Most colonies were punctiform with some larger aggregate mucoid colonies 1.20 - 3.00 mm diameter</td>
<td>Soft and rubbery to pasty consistency. Colony adheres to loop</td>
<td>Rubbery consistency. Colony adheres to loop</td>
<td>Rubbery consistency. Entire colony adheres to loop when touched or either snaps back</td>
</tr>
<tr>
<td>Observation</td>
<td>TSAP</td>
<td>VRBG</td>
<td>OKA</td>
<td>LDBC</td>
<td>EE</td>
<td>DFI</td>
<td>FCA</td>
<td>RF</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>------</td>
<td>------</td>
<td>-----</td>
<td>------</td>
<td>----</td>
<td>-----</td>
<td>-----</td>
<td>----</td>
</tr>
<tr>
<td>Ave. colony diameter (mm)</td>
<td>2.08</td>
<td>3.70</td>
<td>1.20</td>
<td>1.00</td>
<td>1.00</td>
<td>1.90</td>
<td>2.90</td>
<td>1.30</td>
</tr>
<tr>
<td>Colony color</td>
<td>Matte pale yellow pigmentaion</td>
<td>Matte, rough, leathery appearance. Light to medium yellow with purple edge</td>
<td>Shiny yellow transluscent</td>
<td>Cream colored</td>
<td>Rough, matte, creamy white</td>
<td>Dark blue center surrounded by cream colored outer ring.</td>
<td>Gray-cream colored. No lightening of the media</td>
<td>Black shiny</td>
</tr>
<tr>
<td>Form</td>
<td>Circular yet filamentous within the colony.</td>
<td>Irregular</td>
<td>Circular</td>
<td>Circular and filamentous within the colony</td>
<td>Circular</td>
<td>Circular, yet filamentous within the colony</td>
<td>Circular</td>
<td>Circular</td>
</tr>
<tr>
<td>Elevation</td>
<td>Umbonate with high pointed peaks.</td>
<td>Pulvinate</td>
<td>Convex</td>
<td>Umbonate</td>
<td>Convex</td>
<td>Umbonate</td>
<td>Umbonate</td>
<td>Convex</td>
</tr>
<tr>
<td>Margin</td>
<td>Undulate</td>
<td>Undulate</td>
<td>Entire</td>
<td>Entire</td>
<td>Undulate</td>
<td>Entire</td>
<td>Entire</td>
<td>Entire</td>
</tr>
<tr>
<td>Observations when touched with inoculating loop.</td>
<td>Soft rubbery consistency. Adheres to plate and snaps back when touched</td>
<td>Rubbery. Snaps back to plate and retains shape when touched</td>
<td>Rubbery consistency. Entire colony adheres to loop when touched or either snaps back to plate</td>
<td>Pasty/ rubbery consistency</td>
<td>Rubbery. Entire colony removed when touched</td>
<td>Soft rubbery pasty consistency</td>
<td>Rigid rubbery consistency embedded in media. Cannot be removed without breaking agar</td>
<td>Rubbery pasty Consistency</td>
</tr>
</tbody>
</table>
TABLE 2-22. Morphological characterization of *Enterobacter sakazakii* strain FRM-TN on TSAP and differential, selective media.

<table>
<thead>
<tr>
<th>Observation</th>
<th>TSAP</th>
<th>VRBG</th>
<th>OKA</th>
<th>LDBC</th>
<th>EE</th>
<th>DFI</th>
<th>FCA</th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ave. colony diameter (mm)</td>
<td>1.89</td>
<td>2.35</td>
<td>2.65</td>
<td>1.20</td>
<td>1.94</td>
<td>1.60</td>
<td>1.10</td>
<td>1.10</td>
</tr>
<tr>
<td>Colony color</td>
<td>Mucoid light yellow pigmentation</td>
<td>Mucoid. Bright yellow center with purple/ translucent pigmentation</td>
<td>Mucoid yellow translucent</td>
<td>Yellow translucent</td>
<td>Mucoid translucent slightly yellow</td>
<td>Dark blue center surrounded by cream colored outer ring</td>
<td>Gray</td>
<td>Black</td>
</tr>
<tr>
<td>Form</td>
<td>Circular</td>
<td>Circular</td>
<td>Circular, yet filamentous within colony</td>
<td>Circular</td>
<td>Circular</td>
<td>Circular</td>
<td>Circular</td>
<td>Circular</td>
</tr>
<tr>
<td>Elevation</td>
<td>Convex</td>
<td>Slightly umbonate</td>
<td>Convex</td>
<td>Raised</td>
<td>Pulvinate</td>
<td>Umbonate</td>
<td>Umbonate</td>
<td>Convex</td>
</tr>
<tr>
<td>Margin</td>
<td>Entire</td>
<td>Undulate</td>
<td>Entire</td>
<td>Entire</td>
<td>Entire</td>
<td>Entire</td>
<td>Entire</td>
<td>Entire</td>
</tr>
<tr>
<td>Observations when touched with inoculating loop</td>
<td>Soft pasty consistency</td>
<td>Soft pasty consistency</td>
<td>Soft creamy consistency</td>
<td>Soft pasty consistency</td>
<td>Pasty rubbery consistency</td>
<td>Soft creamy consistency</td>
<td>Soft creamy consistency</td>
<td></td>
</tr>
</tbody>
</table>
better than, VRBG, OK, DFI, and EE in recovering injured *E. sakazakii* cells. Colonies of *E. sakazakii* formed on orange-colored RF agar have a distinct black/dark blue color. Based on ease of detecting colonies due to their chromogenic/fluorogenic characteristics, RF agar, along with LBDC and DFI, may be preferred for recovering *E. sakazakii*.

Iversen et al. (2004a) tested 17 separate genera comprising a total of 148 non-*E. sakazakii* strains from the family Enterobacteriaceae for their growth and colony appearance on DFI medium. They observed that 12.8% of the strains were false positive for *E. sakazakii* on DFI. False positives included 16/18 strains of *Escherichia vulneris*, 2/3 *Pantoea* spp., and 1/8 *Citrobacter koseri*. All 95 strains of *E. sakazakii* tested positive by forming blue-green colonies on DFI. R & F Laboratories (company data sheet) stated that RF *E. sakazakii* medium, when used to evaluate 49 non-*E. sakazakii* strains, presented false positives (6.1%) for three strains of *Shigella sonnei*, which formed typical blue-black or blue gray colonies. *Shigella flexneri*, *S. boydii*, and *S. dysenteriae*, however, tested negative for presumptive-positive *E. sakazakii* on RF medium. No *Shigella* spp. were included among the 148 Enterobacteriaceae strains tested on DFI agar (Iversen et al. 2004a). We evaluated DFI agar for supporting the growth of *Shigella sonnei* and *S. flexneri*. One of eight (12.5%) *Shigella* isolates (*Sonnei* 6129) was false positive for *E. sakazakii* on DFI. The dark blue deoxyribonuclease-positive presumptive colony of *Shigella* was confirmed as *S. sonnei* (98% certainty) using the Microbact 12A/B 24-well biochemical test system (Oxoid). *E. sakazakii* strain 4921, which was recovered from powdered infant formula, formed a non-deoxyribonuclease-producing (white) colony on DFI agar. *E. sakazakii* was confirmed using the Microbact 12A/B 24-well biochemical test system. Although two of three strains of *Pantoea agglomerans* produced false-positives on DFI, neither of two *Pantoea* spp. that R & F Laboratories tested on RF *E. sakazakii* chromogenic plating medium
produced false positive colonies (company data sheet). *E. vulneris*, and *C. koseri*, both producing false positive colonies on DFI, were not tested on RF. All strains of *E. sakazakii* tested positive (40/40) on RF medium. Based on these results, DFI medium had a specificity of 87.2% and RF had a 93.9% specificity. Excluding the ten non-Enterobacteriaceae tested on RF medium, the specificity fell from 93.9% to 92.3%. Only one of the non-Enterobacteriaceae, *Pseudomonas aeruginosa*, presented any growth on RF medium, creating clear, flat colonies < 1.0 mm in diameter. None of the other non-Enterobacteriaceae (*Listeria, Bacillus, Streptococcus, Enterococcus, and Staphylococcus*) grew on RF. DFI medium was not tested for its ability to support the growth of non-Enterobacteriaceae.

Iversen and Forsythe (2004) surveyed 82 powdered infant formula products and 404 other food products for the presence of *E. sakazakii* using DFI medium. False positive presumptive colonies of *E. sakazakii* were observed at a level of 38.5% on DFI medium compared to 72.9% on TSA after enrichment in BPW and EE broth, and plating on VRBG agar. The use of chromogenic agars such as RF and DFI medium, or fluorogenic media such as the OK and LBDC, decreases the time to detection of presumptive positive *E. sakazakii* by 48 - 72 h over the traditional media recommended in the FDA method “Isolation and Enumeration of *Enterobacter sakazakii* from Dehydrated Powdered Infant Formula” (USFDA, 2002). This method requires incubating VRBG-streaked agar for 24 h at 36°C followed by streaking colonies on TSA and incubating at 25°C for 24 - 48 h prior to examining colonies for yellow pigment. The use of fluorogenic or chromogenic *E. sakazakii* media would replace the use of VRBG (24 h) and TSA (24 - 48 h) in the FDA method. The EE broth (24 h) step may also be eliminated under certain circumstances when rehydrated powdered infant formula is streaked directly onto fluorogenic or chromogenic *E. sakazakii* media.
ACKNOWLEDGEMENTS

The authors are grateful to Oxoid Inc., Basingstoke, Hampshire, England, and R & F Laboratories, West Chicago, Illinois for providing media for evaluation.
REFERENCES


CHAPTER 3

SURVIVAL OF *ENTEROBACTER SAKAZAKII* IN POWDERED INFANT FORMULA AS AFFECTED BY FORMULA COMPOSITION, WATER ACTIVITY, AND TEMPERATURE\(^1\)

\(^1\) Gurtler, J.B., and L.R. Beuchat. 2007. To be submitted to the *Journal of Food Protection.*
ABSTRACT

A study was done to determine survival characteristics of Enterobacter sakazakii in milk-based and soybean-based powdered infant formulas. Powdered milk containing a mixture of ten strains of E. sakazakii was added to six infant formulas (aw 0.25 – 0.30, 0.31 – 0.33, and 0.43 – 0.50) to give low (0.80 log CFU/g) and high (4.66 – 4.86 log CFU/g) populations. Formulas were stored at 4, 21, and 30°C for up to 12 months. With the exception of two formulas at aw 0.25 – 0.30, initially high populations decreased significantly (p ≤ 0.05), although by less than 1 log CFU/g, within 6 months at 4°C. Populations decreased significantly in all formulas at aw 0.25 – 0.50 during storage for 1 month at 21 or 30°C, and again between 1 and 6 months in most formulas. Significant reductions occurred between 6 and 12 months in some formulas. At all storage temperatures, reductions in populations tended to be greater in formulas at aw 0.43 – 0.50 than in formulas at aw 0.25 – 0.30. E. sakazakii, initially at 0.80 log CFU/g, was detected in all formulas at one or more aw after storage for 6 months at 21 or 30°C, and in all formulas stored for 12 months at 4°C. After storing for 9 months at 21 or 30°C, E. sakazakii was detected in 3 of 6 and 1 of 6 formulas, respectively, by enrichment. The pathogen was detected in 4 of 6 and 1 of 6 formulas stored for 12 months at 21 and 30°C, respectively. The composition of formulas did not have a major effect on the rate of inactivation of E. sakazakii at aw 0.25 – 0.50. Strain 2855, which produces mucoid colonies, and strain 3396, which does not produce mucoid colonies on violet red bile glucose agar supplemented with pyruvate, were spray-inoculated into a milk-based powdered infant formula and a soybean-based powdered infant formula at aw 0.43 – 0.86 and stored at 4, 21, and 30°C for up to 36 weeks. With few exceptions, populations of both strains decreased significantly in both formulas within 2 weeks at all temperatures; rates of death increased with increased storage temperature and aw. The ability of E. sakazakii to form
extracellular mucoidal materials did not correlate with protection against death caused by desiccation.

**INTRODUCTION**

*Enterobacter sakazakii*, a yellow-pigmented coliform occasionally found in powdered infant formula, has been recognized as the cause of neonatal septicemia and meningitis, and has been associated with necrotizing enterocolitis in neonates, as well as infections in other immunocompromised individuals (Bowen and Braden, 2006; Gurtler et al., 2005; Hawkins et al., 1991; Iversen and Forsythe, 2003; Lai, 2001). While the pathogen is known to be exceptionally resistant to desiccation (Breeuwer et al., 2003, Edelson-Mammel et al., 2005), factors affecting its rate of death upon exposure to dry environments at various aw values have not been extensively studied.

Consumption of powdered infant formula has been associated with outbreaks of *E. sakazakii* infections. The pathogen has been isolated from commercially manufactured formulas (Biering et al., 1989; Block et al., 2002; Clark et al., 1990; Himelright et al., 2002; Iversen et al., 2004; Muytgens et al., 1983; 1988; Simmons et al., 1989; Smeets et al., 1998; Van Acker et al., 2001). In one case of infant bacteremia, *E. sakazakii* infection was linked to cross-contamination from a blender used to prepare reconstituted powdered infant formula, although the pathogen was not isolated from the powdered formula (Noriega, 1990). Although the blender was washed in a commercial dish-washing machine daily, the authors surmised that *E. sakazakii* may have adhered to and survived on surfaces of the blender. In an outbreak of *E. sakazakii* meningitis, the pathogen was isolated from a stirring spoon and a dish brush but the powdered infant formula tested negative (Muytjens et al., 1983). Biering et al. (1989) isolated *E. sakazakii* from a blender that had been used for formula preparation in a neonatal intensive care unit of a hospital in which
an outbreak of neonatal meningitis involving *E. sakazakii* occurred. These reports suggest that reconstituted infant formula that has subsequently dried may provide a suitable harbor whereby *E. sakazakii* survive and pose the risk of cross-contamination.

Breeuwer et al. (2004) determined that upon desiccation of *E. sakazakii* at \( a_w 0.23 \), at least 17 genes were expressed from the heat shock regulon, CRP (cyclic AMP receptor protein) regulon, and stringent response regulon, along with an unspecified number of genes involved in trehalose synthesis and cell wall functions such as in the synthesis of lipopolysaccharide and lipid A. Breeuwer et al. (2003) desiccated *E. sakazakii* and *Escherichia coli* cells in phosphate buffer under an atmospheric relative humidity of 20.7% at 25°C. Exponential phase *E. sakazakii* cells decreased by ca. 7 logs within 10 days, while stationary phase cells decreased by only 1 - 1.5 logs during a 46-day period. This is in contrast to stationary phase *E. coli* cells, which declined by >4 logs within 46 days. However, when trehalose was added to the *E. sakazakii* cell suspension prior to desiccation, viable exponential growth phase cells were reduced by only ca. 2.5 logs versus ca. 7 logs in the absence of the polyhydroxyl solute, within 10 days. The trehalose content in stationary phase *E. sakazakii* cells was five times higher than in cells in the exponential phase. These observations suggest that survival of *E. sakazakii* under dry conditions may be due, in part, to synthesis of trehalose, a compatible solute.

Caubilla-Barron and Forsythe (2006) studied survival characteristics of *E. sakazakii*, *Pantoea* spp., *Salmonella* spp., and *Klebsiella* spp in infant formula. Upon desiccation of reconstituted infant formula, *E. sakazakii* and *Pantoea* survived in the formula for up to two years at room temperature, while other Enterobacteriaceae were not recovered after six months. Edelson-Mammel et al. (2005) inoculated powdered infant formula (\( a_w 0.14 \)) drop-wise with a one-strain suspension of *E. sakazakii* to a give a population of ca. 6 log CFU/ml upon
reconstitution. After storage for two years at 20 – 22°C, the $a_w$ of the powdered formula was 0.27 and upon reconstitution the population was ca. 2.5 log CFU/ml.

The conditions to which infant formulas are exposed, whether in the container in which they are manufactured or in open containers under environments with fluctuating relative humidity and temperature, may affect the viability of $E. sakazakii$. However, the interacting effects of composition of formulas, $a_w$, and temperature on survival of $E. sakazakii$ are unknown. The purpose of this study was to determine the ability of $E. sakazakii$ to survive in commercially manufactured milk-based and soy-based powdered infant formulas at $a_w$ of 0.25 – 0.86. Inoculated formulas were held at 4, 21, and 30°C and monitored for populations of the pathogen for up to 12 months.

**MATERIALS AND METHODS**

**Bacterial strains used.** Ten strains of $E. sakazakii$ were examined for their ability to survive in six commercially manufactured powdered infant formulas as affected by $a_w$ and temperature. Five isolates from clinical specimens (strains 2855, 3231, 3234, 3290, and 3295), four isolates from foods (strains 2871, 3437, 3439, 3270), and one environmental isolate (strain 3396) provided by from Dr. Jeffrey Farber (Health Canada, Ottawa, Ont., Canada) were used.

**Powdered infant formulas evaluated.** Four commercially manufactured milk-based powdered infant formulas (codes A, B, D, and F) and two soy-based powdered infant formulas (codes C and E) were used. The major constituents in these formulas are listed in Table 3.1. Three different lots of each formula were used in three replicate trials.

**Measurement of $a_w$.** The $a_w$ of the powdered milk and powdered infant formulas was measured with an AquaLab Model CX2 Water Activity Meter (Decagon Devices, Inc., Pullman, Wash.).
<table>
<thead>
<tr>
<th>Code</th>
<th>Name</th>
<th>Manufacturer</th>
<th>Ingredient label</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Enfamil with Iron Infant Formula. Milk-Based</td>
<td>Mead Johnson Nutritional, Evansville, Ind.</td>
<td>Nonfat milk, lactose, vegetable oil (palm olein, soy, coconut, and high oleic sunflower oils), whey protein concentrate, and less than 1%: vitamin A palmitate, vitamin D₂, vitamin E acetate, vitamin K₁, thiamin hydrochloride, riboflavin vitamin B₁₂, niacinamide, folic acid, calcium pantothenate, biotin, ascorbic acid, choline chloride, inositol, calcium phosphate, magnesium phosphate, ferrous sulfate, zinc sulfate, manganese sulfate, cupric sulfate, potassium chloride, potassium citrate, postassium hydroxide, sodium selenite, taurine, L-carnitine, nucleotides (adenosine 5’-monophosphate, cytidine 5’-monophosphate, disodium guanosine 5’-monophosphate, disodium uridine 5’-monophosphate).</td>
</tr>
<tr>
<td>B</td>
<td>Enfamil LIPIL® with Iron. Milk-Based Formula Powder</td>
<td>Mead Johnson Nutritional</td>
<td>Nonfat milk, lactose, vegetable oil (palm olein, soy, coconut, and high oleic sunflower oils), whey protein concentrate, and less than 1%: Mortierella alpina oil*, Cryptothecodinium cohnii oil**, vitamin A palmitate, vitamin D₂, vitamin E acetate, vitamin K₁, thiamin hydrochloride, riboflavin, vitamin B₁₂, ferrous sulfate, vitamin B₆, niacinamide, folic acid, calcium pantothenate, biotin, ascorbic acid, choline chloride, inositol, calcium phosphate, magnesium phosphate, ferrous sulfate, zinc sulfate, manganese sulfate, cupric sulfate, potassium chloride, potassium citrate, postassium hydroxide, sodium selenite, taurine, L-carnitine, nucleotides (adenosine 5’-monophosphate, cytidine 5’-monophosphate, disodium guanosine 5’-monophosphate, disodium uridine 5’-monophosphate). **A source of arachidonic acid (ARA). **A source of docosahexaenoic acid (DHA).</td>
</tr>
<tr>
<td>C</td>
<td>Similac® Isomil® Advance Soy Formula With Iron</td>
<td>Ross Pediatrics, Ross Products Division, Abbot Laboratories, Columbus, Ohio</td>
<td>Corn syrup solids, 14.6 % soy protein isolate, 11.5% oleic safflower oil, 10.3 % sugar (sucrose), 8.4% soy sil, 7.8% coconut oil; less than 2% of: C. cohnii oil, M. alpine oil, calcium phosphate, potassium citrate, potassium chloride, magnesium chloride, sodium chloride, ferrous sulfate, zinc sulfate, manganese sulfate, cupric sulfate, thiamine chloride hydrochloride, vitamin A palmitate, riboflavin, pyridoxine hydrochloride, folic acid, potassium iodide, phylloquinone, biotin, sodium selenate, beta-carotene, vitamin D₂, and cyanocobalamin. **PAREVE contains no dairy ingredients. Manufactured on dairy equipment. **A source of docosahexaenoic acid (DHA). **A source of arachidonic acid (ARA).</td>
</tr>
<tr>
<td>F</td>
<td>Nestlé Good Start 2 Supreme. Infant Formula with Iron, DHA, and ARA</td>
<td>Nestlé USA, Inc., Glendale, Calif.</td>
<td>Enzymatically hydrolyzed reduced minerals whey protein concentrate (from cow’s milk), vegetable oils (palm olein, soy, coconut, and high-oleic safflower or high-oleic sunflower), lactose, corn maltodextrin, and less than 1.5% of: potassium citrate, potassium phosphate, calcium carbonate, calcium chloride, sodium citrate, magnesium chloride, ferrous sulfate, zinc sulfate, sodium chloride, copper sulfate, potassium iodide, manganese sulfate, M. alpine oil*, C. cohnii oil**, sodium ascorbate, inositol, choline bitartrate, alpha-tocopheryl acetate, niacinamide, calcium pantothenate, riboflavin, vitamin A acetate, pyridoxine hydrochloride, thiamine mononitrate, folic acid, phylloquinone, biotin, vitamin D₂, vitamin B₁₂, ascorbyl palmitate, mixed tocopherols, taurine, L-carnitine. **A source of arachidonic acid (ARA). **A source of docosahexaenoic acid (DHA). Naturally found in breastmilk.</td>
</tr>
</tbody>
</table>
Preparation of infant formulas at low $a_w$ (0.25 – 0.50) for inoculation. Powdered infant formulas ($a_w$ 0.17 – 0.22, 200 g) were placed in plastic trays (23 cm long x 15 cm wide x 5 cm high) (Rubbermaid, Wooster, Ohio). Two trays of each formula were placed on a galvanized steel rack above the surface of ca. 6 liters of one of three saturated salt solutions (potassium acetate, magnesium chloride, or potassium carbonate), which equilibrated with the atmosphere to create relative humidities of ca. 23, 33, and 43%, respectively, in 90-liter plastic tubs (83 cm long x 38 cm wide x 40 cm high) (Sterilite Corporation, Townsend, Mass.). Two brushless 12VDC cooling fans (5.9 cm x 5.9 cm x cm 2.5 cm) (Radio Shack, Forth Worth, Tex.) were attached to internal opposite sides in each tub to circulate the air. Formulas were held in tubs for 10 days at 21°C to allow the $a_w$ to equilibrate at 0.25 – 0.30, 0.31 – 33, 0.43 – 0.50.

Preparation of infant formulas at high $a_w$ (0.43 – 0.86) for inoculation. Two powdered infant formulas (formula A, a milk-based formula and formula C, a soybean-based formula) were used in studies to determine the survival of *E. sakazakii* at high $a_w$ (ca. $a_w$ 0.43 – 0.86). The $a_w$ of both formulas was ca. 0.20, which was not adjusted before spray inoculating with suspensions of strain 2855 or 3396, as described below.

Preparation of inoculum for infant formulas at low $a_w$ (0.25 – 0.50). Stock cultures of *E. sakazakii* were stored in aqueous glycerol (15%) solution at -20°C. Thawed stock suspensions were streaked on tryptic soy agar (TSA; Difco, Becton Dickinson and Co., Sparks, Md.) supplemented with 0.1% sodium pyruvate (TSAP, pH 7.2) and incubated at 37°C for 24 h.

Cells from isolated colonies were inoculated into brain heart infusion broth (BHI, pH 7.4; Difco, Becton Dickinson) (10 ml) and incubated at 37°C for 24 h, with 10-µl loop transfers at 24-h intervals. The final loop transfer was made in 400 ml of BHI in a 500-ml Pyrex screw-cap bottle followed by incubation at 37°C for 24 h on a rotary shaker (New Brunswick Scientific,
Edison, N.J.) set at 62 rpm. Cultures were centrifuged for 15 min at 2,700 x g in a Marathon 12KBR Benchtop Centrifuge (Fisher Scientific, Pittsburgh, Pa.) and the supernatant fluid was decanted. Cells were suspended in 100 ml of sterile deionized water and centrifuged a second time. The supernatant fluid was decanted and cells were resuspended in 10 ml of sterile deionized water. To determine populations of *E. sakazakii* in suspensions, samples were serial diluted in 9 ml of sterile 0.1% peptone solution and surface plated (0.1 ml in duplicate) on TSAP and violet red bile glucose (VRBG) agar (Oxoid, Basingstoke, U.K.) supplemented with 0.1% sodium pyruvate (VRBGP agar). TSAP plates were incubated at 25°C for 24 - 48 h and VRBGP agar plates were incubated at 37°C for 24 h before colonies were counted. Colonies formed on TSAP were examined for yellow pigmentation.

Non-fat powdered milk (Carnation Instant Nonfat Dry Milk, Nestlé USA, Inc., Solon, Ohio) was used as a carrier to prepare inoculum for powdered infant formulas at low *a*<sub>w</sub>. Powdered milk (100 g) was distributed in a layer (ca. 0.75 cm deep) in a sterile 30-cm diameter stainless steel bowl. A cell suspension of each strain, prepared as described above, was applied to each of ten 100-g powdered milk samples using a chromatography reagent sprayer (Model 422530-0050, Kontes Glass Company, Vineland, N.J.). The sprayer was held ca. 35 cm above the surface of the powdered milk formula and sprayed at ca. 2 psi using nitrogen gas as a carrier. The milk powder was mixed intermittently between applications of ca. 0.1 ml of cell suspension over a 150-sec period. A total of ca. 1.0 ml of suspension was added to 100 g of powdered milk. Inoculated powdered milk was mixed, deposited in a plastic bag, sealed, mixed for 2 min, and stored in sterile trays (23 x 15 x 5 cm) at 21°C in a hermetically sealed plastic tub (83 cm long x 38 cm wide x 40 cm high) on a galvanized steel rack above the surface of ca. 6 liters of a saturated potassium acetate solution, which equilibrated with air inside the tub to give an
atmospheric relative humidity of ca. 23%. As described above, fans were used to circulate air within the tubs. Milk powder was stored for 7 days at 21°C before using as an inoculum for powdered infant formulas.

Within 1 h after inoculating powdered milk (day 0) and after storage for 6 days at 21°C, samples were analyzed to determine populations of \textit{E. sakazakii}. Ten grams of inoculated milk powder were combined with 90 ml of sterile deionized water at 45°C, serially diluted in sterile 0.1% peptone, and surface plated (0.1 ml in duplicate) on TSAP and VRBGP agar. TSAP plates were incubated for 24 – 48 at 25°C and VRBGP agar plates were incubated for 24 h at 37°C before colonies were counted. Appropriate amounts of each of the ten inoculated powdered milk samples were combined in a plastic bag to give ca. equal populations of each strain (6 log CFU/g) and thoroughly mixed for 5 min. To determine the population of \textit{E. sakazakii} in the composite mixture of ten strains, 10 g of the powder were combined with 90 ml of sterile deionized water at 45°C, mixed thoroughly, serially diluted in 0.1% peptone, and surface plated (0.1 ml in duplicate) on TSAP and VRBGP agar. TSAP plates were incubated for 48 h at 25°C and VRBGP agar plates were incubated for 24 h at 37°C and colonies were counted.

**Preparation of inoculum for infant formulas at high \(a_w\) (0.43 – 0.86).** Strain 2855, which produces mucoid colonies on VRBGP agar, and strain 3396, which does not produce mucoid colonies on VRBGP agar were grown in BHI for 24 h at 37°C, serially diluted, plated (0.1 ml in duplicate) on TSAP and VRBGP agar, and incubated at 21°C for 3 days. Cells were harvested by depositing 2 ml of sterile deionized water on the surface of each VRBGP agar plate, gently rubbing with a sterile bent glass rod so as to minimize disruption of extracellular materials that might adhere to cells, and removing the cell suspension with a sterile pipette. This
procedure was repeated twice. Suspensions diluted 100-fold in sterile deionized water served as inocula for high-a_w powdered infant formulas.

**Inoculation of powdered infant formulas at low a_w (0.25 – 0.50).** Two grams of the powdered milk inoculated with ten strains of *E. sakazakii*, as described above, were combined with 200 g of the six powdered infant formulas at a_w 0.25 – 0.30, 0.31 – 0.33, and 0.43 – 0.50, sealed in a plastic bag, and mixed for 2 min, resulting in a population of 4.66 – 4.86 log CFU/g (high-population inoculum). After adding 2 g to 200 g of formula. To prepare a low-population inoculum, the inoculated powdered milk was serially diluted 10,000-fold in uninoculated powdered milk (a_w 0.23) before combining 2 g with 200 g of powdered infant formula, resulting in a population of ca. 0.80 log CFU/g (6.31 CFU/g). Inoculated formulas were stored in hermetically sealed containers at 4, 21, and 30°C until analyzed for the presence (by enrichment) and populations of *E. sakazakii*.

**Inoculation of powdered infant formula at high a_w (0.43 – 0.86).** One-hundred grams of each of two powdered infant formulas (formulas A and C, a_w 0.20) were inoculated with cell suspensions of strain 2855 or 3396 by means of a chromatography reagent sprayer. The sprayer was held ca. 35 cm above the surface of the powdered milk formula and suspension was applied at ca. 2 psi using nitrogen gas as a carrier with intermittent stirring. Approximately 2.5 min was required to spray-inoculate each formula. Volumes of inoculum required to achieve target a_w values in test formulas were determined in preliminary experiments. The milk-based formula (code A) required 15, 28, 30, and 36 ml of a suspension of strain 2855 per 100 g of formula to give a_w values of ca. 0.52, 0.75, 0.81 and 0.86, respectively. The soy-based product (code C) required 30, 45, and 78 ml of a suspension of strain 2855 per 100 g to give a_w values of ca. 0.61, 0.72, and 0.80, respectively. The milk-based formula (code A) required 15, 28, 30, and 36 ml of
a suspension of strain 3396 per 100 g of formula to give \(a_w\) values of ca. 0.43, 0.50, 0.65 and 0.81, respectively. The soy-based product (code C) required 30, 45, and 78 ml of suspension of strain 3396 per 100 g to give \(a_w\) values of ca. 0.53, 0.57, and 0.72, respectively.

**Storage of inoculated powdered infant formulas and inoculum.** Ten grams of each low-\(a_w\) (0.25 – 0.50) powdered infant formula inoculated to contain high and low populations of *E. sakazakii* and 10 g of each high-\(a_w\) (0.43 – 0.86) powdered infant formula inoculated to a high population were placed in 20-ml borosilicate liquid scintillation vials and hermetically sealed with polypropylene caps with Poly-Seal™ cap liner cones (Wheaton Scientific, Millville, N.J.). Vials containing inoculated formulas at each low \(a_w\) were double bagged in Ziploc® bags (S.C. Johnson & Son, Inc., Racine, Wisc.) and hermetically sealed in plastic storage tubs and stored at 4, 21, and 30°C. Powdered infant formulas at each high \(a_w\) were stored in hermetically sealed glass jars at 4, 21, or 30°C. Following adjustment of the powdered milk inocula to \(a_w\) 0.28 at 21°C for 7 days, it was double bagged, placed in glass jars, hermetically sealed, and stored at 3°C.

**Microbiological analysis of stored, inoculated, powdered infant formulas, and inocula.** Samples (10 g) of the six inoculated powdered infant formulas adjusted to \(a_w\) 0.25 – 0.30, 0.31 – 0.33, and 0.43 – 0.50 and inoculated with high or low populations of *E. sakazakii* were analyzed for the presence (by enrichment) and populations of the pathogen on day 0 (within 1 h after inoculation) and after storage for 1, 3, 6, 9, and 12 months at 4, 21, and 30°C. Two inoculated infant formulas (codes A and C) with \(a_w\) values of 0.43 – 0.86 were analyzed on day 0 (within 1 h after inoculation) and after storage for 1, 2, 4, 12, 24, and 36 weeks at 4, 21, and 30°C. The milk powder inoculum was analyzed after storage for 0.5, 1, 3, 6, 9, and 12 months at 3°C.
Formulas adjusted at low $a_w$ and milk powder inoculum were pre-enriched by combining 10 g with 90 ml of sterile deionized water at 45°C, shaking for 1 min, and incubating at 37°C for 24 h. Samples adjusted to high $a_w$ (10 g) were placed in bags (BA6041 Stomacher 400 Classic Bags, Seward Ltd., London, U.K.) with 90 of sterile deionized water at 50°C and pummeled in a Stomacher 400 Laboratory Blender (Seward Ltd.) for 2 min. Undiluted samples of these suspensions (0.25 ml in quadruplicate and 0.1 ml in duplicate) and samples (0.1 ml in duplicate) serially diluted in sterile 0.1% sterile peptone were surface plated on TSAP and VRBGP agar and incubated at 25°C for 48 h and 37°C for 24 h, respectively, before colonies were counted. Pre-enrichments (24-h) of high- and low-inoculum infant formulas that did not yield *E. sakazakii* colonies on TSAP or VRBGP agar were enriched by combining 10 ml with 90 ml of Enterobacteriaceae enrichment broth (Difco) supplemented with 0.1% sodium pyruvate (EEP) and incubating at 37°C for 24 h. Enriched samples were streaked on TSAP and VRBGP agar and incubated at 25°C for 48 h and 37°C for 24 h, respectively. Cells from random colonies presumptive for *E. sakazakii* were subjected to confirmation tests using the API 20E Identification System (BioMérieux, Hazelwood, Mo.) and the Microbact 12A/B Identification Kit (Oxoid, Basingstoke, U.K.).

**Colony preservation.** Cells from random colonies presumptive positive for *E. sakazakii* that were isolated from formulas A and C stored at 21 and 30°C were preserved at -20°C in a 15% glycerol solution for future analyses for the purpose of determining if one or more strains persisted over time. These colonies were picked from both TSAP and VRBGP agar.

**Statistical analysis.** Three replicate experiments were conducted. Data were analyzed using the general linear model on SAS software (Version 8.0). The least significant difference
test was used to determine significant differences \( (p \leq 0.05) \) in populations of \emph{E. sakazakii} detected in formulas as affected by composition, \( a_w \), storage temperature, and storage time.

**RESULTS AND DISCUSSION**

**Changes in \( a_w \) of powdered infant formula stored at low \( a_w \).** The initial \( a_w \) ranges of powdered infant formula containing low and high inocula were 0.25 – 0.30, 0.31 – 0.33, and 0.43 – 0.50 (Figs. 3.1 and 3.2). The \( a_w \) of most formulas remained constant over time. Greater fluctuations occurred in formulas A and B at 21 and 30°C than in other formulas. This fluctuation may have been due, in part, to the composition of formulas A and B, the only formulas containing non-fat dry milk and lactose, both hygroscopic, as the two primary ingredients.

**Survival in formulas at low \( a_w \) (0.25 – 0.50) and containing low inoculum.** In powdered infant formulas inoculated with a low population of \emph{E. sakazakii} (0.80 log CFU/g), the pathogen was detected by enrichment of 17, 7, and 2 of 18 formula/\( a_w \) combinations stored at 4, 21, and 30°C, respectively, for 12 months (Table 3.2). Survival was favored by low \( a_w \) and low storage temperature. Formula composition did not have a marked affect on survival of the pathogen. The ability of \emph{E. sakazakii} to persist in a desiccated state was not unexpected. Edelson-Mammel et al. (2005) reported that powdered infant formula (\( a_w \) 0.27) inoculated with \emph{E. sakazakii} (6 log CFU/ml after reconstitution) and stored for 2 years at room temperature (20 – 22°C) declined by only 3.4 logs. The mechanism whereby \emph{E. sakazakii} is tolerant to desiccation may be attributed to the uptake or synthesis of solutes compatible with metabolic processes in microorganisms (Corry, 1987).

Breeuwer et al. (2003) reported that the trehalose concentration in exponential phase \emph{E. sakazakii} cells was less than 0.003 \( \mu \text{mol/mg} \) of protein, regardless of drying treatment.
Water activity of powdered infant formulas (codes A, B, and C) inoculated with *E. sakazakii* at 0.80 log CFU/g (low inoculum) and 4.69 – 4.86 log CFU/g (high inoculum) and stored at 4 (○), 21 (□), and 30°C (△) for up to 12 months.
Water activity of powdered infant formulas (codes D, E, and F) inoculated with *E. sakazakii* at 0.80 log CFU/g (low inoculum) and 4.66 – 4.81 log CFU/g (high inoculum) and stored at 4 (○), 21 (□), and 30°C (△) for up to 12 months.

FIGURE 3.2.
TABLE 3.2. Detection of *E. sakazakii* by enrichment of powdered infant formulas inoculated with a low population of *E. sakazakii* (0.80 log CFU/g) and stored at 4, 21, or 30°C for up to 12 months

<table>
<thead>
<tr>
<th>Formula code</th>
<th>Initial log CFU/g</th>
<th>0&lt;sup&gt;th&lt;/sup&gt; month</th>
<th>4°C</th>
<th>21°C</th>
<th>30°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.26</td>
<td>2 2 3 1 0 2 2 0 0 0 0 1</td>
<td>0 0 0 0 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.27</td>
<td>1 1 0 2 1 2 0 0 0 0 1</td>
<td>1 0 0 0 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.30</td>
<td>2 1 1 2 2 2 0 0 0 1 2</td>
<td>0 0 0 1 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>0.26</td>
<td>2 2 2 1 1 1 0 1 1 1 0</td>
<td>0 0 0 0 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>0.26</td>
<td>1 2 2 1 2 1 1 0 1 0 1</td>
<td>1 1 0 0 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>0.25</td>
<td>0 1 0 1 1 1 1 0 0 0 2</td>
<td>0 0 0 0 0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Formulas were pre-enriched in deionized water at 37°C for 24 h, enriched in EEP at 37°C for 24 h, streaked on VRBGP agar and TSAP, and incubated at 37°C for 24 h and 25°C for 48 h, respectively. Numbers indicate positive samples (by enrichment) out of two tested (0 month) or three tested (1 - 12 months). Detection limit was 1 CFU/10 g.

<sup>b</sup>Values for month 0 enrichments of formulas were the same for respective formulas to be stored at 4, 21, and 30°C.
When cells in the stationary phase were dried at 25°C, the concentration increased to 0.23 µmol/mg of protein. In contrast, they were not able to detect any trehalose in *E. coli* 219. Our findings that *E. sakazakii* survives under desiccated conditions for extended periods of time (e.g., at 4, 21, and 30°C up to 12 months) may be explained by these intracellular metabolic processes.

**Survival in formulas at low aw (0.25 – 0.50) and containing high inoculum.** Initial high populations of *E. sakazakii* in formulas were 4.66 – 4.86 log CFU/g. Populations in formulas containing a high inoculum decreased significantly over time at all aw/temperature combinations (Figs. 3.3 – 3.8). Rates of death of *E. sakazakii* were similar in all formulas at the same aw and stored at the same temperature. *E. sakazakii* was recovered in greater numbers on TSAP than on VRBGP agar in 45 of 324 (13.9%) formula/aw /temperature combinations. In no instance was a significantly higher number of *E. sakazakii* recovered on VRBGP agar than on TSAP. This corroborates observations that VRBGP agar is an inferior medium to TSAP in recovering stressed *E. sakazakii* cells (Gurtler and Beuchat, 2005). Caubilla-Barron and Forsythe (2006) reported that 1 – 4 log fewer cells were recovered on VRBG agar than on TSA.

When examining the effects of aw of formulas on survival of *E. sakazakii*, significant reductions (*p* ≤ 0.05) occurred within 6 months at 4°C in 5 of 6 formulas at aw 0.43 – 0.50 compared to formulas at aw 0.25 – 0.30. Decreases in populations were greater in formulas at 21 and 30°C than at 4°C, and greater at 30 than at 21°C. In 3 of 6 formulas (aw 0.43 – 0.50) stored for 6 months, 5 of 6 formulas stored for 9 months, and 6 of 6 formulas stored for 12 months, initially high populations decreased to levels undetectable by direct plating. *E. sakazakii* was not detected by direct plating of 2 of 6 high-inoculum formulas (aw 0.43 – 0.50) stored at 30°C for 1 month and 4 of 6 formulas stored for 3 months; however, the pathogen was detected (≥ 1 CFU/10 g) by enrichment. Edelson-Mammel et al. (2005) reported ca. 1-log CFU/ml (after
Populations of *E. sakazakii* recovered from powdered infant formula (code A) as affected by initial aw 0.26 (○), 0.34 (□), and 0.49 (△), and storage temperature. Bars indicate standard deviations. Detection limit was 1 log CFU/g (10 CFU/g). Values less than 1 log CFU/g but more than 0 log CFU/g indicate that *E. sakazakii* was detected in one or more replicate samples.
FIGURE 3.4. Populations of *E. sakazakii* recovered from powdered infant formula (code B) as affected by initial $a_w$ 0.27 (○), 0.33 (□), and 0.50 (Δ), and storage temperature. Bars indicate standard deviations. Detection limit was 1 log CFU/g (10 CFU/g). Values less than 1 log CFU/g but more than 0 log CFU/g indicate that *E. sakazakii* was detected in one or more replicate samples.
FIGURE 3.5. Populations of *E. sakazakii* recovered from powdered infant formula (code C) as affected by initial water activity (aw) 0.30 (○), 0.32 (□), and 0.44 (△), and storage temperature. Bars indicate standard deviations. Detection limit was 1 log CFU/g (10 CFU/g). Values less than 1 log CFU/g but more than 0 log CFU/g indicate that *E. sakazakii* was detected in one or more replicate samples.
FIGURE 3.6. Populations of \textit{E. sakazakii} recovered from powdered infant formula (code D) as affected by initial $a_w$ 0.26 (○), 0.31 (□), and 0.44 (△), and storage temperature. Bars indicate standard deviations. Detection limit was 1 log CFU/g (10 CFU/g). Values less than 1 log CFU/g but more than 0 log CFU/g indicate that \textit{E. sakazakii} was detected in one or more replicate samples.
FIGURE 3.7. Populations of *E. sakazakii* recovered from powdered infant formula (code E) as affected by initial *a*<sub>w</sub> 0.26 (○), 0.33 (□), and 0.44 (△), and storage temperature. Bars indicate standard deviations. Detection limit was 1 log CFU/g (10 CFU/g). Values less than 1 log CFU/g but more than 0 log CFU/g indicate that *E. sakazakii* was detected in one or more replicate samples.
FIGURE 3.8. Populations of *E. sakazakii* recovered from powdered infant formula (code F) as affected by initial $a_w$ 0.25 (○), 0.32 (□), and 0.43 (Δ), and storage temperature. Bars indicate standard deviations. Detection limit was 1 log CFU/g (10 CFU/g). Values less than 1 log CFU/g but more than 0 log CFU/g indicate that *E. sakazakii* was detected in one or more replicate samples.
Reconstitution) reduction in population of \textit{E. sakazakii} in powdered infant formula (aw 0.27) inoculated with stationary phase cells 6 log CFU/ml and stored at 21 – 22°C for 100 days. The population declined another ca. 1.3 log CFU/ml by day 153 days and an additional 1-log reduction occurred within 687 days. Breeuwer et al. (2003) reported that populations of three strains of dried stationary phase cells of \textit{E. sakazakii} stored at 25°C and 20.7% relative humidity for 46 days decreased by 1 – 1.5 log CFU/ml. Reductions of 1.5 – 3 log CFU/ml occurred when cells were stored at 45°C.

Differences in the number of \textit{E. sakazakii} recovered from formulas at low aw (0.25 – 0.50) as affected by composition were minor. A comparison of the number of \textit{E. sakazakii} recovered from various formulas stored for 1, 3, and 6 months revealed that formulas A and B contained lower populations than other formulas in 14 of 27 (52\%) of the aw/temperature combinations. When formulas (aw 0.43 – 0.50) were stored at 21°C for three months, the pathogen was undetectable in formula A by direct plating; was at 0.33 log CFU/g in formula B by direct plating, and was recovered at consistently high populations (2.45 – 2.56 log CFU/g) in formulas C – F. \textit{E. sakazakii} was not detected by direct plating of formulas A and B that had been stored for 1 month at 30°C; however, populations of 0.53 – 1.44 log CFU/g were detected in formulas C – F.

**Survival in powdered milk inocula.** Populations of \textit{E. sakazakii} in the powdered milk inoculum (aw 0.28) stored at 21°C for 7 days followed by storage at 3°C up to 12 months decreased significantly ($p \leq 0.05$) but only by 1 log CFU/g (Table 3.3). Recovery of the pathogen on TSAP was significantly greater than recovery on VRBGP agar at all sampling times except at 0.5 months, again confirming the inferior performance of VRBGP in recovering desiccation-stressed \textit{E. sakazakii} (Gurtler and Beuchat, 2005).
### TABLE 3.3. Populations of *E. sakazakii* (log CFU/g) recovered from spray-inoculated powdered milk

<table>
<thead>
<tr>
<th>Storage time (months)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Recovery medium</th>
<th>Number recovered (log CFU/g)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>TSAP</td>
<td>a 6.82 a</td>
</tr>
<tr>
<td></td>
<td>VRBGP</td>
<td>b 6.57 a</td>
</tr>
<tr>
<td>0.5</td>
<td>TSAP</td>
<td>a 6.64 b</td>
</tr>
<tr>
<td></td>
<td>VRBGP</td>
<td>a 6.65 a</td>
</tr>
<tr>
<td>1</td>
<td>TSAP</td>
<td>a 6.53 bc</td>
</tr>
<tr>
<td></td>
<td>VRBGP</td>
<td>b 6.34 b</td>
</tr>
<tr>
<td>3</td>
<td>TSAP</td>
<td>a 6.54 bc</td>
</tr>
<tr>
<td></td>
<td>VRBGP</td>
<td>b 6.22 bc</td>
</tr>
<tr>
<td>6</td>
<td>TSAP</td>
<td>a 6.40 cd</td>
</tr>
<tr>
<td></td>
<td>VRBGP</td>
<td>b 6.12 c</td>
</tr>
<tr>
<td>9</td>
<td>TSAP</td>
<td>a 6.31 d</td>
</tr>
<tr>
<td></td>
<td>VRBGP</td>
<td>b 6.10 c</td>
</tr>
<tr>
<td>12</td>
<td>TSAP</td>
<td>a 5.82 e</td>
</tr>
<tr>
<td></td>
<td>VRBGP</td>
<td>b 5.52 d</td>
</tr>
</tbody>
</table>

<sup>a</sup> Inoculated powdered milk was stored at 21°C for 7 days, followed by storing at 3°C for up to 12 months.

<sup>b</sup> Within storage time, values that are not preceded by the same letter are significantly different (p ≤ 0.05). Within each recovery medium, values (log CFU/g) that are not followed by the same letter are significantly different (p ≤ 0.05). Minimum detection level was 1 CFU/ml (0 log CFU/ml).
Changes in aw of powdered infant formula stored at high aw. The initial aw ranges of powdered infant formulas inoculated with strain 2855 were 0.52 – 0.86 (formula A) and 0.61 – 0.80 (formula C) (Fig. 3.9). The initial aw ranges of powdered infant formulas inoculated with strain 3396 were 0.43 – 0.81 (formula A) and 0.53 – 0.72 (formula C) (Fig. 3.9). Fluctuation in aw of the two formulas, particularly formula A, is attributable, in part, to the hygroscopic nature of the two primary ingredients, non-fat powdered milk and lactose.

Survival in formulas at high aw (0.43 – 0.86). When plated on TSAP, initial populations of *E. sakazakii* were 4.98 – 5.76 log CFU/g (formula A, strain 2855), 5.51 – 6.74 log CFU/g (formula C, strain 2855) (Figs. 3.10 and 3.11), 6.56 – 7.07 log CFU/g (formula A, strain 3396), and 6.82 – 6.95 (formula C, strain 3396) (Figs. 3.12 and 3.13). The rate of death of *E. sakazakii* increased with increased storage temperature. With one exception (strain 3396 in formula C stored at 4°C), initial populations of the two strains of *E. sakazakii* (4.98 – 7.07 log CFU/g) in powdered infant formulas A and C initially at aw 0.43 – 0.86 decreased significantly at all aw/temperature combinations during the 36-week storage period (Figs. 3.10 – 3.13). At a given storage time, populations were often significantly lower in formulas at higher aw that were stored at higher storage temperatures. Strains 2855 and 3396 were detected in formulas A and C stored at 4°C for 36 weeks, regardless of aw of the formulas. Strain 2855 was not detected in formulas A or C held at 21 or 30°C for 24 weeks. In contrast, strain 3396 was detected in both formulas at one or more aw values when formulas were stored at 21 or 30°C for 36 weeks.

In formulas stored at 4°C, death of *E. sakazakii* was generally more rapid as the aw of the formulas was increased from 0.43 to 0.86. This trend was not as evident when formulas were stored at 21 or 30°C. Regardless of formula composition, aw or storage temperature, however, death of *E. sakazakii* was more rapid in the aw 0.43 – 0.86 range than at aw 0.25 – 0.50.
FIGURE 3.9. Water activity of powdered infant formula (codes A and C) inoculated with *E. sakazakii* (4.98 – 7.07 CFU/g) and stored at 4 (○), 21 (□), and 30°C (△) for up to 24 weeks.
FIGURE 3.10. *Population of E. sakazakii strain 2855 recovered from powdered infant formula (code A) as affected by initial a_w 0.52 (○), 0.75 (□), 0.81 (△), and 0.86 (△) and storage temperature. Bars indicate standard deviations. Detection limit was 1 log CFU/g (10 CFU/g). Values less than 1 log CFU/g but more than 0 log CFU/g indicate that E. sakazakii was detected in one or more replicate samples.*
FIGURE 3.11. Population of E. sakazakii strain 2855 recovered from powdered infant formula (code C) as affected by initial $a_w$ 0.61 (○), 0.72 (□), and 0.80 (△) and storage temperature. Bars indicate standard deviations. Detection limit was 1 log CFU/g (10 CFU/g). Values less than 1 log CFU/g but more than 0 log CFU/g indicate that E. sakazakii was detected in one or more replicate samples.
FIGURE 3.12. Population of *E. sakazakii* strain 3396 recovered from powdered infant formula (code A) as affected by initial $a_w$ 0.43 (○), 0.50 (□), 0.65 (Δ), and 0.81 (▲) and storage temperature. Bars indicate standard deviations. Detection limit was 1 log CFU/g (10 CFU/g). Values less than 1 log CFU/g but more than 0 log CFU/g indicate that *E. sakazakii* was detected in one or more replicate samples.
FIGURE 3.13. Population of *E. sakazakii* strain 2855 recovered from powdered infant formula (code C) as affected by initial *a*$_w$ 0.53 (○), 0.57 (□), and 0.72 (△) and storage temperature. Bars indicate standard deviations. Detection limit was 1 log CFU/g (10 CFU/g). Values less than 1 log CFU/g but more than 0 log CFU/g indicate that *E. sakazakii* was detected in one or more replicate samples.
In contrast to strain 2855, strain 3396 produced no visibly apparent mucoidal material when grown on VRBGP agar at 21°C for 3 days. Strain 3396 formed crinkled, matte colonies with a tough, rubbery texture on VRBGP agar, and cells were not as easily dispersed in deionized water as were cells of 2855. In contrast to strain 2855, suspensions of strain 3396 became viscous over time. Extracellular materials contributing to the distinct visual appearance of colonies of strain 3396 may have gelling and water-binding properties, which necessitated us using a greater volume of inoculum to achieve the target \(a_w\) values, and resulted in higher populations in formulas (6.49 – 7.07 log CFU/g) versus populations of strain 2855 (4.98 – 5.76 log CFU/g). Other studies (Kim et al., 2006a) revealed that strain 2855 is capable of forming mucoid colonies on TSAP and other media at 12 or 25°C. Colonies of strain 3396 grown on TSAP at 21°C for 24 h had either a yellow matte colony or a lighter cream colored mucoid appearance. However, when incubated for 24 h at 37°C on TSAP, strain 3396 forms only colonies that were yellow and mucoid in appearance.

**CONCLUSIONS**

*E. sakazakii* is resistant to desiccation in powdered infant formulas over a wide range of \(a_w\) (0.25 – 0.86) and temperatures (4 – 30°C). Although death is more rapid as the \(a_w\) and storage temperature are increased, survival of the pathogen in formulas (initial \(a_w\) 0.25 – 0.50) stored for up to 12 months at 4, 21, and 30°C was not markedly affected by the composition of powdered infant formula. Exceptions were that populations of *E. sakazakii* decreased more rapidly at some \(a_w/\)temperature/storage time combinations for formulas A and B than in formulas C – F at \(a_w\) 0.25 – 0.50.

*E. sakazakii* strain 2855 survived in higher numbers in powdered infant formula C (initial \(a_w\) 0.43 – 0.86), a soy-based formula, stored for 36 weeks at 4°C than in formula A, a milk-based
formula. *E. sakazakii* strain 3396 (non-mucoidal) survived longer (36 weeks) than did strain 2855 (mucoidal strain) in formulas at high initial $a_w$ (0.43 – 0.86) at all storage temperatures, suggesting that the ability of *E. sakazakii* to form extracellular mucoidal materials does not necessarily correlate with protection against death caused by desiccation. Further work is needed to determine if the amount of extracellular polymeric materials produced by various strains of *E. sakazakii* affects the survival of *E. sakazakii* upon exposure to desiccation.

**ACKNOWLEDGMENTS**

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REFERENCES


CHAPTER 4

GROWTH OF *ENTEROBACTER SAKAZAKII* IN RECONSTITUTED POWDERED INFANT FORMULA AS AFFECTED BY FORMULA COMPOSITION AND TEMPERATURE

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1 Gurtler, J.B., and L.R. Beuchat. 2007. To be submitted to the *Journal of Food Protection.*
ABSTRACT

Because of its ability to cause infections in infants and its occasional presence in powdered infant formula, Enterobacter sakazakii has been classified as one of two category-A pathogens in powdered infant formula. A study was done to determine survival and growth characteristics of E. sakazakii initially at populations of 0.02 and 0.53 CFU/ml in six commercial infant formulas reconstituted with water. Reconstituted formulas were stored at 4, 12, 21, and 30°C and populations were monitored up to 72 h. E. sakazakii did not grow in formulas stored at 4°C, although it was detected by enrichment in all formulas 72 h after reconstitution. E. sakazakii, initially at a population of 0.02 CFU/ml, can grow to populations of > 1 log CFU/ml of reconstituted formula held at 12, 21, and 30°C for 48, 12, and 8 h, respectively. At an initial population of 0.53 CFU/ml, the pathogen grew to populations of > 1 log CFU/ml in reconstituted infant formula held at 12 and 21°C for 24 and 8 h, respectively, and to populations of > 3 log CFU/ml when held at 30°C for 8 h. When present in powdered infant formula, E. sakazakii is at low numbers (e.g., 0.36 CFU/100g); however, because the infectious dose for immunocompromised, premature neonates is unknown, results support the U.S. Food and Drug Administration recommendation that the hang time for reconstituted infant formula in neonatal intensive care units should be no longer than 4 h in an attempt to reduce the potential for growth and risk of infections.

INTRODUCTION

Enterobacter sakazakii has been detected in commercially manufactured powdered infant formula (Biering et al., 1989, Block et al., 2002, Clark et al., 1990, Himelright et al., 2002, Iversen, et al., 2004a; Jung and Park, 2006; Muyltjens et al., 1983, Muyltjens et al., 1988, Simmons et al., 1989, Smeets et al., 1998, Van Acker et al., 2001). The pathogen is known to
have caused several cases of neonatal septicemia and meningitis, and has been associated with necrotizing enterocolitis in neonates, as well as infections in older immunocompromised individuals (Urmenyi and Franklin, 1961; Pangalos, 1929; Joker et al., 1965; Monroe and Tift, 1979; Kleiman et al., 1981; Muytjens et al., 1983; Muytjens, 1985; Muytjens and Kollee, 1990; Arseni et al., 1985; Biering et al., 1989; Clark et al., 1990; Willis and Robinson, 1988; Simmons et al., 1989; Noriega et al., 1990; Gallagher and Ball, 1991; Lai, 2001; Van Acker et al., 2001; Burdette and Santos, 2000; Bar-Oz et al., 2001; Block et al., 2002; Himelright et al., 2002; Weir, 2002). At least 76 cases of *E. sakazakii* infections and 19 deaths of infants and children have been documented (Iversen and Forsyth, 2003).

The first outbreak of *E. sakazakii* infection linked to powdered infant formula obtained from a previously unopened can was in 2001 (Centers for Disease Control and Prevention, 2002; Himelright et al., 2002; Weir, 2002). In another outbreak, powdered formula tested negative for *E. sakazakii* but the blender used to prepare the reconstituted formula was positive for the pathogen (Noriega et al., 1990). It was suggested that contamination of the blender could have resulted from contact with a previous batch of powdered infant formula that contained the pathogen. The blender was washed in a dishwashing machine daily but the cleaning procedure was apparently not sufficient to eliminate *E. sakazakii*. The Food and Agricultural Organization and the World Health Organization (2006) lists *E. sakazakii* as one of only two category-A pathogens in powdered infant formula, based on its ability to cause infections in infants and because powdered infant formula has been demonstrated to be a vehicle of infection.

Farmer et al. (1980) examined 57 isolates of *E. sakazakii* and observed that all grew at 25°C and 45°C on D-glucose and citrate without any other added source of carbon or energy. None of the isolates grew at 4 or 50°C, whereas at 47°C, 7 (12%) of the isolates failed to grow.
Breeuwer et al. (2003) examined 22 strains of *E. sakazakii*, all capable of growing in brain heart infusion broth at 47°C. Iversen et al. (2004b) reported that six clinical and food strains of *E. sakazakii* grew between 6 and 45°C in infant formula, with an optimum growth temperature of 37 – 43°C, and Kandhai et al. (2006) reported that the bacterium grew in reconstituted infant formula at 8 – 47°C, with an optimum at 39.4°C. Nazarowec-White and Farber (1997) reported that twelve strains of *E. sakazakii* incubated in brain heart infusion broth grew at maximum temperatures of between 41 – 45°C, and minimum temperatures of 5 – 8°C. Because powdered infant formula presents an abundance of nutrients to support the growth of *E. sakazakii*, appropriate temperature control of reconstituted formula is critical to inhibiting multiplication and minimizing the risk of illness. Holding temperatures after reconstitution, as well as differences in infant formula composition, may also affect the rate of growth.

The objective of this study was to determine the effects of composition (six formulas representing four companies) holding temperature (4, 12, 21, and 30°C) on survival and growth of *E. sakazakii* in formulas reconstituted with water.

**MATERIALS AND METHODS**

**Bacterial strains used.** Ten strains of *E. sakazakii* were examined for their ability to survive and grow in six types of reconstituted powdered infant formula as affected by temperature. Five isolates from clinical specimens (strains 2855, 3231, 3234, 3290, and 3295), four isolates from foods (strains 2871, 3437, 3439, 3270), and one environmental isolate (strain 3396) obtained from Dr. Jeffrey Farber (Health Canada, Ottawa, Ont., Canada) were used.

**Powdered infant formulas evaluated.** Four commercially manufactured milk-based powdered infant formulas and two soy-based powdered infant formulas manufactured by four
companies were used. The major ingredients in these formulas are listed in Table 4.1. Three different lots of each formula were used in three replicate trials.

**Preparation of infant formulas.** Powdered infant formulas (34 - 38.4 g) were combined with eight fluid ounces (237 ml) of sterile deionized water (22°C) in 500-ml bottles. Powder:water ratios were specified on formula labels. The mixture was vigorously shaken for 1-2 min, resulting in a volume of ca. 270 ml of reconstituted formula.

**Preparation of high-population inoculum.** Stock cultures of *E. sakazakii* were streaked on tryptic soy agar (TSA; Difco, Becton Dickinson and Co., Sparks, Md.) supplemented with 0.1% sodium pyruvate (TSAP, pH 7.2) and incubated at 37°C for 24 h. Cells from isolated colonies were inoculated into 10 ml of brain heart infusion (BHI) broth (pH 7.4; Difco, Becton Dickinson) and incubated at 37°C for 24 h, with three 10-µl loop transfers at 24-h intervals. Cultures were centrifuged for 10 min at 2,700 x g in a Marathon 12KBR Benchtop Centrifuge (Fisher Scientific, Pittsburgh, Pa.) and the supernatant fluid was decanted. Cells were suspended in 10 ml of sterile deionized water and centrifuged a second time. The supernatant fluid was decanted and cells were resuspended in 10 ml of sterile deionized water.

To determine populations of *E. sakazakii* in suspensions of each of the ten strains, samples were serially diluted in 0.1% peptone solution and spiral plated (50 µl) in duplicate on TSAP and violet red bile glucose (VRBG) agar (Oxoid, Basingstoke, U.K.) supplemented with 0.1% sodium pyruvate (VRBGP). TSAP plates were incubated at 25°C for 24 - 48 h and VRBGP plates were incubated at 37°C for 24 h before colonies were counted. Colonies formed on TSAP had typical yellow pigmentation. To obtain a high-population inoculum (2 – 3 log CFU/100 g of powdered infant formula), 1 ml of suspension of each of the ten strains of *E. sakazakii* were combined to give a ten-strain mixture and diluted in sterile deionized water to
<table>
<thead>
<tr>
<th>Code</th>
<th>Name</th>
<th>Manufacturer</th>
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<tr>
<td>A</td>
<td>Enfamil with Iron Infant Formula. Milk-</td>
<td>Mead Johnson Nutrionals, Evansville, Ind.</td>
<td>Nonfat milk, lactose, vegetable oil (palm olein, soy, coconut, and high oleic sunflower oils), whey protein concentrate, and less than 1%: vitamin A palmitate, vitamin D₃, vitamin E acetate, vitamin K₃, thiamin hydrochloride, riboflavin, vitamin B₆, hydrochloride, vitamin B₁₂, niacinamide, folic acid, calcium pantothenate, biotin, ascorbic acid, choline chloride, inositol, calcium phosphate, magnesium phosphate, ferrous sulfate, zinc sulfate, manganese sulfate, cupric sulfate, potassium chloride, potassium citrate, postassium hydroxide, sodium selenite, taurine, L-carnitine, nucleotides (adenosine 5’-monophosphate, cytidine 5’-monophosphate, disodium guanosine 5’-monophosphate, disodium uridine 5’-monophosphate).</td>
</tr>
<tr>
<td>B</td>
<td>Enfamil LIPIL® with Iron. Milk-Based Infant Formula Powder</td>
<td>Mead Johnson Nutrionals</td>
<td>Nonfat milk, lactose, vegetable oil (palm olein, soy, coconut, and high oleic sunflower oils), whey protein concentrate, and less than 1%: Mortierella alpina oil*, Cryptococcus cohnii oil**, vitamin A palmitate, vitamin D₃, vitamin E acetate, vitamin K₃, thiamin hydrochloride, riboflavin, vitamin B₆, hydrochloride, vitamin B₁₂, niacinamide, folic acid, calcium pantothenate, biotin, ascorbic acid, choline chloride, inositol, calcium phosphate, magnesium phosphate, ferrous sulfate, zinc sulfate, manganese sulfate, cupric sulfate, potassium chloride, potassium citrate, potassium hydroxide, sodium selenite, taurine, L-carnitine, nucleotides (adenosine 5’-monophosphate, cytidine 5’-monophosphate, disodium guanosine 5’-monophosphate, disodium uridine 5’-monophosphate).</td>
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give a population of ca. 2 log CFU/ml. One milliliter (ca. 100 CFU) of the ten-strain mixture was added to 270 ml of reconstituted infant formula and shaken for 1 min. This procedure resulted in a population of ca. 0.53 CFU/ml of reconstituted formula (ca. 409 CFU/100 g of powdered formula).

**Preparation of low-population inoculum.** Inoculum preparation was done as described for the high-population inoculum, with the following exceptions. To create a low-population inoculum (1 – 2 log CFU/100 g of powdered infant formula), 1 ml from each of the suspensions of the ten *E. sakazakii* strains was combined to give a ten-strain mixture, followed by diluting in sterile water to give a population of ca. 13 CFU/ml. From this diluted ten-strain mixture, 0.35 ml was added to 270 ml of reconstituted infant formula and shaken for 1 min. This procedure resulted in a population of ca. 0.02 CFU/ml of reconstituted formula (ca. 13 CFU/100 g of powdered formula).

**Storage of reconstituted infant formulas.** Following reconstitution and inoculation, each of the six formulas containing low or high numbers of *E. sakazakii* was stored in sterile 500-ml screw cap bottles at 4, 12, 21, and 30°C. Samples were subjected to microbiological analyses at 4- to 24-h intervals.

**Microbiological analyses of reconstituted formulas.** Samples (10 ml) of the six reconstituted, inoculated formulas were analyzed for populations of mesophilic, aerobic microorganisms (total plate count) and *E. sakazakii*, and the presence of *E. sakazakii* (by enrichment) at time 0 (within 30 min after inoculating) as well as after storage for 4, 8, 12, 24, 48, and 72 h at 4, 12, 21, and 30°C. Undiluted samples (0.25 ml in quadruplicate and 0.1 ml in duplicate) and samples (0.1 ml in duplicate) serially diluted in sterile 0.1% sterile peptone were surface plated or spiral plated (50 µl) on TSAP and VRBGP agar and incubated at 25°C for 48 h.
and 37°C for 24 h, respectively, before colonies were counted. Total plate counts as well as populations of \textit{E. sakazakii} in formulas were determined by counting colonies formed on TSAP. Samples were also pre-enriched by incubating 10 ml of formula at 37°C for 24 h. Samples (10 ml) of pre-enriched formula that did not yield \textit{E. sakazakii} colonies on TSAP or VRBGP agar were combined with 90 ml of Enterobacteriaceae enrichment broth (Difco) supplemented with 0.1% sodium pyruvate (EEP) and incubated at 37°C for 24 h. Enriched samples were streaked on TSAP and VRBGP and incubated at 25°C for 48 h and 37°C for 24 h, respectively. Random colonies presumptive for \textit{E. sakazakii} were subjected to confirmation tests using the API 20E Identification System (BioMérieux, Hazelwood, Mo.) and the Microbact 12A/B Identification Kit (Oxoid, Basingstoke, U.K.).

**Preservation of isolates.** Cells from random colonies that were presumptive positive for \textit{E. sakazakii} were picked from plates on which the highest dilution of reconstituted formulas A and C that had been incubated at 21°C and 30°C were formed, deposited in 15% glycerol solution, and stored at -20°C. Isolates may be subjected to pulsed field gel electrophoresis to determine if one or more strains predominated in reconstituted formulas.

**Statistical analysis.** Three independent replicate trials using three lots of each formula were conducted. Data were analyzed using the general linear model of Statistical Analysis Systems procedure (SAS Version 8.0; SAS Institute, Cary, N.C.). The least significant differences test was used to determine if changes in populations of \textit{E. sakazakii} and mesophilic aerobic bacteria in reconstituted infant formulas was significant ($P \leq 0.05$).

**RESULTS AND DISCUSSION**

**Survival and growth in infant formulas.** Survival and growth of \textit{E. sakazakii} in six reconstituted infant formulas stored at 4, 12, 21, and 30°C for up to 72 h after inoculation at
populations of 0.02 CFU/ml and 0.53 CFU/ml are shown in Figures 4.1 and 4.2. Data shown in these figures were obtained from counts obtained on TSAP. Although rarely significant, populations of *E. sakazakii* recovered on TSAP were higher than on VRBGP agar, which is in agreement with observations reported by Caubilla-Baron and Forsythe (2006). In a previous study (Gurtler and Beuchat, 2005), we also observed the inability of VRBGP agar to support resuscitation and colony formation by stressed cells of *E. sakazakii*. Initial *E. sakazakii* populations of 0.02 or 0.53 CFU/ml increased significantly (*p* ≤ 0.05) in formulas held at 12, 21, and 30°C. Aberrant counts from some formulas incubated at 4°C are not interpreted as an indication of growth. Formulas inoculated at a population of 0.02 CFU/ml tested positive for *E. sakazakii* by enrichment of one or more replicate samples of all formulas stored for 72 h at 4°C. Populations of *E. sakazakii* in all formulas inoculated to 0.02 CFU/ml and stored at 12°C increased to ≥ 1.07 and 1.83 log CFU/ml within 48 and 72 h, respectively. Slightly higher populations were reached in formulas inoculated with 0.53 CFU/ml. Populations of *E. sakazakii* in all formulas inoculated to 0.02 CFU/ml and stored at 21°C increased to populations as high as 0.20 log CFU/ml within 8 h. Populations in all formulas inoculated at 0.53 CFU/ml and stored at 21°C increased to maximum populations of 0.26 and 1.30 log CFU/ml within 4 and 8 h, respectively. Telang et al. (2005) reported that fresh human milk and infant formula inoculated with *E. sakazakii* at 2 – 3 log CFU/ml and incubated at 22°C supported negligible growth (ca. 0.50 log CFU/g) within 6 h. In our study, populations of *E. sakazakii* in all formulas inoculated at 0.53 CFU/ml and stored at 21°C increased to ≥ 1.70, 4.59, 8.40, and 8.99 log CFU/ml within 12, 24, 48, and 72 h, respectively, while populations of *E. sakazakii* in all formulas inoculated at 0.02 CFU/ml and stored at 21°C were 2.25 – 4.23 log CFU/ml at 24 h, 5.67 – 8.60 log CFU/ml at 48 h, and 6.60 – 8.72 log CFU/ml at 72 h of storage.
FIGURE 4.1  Populations (log CFU/ml) of E. sakazakii recovered from reconstituted infant formulas (codes A – C) inoculated at low (0.02 CFU/ml) or high (0.53 CFU/ml) populations of the pathogen and stored at 12 (□), 21 (∆), or 30°C (○) for up to 72 h. Samples were surface plated on TSAP. Bars indicate standard deviations. The detection limit was 1 CFU/ml (0 log CFU/ml).
FIGURE 4.2  Populations (log CFU/ml) of E. sakazakii recovered from reconstituted infant formulas (codes D – F) inoculated at low (0.02 CFU/ml) or high (0.53 CFU/ml) numbers of the pathogen and stored at 12 (□), 21 (△), or 30°C (○) for up to 72 h. Samples were surface plated on TSAP. Bars indicate standard deviations. The detection limit was 1 CFU/ml (0 log CFU/ml).
We found that after 12 h at 21°C, populations of *E. sakazakii* in infant formulas inoculated with either 0.02 CFU/ml or 0.53 CFU/ml increased to populations of only 1.16 and 2.40 log CFU/ml, respectively. After storing formulas for 12 h at 30°C, populations in infant formulas inoculated to 0.02 CFU/ml and 0.53 CFU/ml did not exceed 4.78 and 5.63 log CFU/ml, respectively. When incubated at 30°C for 12, 24, 48, and 72 h, initial populations of 0.02 CFU/ml increased to 2.05 – 4.78, 5.77 – 8.99, 5.51 – 8.84, and 4.29 – 8.88 log CFU/ml of storage, respectively. Initial populations of 0.53 CFU/ml of formula were 2.55 – 3.14, 2.48 – 5.63, 8.41 – 8.62, 8.83 – 9.11, and 5.21 – 9.24 log CFU/ml after 8, 12, 24, 48, and 72 h, respectively.

Survival of the pathogen at 4°C was largely unaffected by formula composition. An initial population of 0.53 CFU/ml grew in formulas A, B, and D (milk-based formulas) stored at 12°C for 72 h to populations as high as 3.62 log CFU/ml which were significantly higher than the population in formula E (soy-based formula). When inoculated at 0.02 CFU/ml and stored at 30°C, formulas B and F supported the growth of *E. sakazakii* to maximum populations of only 5.68 and 5.51 log CFU/ml, respectively, after 48 h, and 4.29 and 5.65 log CFU/ml, respectively, after 72 h. These populations are less than those in formulas A, C, D, and E, which increased to ≥ 8.65 log CFU/ml within 72 h. This trend did not occur in formulas inoculated with *E. sakazakii* at 0.53 CFU/ml and incubated at 30°C. Reduction in populations occurred between 48 and 72 h in some formulas. This is attributed in part to decreases in pH caused by fermentative activity.

Changes of pH of formulas containing initial inocula of 0.02 and 0.53 CFU/ml are shown in Figures 4.3 and 4.4. The initial pH values of formulas receiving both levels of inoculum were 6.74 – 7.12. When formulas were stored at 4 or 12°C for 72 h, pH values were in the range of 6.59 – 7.30. Little fluctuation in pH occurred in formulas stored at 21°C for up to 24 h.
regardless of inoculum population. However, formulas inoculated at 0.53 CFU/ml and held at 21°C for 48 and 72 h were in the pH ranges of 5.89 – 6.10 and 5.72 – 6.20, respectively.

Formulas inoculated at a population of 0.02 CFU/ml and stored at 21°C for 48 and 72 h were at pH 6.01 – 6.35 and 5.32 – 6.31, respectively. Formulas inoculated with low or high populations of *E. sakazakii* and stored at 30°C for up to 24 and 48 h were in the pH range of 5.69 – 6.22. When stored for 48 h at 30°C, formulas B, C, and F inoculated at a low population had pH values of 4.64, 5.01, and 4.82, respectively, while formulas A, D, and E had pH values of 5.13, 5.63, and 5.56, respectively. Formulas A, B, and F inoculated at a high population had pH values of 4.76, 4.46, and 4.94, respectively, when stored for 48 h at 30°C, while formulas C, D, and E had pH values of 5.16, 5.39, and 5.47, reached in formula E, a soy-based formula. However, when incubated at 21°C for 72 h, significant differences in populations of *E. sakazakii* were not as apparent. When stored up to 72 h at 30°C, formulas D and E decreased to pH 5.14 – 5.58, while the pH range of formulas A, B, C and F was 4.31 – 5.00, regardless of inoculum population. Overall, the inoculum population did not have a marked effect on changes in pH (Figs. 4.3 and 4.4). The pH of formulas A, B, and F declined most rapidly within 72 h at 21 and 30°C, while changes pH of formulas D and E were less.

Populations of mesophilic aerobic bacteria (total plate counts) in reconstituted formulas incubated for up to 72 h at 4, 12, 21, and 30°C are shown in Figures 4.5 and 4.6. With few exceptions, counts did not increase significantly in formulas stored at 4°C. Populations of mesophilic aerobic bacteria in formulas stored at 12°C grew to ≥ 1.11 and 1.84 log CFU/ml when stored for 48 and 72 h, respectively, regardless of the inoculum population. Mesophilic aerobic bacteria grew to at least 7.60 and 8.30 log CFU/ml when formulas were stored at 21°C for 48 and 72 h, respectively. Populations were ≥ 7.94, 7.98, and 7.79 log CFU/ml when
FIGURE 4.3. *Change in pH of reconstituted infant formulas (codes A – C) inoculated with low (0.02 CFU/ml) or high (0.53 CFU/ml) numbers of E. sakazakii and stored at 4 (○), 12 (□), 21 (△), or 30°C (○) for up to 72 h. Bars indicate standard deviations.*
FIGURE 4.4. Change in pH of reconstituted infant formulas (codes D – F) inoculated with low (0.02 CFU/ml) or high (0.53 CFU/ml) numbers of E. sakazakii and stored at 4 (○), 12 (□), 21 (Δ), or 30°C (⊗) for up to 72 h. Bars indicate standard deviations.
Populations of mesophilic aerobic microorganisms (total plate counts) recovered from reconstituted infant formulas (codes A – C) inoculated with low (0.02 CFU/ml) or high (0.53 CFU/ml) numbers of *E. sakazakii* and stored at 4 (○), 12 (□), 21 (∆), or 30°C (○) for up to 72 h. Bars indicate standard deviations. The detection limit was 1 CFU/ml (0 log CFU/ml).

**FIGURE 4.5.**
FIGURE 4.6. Populations of mesophilic aerobic microorganisms (total plate counts) recovered from reconstituted infant formulas (codes D – F) inoculated with low (0.02 CFU/ml) or high (0.53 CFU/ml) numbers of E. sakazakii and stored at 4 (○), 12 (□), 21 (△), or 30°C (☉) for up to 72 h. Bars indicate standard deviations. The detection limit was 1 CFU/ml (0 log CFU/ml).
formulas were stored for 24, 48 and 72 h, respectively at 30°C. In many instances, total counts reflected counts for *E. sakazakii*.

**CONCLUSIONS**

Results of this study show that *E. sakazakii*, initially at a population of 0.02 CFU/ml, can grow to populations of ≥ 1 log CFU/ml in reconstituted infant formulas held at 12, 21, and 30°C for 48, 12, and 8 h, respectively. Initially at a population of 0.53 CFU/ml, the pathogen can grow to ≥ 1 log CFU/ml in reconstituted infant formulas held at 12 and 21°C for 24 and 8 h, respectively, and to populations of > 3 log CFU/ml when held at 30°C for 8 h. Populations of *E. sakazakii* initially at 0.02 and 0.53 CFU/ml increased to 0.25 and 0.40 log CFU/ml, respectively, when in reconstituted formulas stored at 30°C for 4 h. *E. sakazakii* survives for at least 72 h in reconstituted infant formulas inoculated at populations of 0.02 and 0.53 CFU/ml and stored at 4°C. Overall, growth of *E. sakazakii* was not markedly influenced by the composition of formulas. Based on these results, and because the infectious dose of *E. sakazakii* in immunocompromised neonates is unknown but may be extremely low, the FDA (2002) recommendation that reconstituted infant formula be held for no longer than 4 h at room temperature after preparation in neonatal intensive care units for the purpose of reducing the risk of growth of *E. sakazakii* is supported.

**ACKNOWLEDGMENTS**

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REFERENCES


CHAPTER 5

VIABILITY OF \textit{ENTEROBACTER SAKAZAKII} IN RECONSTITUTED INFANT FORMULA CONTAINING THE LACTOPEROXIDASE SYSTEM\textsuperscript{1}

\textsuperscript{1}Gurtler, J.B., and L.R. Beuchat. 2007. To be submitted to the \textit{Journal of Food Protection}.
ABSTRACT

Neonatal bacteremia and meningitis caused by *Enterobacter sakazakii* have been linked to consumption of reconstituted powdered infant formula. A study was done to determine the effects of the lactoperoxidase system (LPOS) and storage temperature on survival and growth of *E. sakazakii* in a milk-based powdered infant formula reconstituted with water. Initially at 0.03 CFU/ml, *E. sakazakii* grew to ≥2.40 log CFU/ml in reconstituted infant formula held at 30 or 37°C for 8 h and to 0.60 log CFU/ml in formula held at 21°C for 12 h. The pathogen was not detected by enrichment of formula treated with 10 - 30 µg/ml LPO and stored for 24 h at 37°C or 30 µg/ml LPO and stored for 24 h at 30°C. Populations of *E. sakazakii*, initially at 4.40 log CFU/ml in reconstituted infant formula containing 5 µg/ml LPO, did not change significantly (*p* > 0.05) for up to 12 h at 21 and 30°C. Populations either decreased significantly or were unchanged in formula containing 10 µg/ml LPO, which was stored for up to 24, 8, and 8 h at 21, 30, or 37°C, respectively. Results indicate that the presence of LPOS in reconstituted infant formula prevents or inhibits the growth of *E. sakazakii*, thereby potentially reducing the risk of neonatal infections resulting from consumption of formula that may be contaminated with the pathogen.
INTRODUCTION

Reconstituted infant formula has been shown to be an excellent substrate for growth of *Enterobacter sakazakii* (Gurtler and Beuchat, 2007; Iversen et al., 2004; Kandhai et al., 2006). The bacterium can cause bacteremia and meningitis in neonatal infants by invading the intestinal epithelium. Increased intestinal permeability and invasion by *E. sakazakii* is enhanced by heat stable toxins produced in the lipopolysaccharide membrane (Townsend et al., 2007).

Bowen and Braden (2006) reviewed 46 cases of invasive infant *E. sakazakii* infections to assess risk factors. They reported that 40% of the infants were delivered via Cesarean section, eliminating the likelihood of *E. sakazakii* contamination via the birth canal. Ninety-two percent of the infants for whom feeding information was available consumed reconstituted powdered infant formula and *E. sakazakii* was recovered from 68% of formulas consumed by 22 of the infants. They also reported that 87% of *E. sakazakii* isolates recovered from these formulas were indistinguishable from patient strains when characterized by biotype or genotype.

Lactoperoxidase (LPO) is a naturally occurring oxidoreductase that protects the neonatal gastrointestinal tract as well as mammary glands against pathogenic microorganisms (Naidu, 2000). The enzyme has been detected in all mammalian milks tested (Seifu, 2005) and is also present in human saliva, nasal fluid, luminal fluid, tears, and vaginal secretions (Ozer, 1999). The LPO enzyme has also been shown to protect somatic cells against the effects of peroxides. It is involved in the degradation of carcinogens (Tenovuo, 1985; Gotheffors and Marklund, 1975; Stanislawski et al., 1989; Odajima et al., 1996), benign to mammalian cells, and serves as an antioxidant (Bjorck, 1990, Reiter and Harnulv, 1984).

The LPO system has three chemical components, viz. LPO, thiocyanate (SCN⁻), and H₂O₂. In the presence of LPO, SCN⁻ is oxidized by H₂O₂, which produces two primary
antibacterial intermediate compounds, the hypothiocyanite ion (OSCN⁻) and hypothiocyanous acid (HOSCN) (Seifu, 2005). Other short-lived intermediates produced by the LPOS that play lesser roles in antimicrobial activity include thiocyanogen (SCN₂), cyanogen thiocyanate (NC-SCN), cyanosulfurous acid (HO₂SCN), and cyanosulfuric acid (HO₃SCN) (Pruitt and Kamau, 1991). These antimicrobial compounds act by oxidizing sulfhydryl groups on enzymes and proteins in cytoplasmic membranes, inhibiting enzyme activity, creating leaky membranes, and preventing the uptake of critical compounds (Reiter and Harnulv, 1984).

The LPOS has been studied as a means to control pathogens in raw milk (Bjorck et al., 1979; FAO and WHO, 1991), pasteurized milk (Marks et al., 2001), skim milk (Vannini et al., 2004; Boussouel et al., 2000), UHT skim milk (Garcia-Graells et al., 2003; Zapico et al., 1998), reconstituted non-fat dry milk (Siragusa and Johnson, 1989), caprine milk (Seifu, et al., 2004), infant formula (Banks and Board, 1985), fruit and vegetable juice (Van Opstal et al., 2006), ground beef (Kennedy et al., 2000), beef cubes (Elliot et al., 2004), marinated broiler drum sticks (Tan and Ockerman, 2006), and fish (Elotmani and Assobhei, 2003). Studies have documented the antimicrobial effects of LPOS against Escherichia coli, Salmonella, Campylobacter jejuni, Shigella spp., Pseudomonas spp., Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes, Brucella melitensis, and Streptococcus spp., as well as fungi and viruses (Tenovuo et al., 1985; Zajac, 1981; Seifu, 2005).

The LPOS has been accepted and recommended for use by the International Dairy Federation (IDF) (1988) as an antibacterial to preserve raw milk during transport in developing countries. Guidelines for preserving raw milk by using LPOS (FAO/WHO, 1991) call for the addition of NaSCN (14 µg/ml) and sodium percarbonate (30 µg/ml).
This concentration of NaSCN is much lower than those occurring in human saliva and cruciferous vegetables. The FAO/WHO (1991) states that when LPOS is activated according to these guidelines, raw milk can be preserved for up to 26 h, depending on ambient temperature. The document notes that when NaSCN is added, the milk should be mixed for 1 min followed by the addition of sodium percarbonate and mixing for an additional 2 – 3 min.

Min et al. (2006) studied the inhibitory activity of LPOS in edible coatings applied to roasted turkey inoculated with *Salmonella* and *E. coli*. These pathogens, at inoculum population of ca. 5.3 log CFU/ml, immediately declined by 2 - 3 log CFU/ml after applying coatings containing 0.036 – 0.063% LPO. Other studies suggest that activation of LPOS in calf milk replacements leads to an increase in weight and to reduced incidence of diarrhea and mortality in calves (Björck, 1990; Reiter et al., 1980, Reiter et al., 1981; van Leeuwen et al., 2000).

Banks and Board (1985) studied the antimicrobial activity of LPOS in infant formula. The LPOS was established by the addition of LPO (1.5 µ/ml), glucose oxidase (0.1 µ/ml), and KSCN at a concentration of 0.52 mM. Enterobacteriaceae, initially at ca. 1.3 log CFU/ml of formula, increased to > 8.0 log CFU/g in untreated formulas but were undetectable for up to 48 h in LPOS-treated formula. Although glucose oxidase was used to generate H₂O₂ used in the LPOS, sodium percarbonate has shown to be a more economically feasible alternative (Banks and Board, 1985) to achieve the same ends (FAO and WHO, 1991).

We undertook a study to determine if the LPOS is effective in controlling the growth of *E. sakazakii* in a commercially manufactured milk-based powdered infant formula upon reconstitution with water. The effect of temperature on inhibitory or lethal activity was examined.
MATERIALS AND METHODS

**Bacterial strains used.** Ten strains of *E. sakazakii* were examined for their ability to survive and grow in reconstituted powdered infant formula as affected by the LPOS and temperature. Five isolates from clinical specimens (strains 2855, 3231, 3234, 3290, and 3295), four isolates from foods (strains 2871, 3437, 3439, 3270), and one environmental isolate (strain 3396) were obtained from Dr. Jeffrey Farber (Health Canada, Ottawa, Ont., Canada).

**Powdered infant formula evaluated.** A commercially manufactured milk-based powdered infant formula was used. The major ingredients in this formula are listed in Chapter 4 (Table 4-1, code A). Three different lots of the formula were used in three replicate trials.

**Preparation of infant formulas.** Powdered infant formula (34 g) was combined with eight fluid ounces (237 ml) of sterile deionized water adjusted to 21, 30, or 37°C in 500-ml bottles. The powder:water ratio was specified on the formula label. The mixture was vigorously shaken, resulting in a volume of ca. 270 ml of reconstituted formula.

**Preparation of low-population inoculum.** Stock cultures of *E. sakazakii* were streaked on tryptic soy agar (TSA; Difco, Becton Dickinson and Co., Sparks, Md.) supplemented with 0.1% sodium pyruvate (TSAP, pH 7.2) and incubated at 37°C for 24 h. Cells from isolated colonies of each strain were separately inoculated into 10 ml of brain heart infusion (BHI) broth (pH 7.4; Difco, Becton Dickinson) and incubated at 37°C for 24 h, with three 10-µl loop transfers at 24-h intervals. Cells were collected by centrifugation for 10 min at 2,700 x g in a Marathon 12KBR Benchtop Centrifuge (Fisher Scientific, Pittsburgh, Pa.) and the supernatant fluid was decanted. Cells were suspended in 10 ml of sterile deionized water and centrifuged a second time. The supernatant fluid was decanted and cells were resuspended in 10 ml of sterile deionized water.
To determine populations of *E. sakazakii* in suspensions of each of the ten strains, samples were serially diluted in 0.1% peptone solution and spiral plated (50 µl) in duplicate on TSAP. TSAP plates were incubated at 25°C for 48 h before colonies were counted. Colonies formed on TSAP had typical yellow pigmentation. To obtain a low-population inoculum (1 – 2 log CFU/100 g of powdered infant formula), 1 ml of suspension of each of the ten strains of *E. sakazakii* were combined to give a ten-strain mixture and diluted in sterile deionized water to give a population of ca. 1 log CFU/ml. To determine populations of *E. sakazakii* in suspensions of the ten-strain mixture, samples were serially diluted in sterile 0.1% peptone solution and spiral plated (50 µl) in duplicate on TSAP and violet red bile glucose agar supplemented with 0.1% sodium pyruvate (VRBGP agar); plates were incubated at 25°C or 37°C for 48 h or 24 h, respectively, before colonies were counted. One milliliter (ca. 10 CFU) of the ten-strain mixture was added to 270 ml of reconstituted infant formula at 21, 30, and 37°C and shaken for 1 min. This procedure resulted in a population of ca. 0.03 CFU/ml of reconstituted formula (ca. 25 CFU/100 g of powdered formula).

**Preparation of high-population inoculum.** Inoculum preparation was done as described for the low-population inoculum, with the following exceptions. To create a high-population inoculum (4 – 5 log CFU/ml of reconstituted formula, equivalent to 7 – 8 log CFU/100 g of powdered infant formula), 1 ml from each of the suspensions of the ten *E. sakazakii* strains was combined to give a ten-strain mixture, followed by diluting in sterile water to give a population of 6.83 log CFU/ml. From this diluted ten-strain mixture, 1.0 ml was added to 270 ml of reconstituted infant formula and shaken for 1 min. This procedure resulted in a population of ca. 4.40 log CFU/ml of reconstituted formula (ca. 7.30 log CFU/100 g of powdered formula).
**Treatment with the lactoperoxidase system.** Reconstituted formula, upon inoculation, was immediately treated with the LPOS. The LPOS was created by adding sodium thiocyanate (NaSCN⁻) (Fisher Chemical, Fisher Scientific, Rochester, NY), sodium percarbonate (Acros Organics, Morris Plains, NJ), and lactoperoxidase (MP Biomedicals, Inc., Irvine, CA) to formula. Stock solutions of SCN⁻ (10 mg/ml of sterile deionized water), sodium percarbonate (10 mg/ml), and lactoperoxidase (10 mg/ml) were prepared less than 24 h prior to the experiment and stored at 4°C. Portions of the lactoperoxidase stock solutions not used within 24 h were stored at -30°C, then held at 4°C less than 24 h prior to use. Lactoperoxidase solution (0 – 0.81 ml) was added to give concentrations of 0, 10, 20, and 30 µg/ml of formula for the low-population inoculum study and 0, 5, or 10 µg/ml of formula for the high-population inoculum study and shaken vigorously. Sodium thiocyanate was then added to give a concentration of 14 µg/ml, followed by vigorous shaking. Sodium percarbonate was added last at a concentration of 30 µg/ml and the formulas were immediately and shaken vigorously for 30 sec. Concentrations of NaSCN⁻ and sodium percarbonate approximated those listed in the FAO/WHO (1991) guidelines for milk.

**Storage of reconstituted infant formulas.** Following reconstitution, inoculation and treatment with the LPOS, formulas containing low or high numbers of *E. sakazakii* were stored in sterile 500-ml screw cap bottles at 21, 30, or 37°C. Samples were subjected to microbiological analyses after storing for 4, 8, 12, and 24 h.

**Measurement of pH.** The pH of formula to be stored at 21°C was measured within 20 min of establishing the LPOS (0 h). The pH of treated and control formulas stored at 21, 30, or 37°C was measured at each subsequent sampling time.
**Microbiological analyses of reconstituted formulas.** Samples of the six reconstituted, inoculated, treated or control formulas stored for 4, 8, 12, and 24 h at 21, 30, and 37°C were analyzed for populations of total mesophilic aerobic microorganisms (total plate count) and *E. sakazakii* and for the presence of *E. sakazakii* by enrichment. Undiluted samples (0.25 ml in quadruplicate and 0.1 ml in duplicate) and samples (0.1 ml in duplicate) serially diluted in sterile 0.1% sterile peptone were surface plated or spiral plated (50 µl) on TSAP and incubated at 25°C for 48 h before colonies were counted.

Samples taken at 4, 8, and 12 h were also pre-enriched at 37°C for 24 h. Samples (10 ml) of formulas pre-enriched at 37°C for 24 h were combined with 90 ml of Enterobacteriaceae enrichment broth (Difco, Becton Dickinson) supplemented with 0.1% sodium pyruvate (EEP) and incubated at 37°C for 24 h. After removing samples from formula stored for 24 h, 220 ml of double-strength EEP was added to the remaining portion (ca. 220 ml) and the mixture was incubated at 37°C for 24 h. Enriched samples were streaked on TSAP and VRBGP and incubated at 25°C for 48 h and 37°C for 24 h, respectively. Random colonies presumptive for *E. sakazakii* were subjected to confirmation tests using the Microbact 12A/B Identification Kit (Oxoid, Basingstoke, U.K.).

**Statistical analysis.** Three independent replicate trials using three different lots of formula were conducted. Data were analyzed using the general linear model of Statistical Analysis Systems procedure (SAS Version 8.0; SAS Institute, Cary, N.C.). The least significant differences test was used to determine if populations of *E. sakazakii* and mesophilic aerobic bacteria in reconstituted infant formulas were significantly (*p* ≤ 0.05) affected by LPOS, temperature, and storage time.
RESULTS AND DISCUSSION

**Survival and growth in infant formulas.** Populations of *E. sakazakii* in the reconstituted control formula stored at 12, 21, and 30°C for up to 24 h after inoculation at a population of 0.03 CFU/ml are shown in Figure 5-1. Counts were obtained using TSAP as an enumeration medium. When stored at 21°C, the population increased to 0.60 and 4.04 log CFU/ml within 12 and 24 h, respectively. Populations in formula held at 30°C for 12 or 24 h increased to 3.77 and 8.46 CFU/ml, respectively; populations in formula held at 37°C increased to 7.97 and 8.89 CFU/ml, respectively. The generation time at 21°C was 62 min between 12 and 24 h. Generation times at 30 and 37°C were 46 and 43 min, respectively, between 4 and 24 h. Between 4 and 12 h, the generation time at 37°C was only 19.5 min.

*E. sakazakii*, at an initial population of 0.03 CFU/ml, was not detected by direct plating formula containing 10, 20, or 30 µg/ml LPO, regardless of storage temperature or time. However, some samples tested positive for the pathogen by enrichment (Table 5-1). After incubating for 24 h at 21°C or 12 h at 30°C, one or more samples out of three samples analyzed were positive for *E. sakazakii* by enrichment. After incubating formula for 24 h at 30°C, one of three samples treated with 10 or 20 µg LPO were positive, but none of the formulas containing 30 µg/ml LPO was positive. None of the samples from formula treated with 10, 20, or 30 µg/ml LPO and stored at 37°C was positive for *E. sakazakii* by enrichment at any sampling time during the 24-h storage period. The inhibitory or lethal activity of the LPOS against *E. sakazakii* is clearly greater at 37°C than at 21 or 30°C.
FIGURE 5-1  Populations (log CFU/ml) of E. sakazakii recovered from reconstituted infant formula inoculated with a low population of the pathogen (0.03 CFU/ml) and stored at 21 (○), 30 (□), or 37°C (△) for up to 24 h. Samples were surface plated on TSAP. Bars indicate standard deviations. The detection limit was 1 CFU/ml (0 log CFU/ml).
TABLE 5-1. *Presence of Enterobacter sakazakii as detected by enrichment of reconstituted infant formula inoculated with a low population of the pathogen (0.03 CFU/ml) treated with the lactoperoxidase system, and incubated for up to 24 h at 21, 30, or 37°C*.a

<table>
<thead>
<tr>
<th>Storage temperature (°C)</th>
<th>Lactoperoxidase concentration (µg/ml)</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>24</th>
</tr>
</thead>
<tbody>
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<td>3</td>
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<td>3</td>
<td>3</td>
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<td>30</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

aSamples (10 ml) of formula stored for 4, 8 and 12 h were pre-enriched for 24 h at 37°C, enriched in 90 ml of EEP for 24 h at 37°C, streaked on TSAP and VRBGP agar, and incubated at 25°C for 48 h or 37°C for 24 h, respectively. Samples (ca. 220 ml) at hour 24 were enriched by combining with 220 ml of double strength EEP, incubating for 24 h at 37°C, streaking on TSAP and VRBGP agar, and incubating at 25°C for 48 h or 37°C for 24 h, respectively. Numbers indicate samples positive for *E. sakazakii* out of three tested (three replicate experiments).
Generation times and maximum populations for control formulas inoculated with a high population (4.40 log CFU/ml) and stored at 21, 30, or 37°C for various storage times are shown in Table 5-2. The generation time of *E. sakazakii* in formula stored at 30°C during the 4 – 8 h incubation period was 32 min. A generation time of 35 min was calculated in a previous study (Gurtler and Beuchat, 2007) for the same formula inoculated with 0.02 CFU/ml *E. sakazakii* held at the same time and temperature. Comparison of generation times in the two studies is difficult, however, because the temperature at the time formula was inoculated in the later study was 21°C whereas formula was at 30°C in the study reported here. Nazarowec-White and Farber (1997) reported an average generation time of 40 min for *E. sakazakii* inoculated in infant formula at 3.04 log CFU/ml and held at 23°C for 24 h.

Untreated formula inoculated with a high population of *E. sakazakii* (4.40 log CFU/ml) and stored at 21°C supported significant increases in populations between each sampling time, reaching 8.25 log CFU/ml within 24 h (Fig. 5-2). Growth of *E. sakazakii* was inhibited for up to 12 h at 21°C in formula containing 5 µg/ml LPO. Populations were significantly reduced for up to 24 h in formula containing 10 µg/ml LPO. Growth at 30°C in formula containing 5 and 10 µg/ml LPO was significantly inhibited for 12 and 8 h, respectively.

Growth at 37°C in formula containing 10 µg/ml LPO was significantly inhibited for 8 h. The extent of control of growth of *E. sakazakii* by the LPOS in reconstituted infant formula is similar to that reported by the FAO/WHO (1991) for controlling microbial growth in milk by activating intrinsically occurring LPO through the addition of NaSCN and sodium percarbonate, viz., 7 – 8 h at 30°C, 11 – 12 h at 25°C, 16 – 17 h at 20°C, and 24 – 26 h at 15°C. Results are also similar to those in a study reported by Vannini et al (2004) in which LPO at 30 µg/ml was
Table 5-2. *Estimated generation times for* E. sakazakii *in infant formula inoculated with a high population of the pathogen* *(4.40 CFU/ml)*

<table>
<thead>
<tr>
<th>Temp. (°C)</th>
<th>Incubation Period (h)a</th>
<th>Generation time (min)</th>
<th>Maximum population (CFU/ml)</th>
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</thead>
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<tr>
<td>21</td>
<td>0 - 4</td>
<td>93</td>
<td>5.07</td>
</tr>
<tr>
<td></td>
<td>4 - 8</td>
<td>68</td>
<td>5.98</td>
</tr>
<tr>
<td></td>
<td>8 - 12</td>
<td>85</td>
<td>6.79</td>
</tr>
<tr>
<td></td>
<td>12 - 24</td>
<td>147</td>
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</tr>
<tr>
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<td>0 - 4</td>
<td>42</td>
<td>6.06</td>
</tr>
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</tr>
<tr>
<td></td>
<td>8 - 12</td>
<td>33</td>
<td>8.67</td>
</tr>
</tbody>
</table>

*a Incubation period indicates segment of growth curve used to calculate generation time*
FIGURE 5-2. Populations (log CFU/ml) of *E. sakazakii* recovered from reconstituted infant formula inoculated at a high (4.40 log CFU/ml) population, treated with 0 (○), 5 (□), or 10 µg/ml (Δ) of lactoperoxidase, and stored for up to 24 h. Samples were surface plated on TSAP. Bars indicate standard deviations.
added to skim milk and inoculated with *E. coli* strain 555 at ca. 4.2 log CFU/ml. After incubating at 37°C for 3 h, the population of *E. coli* decreased to ca. 2.2 log CFU/ml and between 6 and 24 h was undetectable (detection limit was 0.5 CFU/ml).

The pH values of reconstituted infant formula containing LPOS and inoculated with a low population (0.03 CFU/ml) of *E. sakazakii* are shown in Table 5-3. With few exceptions, little change in pH occurred during 24 h, regardless of LPOS treatment. A significant decrease in the pH in the control formula stored at 30°C occurred between 12 h (pH 6.80) and 24 h (pH 6.12). The pH of the control formula stored at 37°C decreased significantly between 8 h (pH 6.79) and 12 h (pH 6.42), as well as between 12 and 24 h (pH 6.07). Reductions in pH paralleled rapid growth of the pathogen (Fig. 5-1).

Total plate counts in reconstituted formula inoculated with a low population of *E. sakazakii* (0.03 CFU/ml) are shown in Figure 5-3. Total plate counts did not exceed 0.56 log CFU/ml of formula treated with 10, 20, or 30 µg/ml LPO and held at 21°C for up to 24 h. Counts in the control formula, however, increased to 0.96 and 4.16 log CFU/ml at 12 and 24 h of storage, respectively. Total plate counts did not exceed 0.73 log CFU/ml in formula containing 10, 20, or 30 µg/ml LPO and held up to 12 h at 30°C, but counts in the control formula increased to 2.44 and 3.88 log CFU/ml at 8 and 12 h, respectively. Total plate counts in formula stored at 30°C for 24 h and treated with LPO at concentrations of 0 µg/ml, 10 or 20 µg/ml, and 30 µg/ml were 8.51, 5.78, 5.49, and 1.86 log CFU/ml, respectively. The only significant difference in counts was in formula containing 0 and 30 µg/ml LPO. Total plate counts in formula containing 10, 20, or 30 µg/ml LPO and stored at 37°C exhibited trends similar to those in formula held at 30°C but exceeded 1.04, 1.87, and 1.93 log CFU/ml, respectively, within 12 h. Counts in the control formula increased to 2.67 and 8.21 log CFU/ml.
TABLE 5-3. pH values of reconstituted powdered infant formulas inoculated with E. sakazakii, (0.03 CFU/ml) treated with the lactoperoxidase system, and stored for up to 24 h at 21, 30, or 37°C

<table>
<thead>
<tr>
<th>Storage temp. (ºC)</th>
<th>Lactoperoxidase concentration (µg/ml)</th>
<th>Storage time (h)&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
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<tr>
<td>21</td>
<td>0</td>
<td>a 6.79 (0.10) a A</td>
<td>a 6.84 (0.11) a A</td>
<td>a 6.85 (0.10) a A</td>
<td>a 6.84 (0.11) a A</td>
<td>a 6.83 (0.14) a A</td>
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<tr>
<td></td>
<td>10</td>
<td>a 6.86 (0.10) a A</td>
<td>a 6.87 (0.10) a A</td>
<td>a 6.88 (0.09) a A</td>
<td>a 6.88 (0.11) a A</td>
<td>a 6.86 (0.10) a A</td>
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<td></td>
<td>20</td>
<td>a 6.85 (0.11) a A</td>
<td>a 6.88 (0.10) a A</td>
<td>a 6.87 (0.10) a A</td>
<td>a 6.88 (0.09) a A</td>
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<td>a 6.79 (0.10) a A</td>
<td>a 6.81 (0.12) a A</td>
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<td>b 6.12 (0.11) b B</td>
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<td>a 6.84 (0.11) a A</td>
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<td>ab 6.80 (0.09) a A</td>
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<td>30</td>
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<td>a 6.84 (0.10) a A</td>
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<tr>
<td>37</td>
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<td>a 6.79 (0.10) a A</td>
<td>a 6.81 (0.11) a A</td>
<td>a 6.79 (0.11) a A</td>
<td>b 6.42 (0.20) b B</td>
<td>b 6.07 (0.15) b C</td>
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<td>a 6.82 (0.10) a A</td>
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<td>b 6.53 (0.13) ab B</td>
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<td>a 6.82 (0.10) a A</td>
<td>a 6.59 (0.33) a A</td>
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</tbody>
</table>

<sup>a</sup>Standard deviations are shown in parentheses. Comparison of effect of lactoperoxidase: within the same storage temperature, values in the same column that are not followed by the same lower case letter are significantly different (p ≤ 0.05). Comparison of the effect of temperature: within the same lactoperoxidase concentration, values in the same column that are not preceded by the same lower case letter are significantly different (p ≤ 0.05). Comparison of the effect of time: values in the same row that are not followed by the same upper case letter are significantly different (p ≤ 0.05).
FIGURE 5-3. Populations (log CFU/ml) of mesophilic aerobic bacteria recovered from reconstituted infant formula inoculated with a low (0.03 CFU/ml) populations of E. sakazakii, and treated with 0 (○), 10 (□), 20 (△) or 30 ( ) µg of lactoperoxidase and stored for up to 24 h. Samples were surface plated on TSAP. Bars indicate standard deviations. The detection limit was 1 CFU/ml (0 log CFU/ml).
within 8 and 12 h, respectively. Formula containing 30 µg/ml LPO and held at 37°C had a significantly lower total plate count (4.82 log CFU/ml) than that in the control formula (8.89 log CFU/ml). Populations in formula containing 10 or 20 µg/ml LPO increased to 5.30 and 6.17 log CFU/ml, respectively, within 24 h at 37°C. Results are similar to those reported by Garcia-Graells et al. (2000) on the growth of *E. sakazakii* in skim milk. The population of four strains of *E. coli* (4.2 – 6.0 log CFU/ml) remained static in skim milk treated with 5 µg/ml LPO and held at 20°C for 24 h. When the milk was inoculated with ca. 6.0 log CFU/ml followed by treatment with 5 µg/ml LPO, hydrostatic pressure at 300 MPa, and incubation at 20°C, populations decreased to less than the detection limit (20 CFU/ml) within 6 h and remained there for up to 24 h (Garcia-Graells et al., 2003).

Populations of total mesophilic aerobic bacteria in reconstituted formulas inoculated with a high population of *E. sakazakii* (4.40 log CFU/ml) are shown in Table 5-4. Slight differences between these counts and *E. sakazakii* counts (Figure 5-2) may reflect initially high populations of *E. sakazakii* (4.40 CFU/ml) out-competing background microflora.

The pH values of reconstituted formula containing LPOS and inoculated with a high population of *E. sakazakii* (4.40 log CFU/ml) are shown in Table 5-5. Formula inoculated with a high population of *E. sakazakii* (4.40 log CFU/ml) and stored at 21, 30, or 37°C underwent reductions in pH commensurate with growth of the pathogen (Fig. 5-2). Significant reductions in pH occurred in formula containing 5 µg/ml LPO and stored at 37°C for 8, 12, and 24, or 10 µg/ml LPO and stored for 12 and 24 h.
TABLE 5-4. *Populations of mesophilic aerobic bacteria in reconstituted infant formula inoculated with a high population of E. sakazakii (4.40 log CFU/ml), treated with the lactoperoxidase system, and incubated for up to 24 h at 21, 30, or 37°C*

<table>
<thead>
<tr>
<th>Storage temperature (°C)</th>
<th>Lactoperoxidase concentration (µg/ml)</th>
<th>Mesophilic aerobic bacteria (log CFU/ml)(^a)</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td>4 h</td>
</tr>
<tr>
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<td>a</td>
</tr>
<tr>
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<tr>
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<td>0</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>5</td>
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<tr>
<td>37</td>
<td>0</td>
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<tr>
<td></td>
<td>5</td>
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<td></td>
<td>10</td>
<td>b</td>
</tr>
</tbody>
</table>

\(^a\)Populations of aerobic bacteria in formula stored for up to 24 h at 21, 30, or 37°C were determined by surface plating on TSAP. Comparison of effect of lactoperoxidase: within the same storage temperature, values in the same column that are not followed by the same lower case letter are significantly different (\(p \leq 0.05\)). Comparison of the effect of temperature: within the same lactoperoxidase concentration, values in the same column that are not preceded by the same lower case letter are significantly different (\(p \leq 0.05\)). Comparison of the effect of time: values in the same row that are not followed by the same upper case letter are significantly different (\(p \leq 0.05\)).
<table>
<thead>
<tr>
<th>Storage temp. °C</th>
<th>Lactoperoxidase concentration (µg/ml)</th>
<th>Storage time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>5</td>
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</tr>
<tr>
<td></td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>21</td>
<td>0 a 6.84 (0.15) a A</td>
<td>A 6.87 (0.13) a A</td>
</tr>
<tr>
<td></td>
<td>5 a 6.89 (0.16) a A</td>
<td>A 6.90 (0.14) a A</td>
</tr>
<tr>
<td></td>
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<td>A 6.90 (0.14) a A</td>
</tr>
<tr>
<td>30</td>
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<td>B 6.48 (0.11) b B</td>
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<td>A 6.89 (0.14) a A</td>
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<td>A 6.91 (0.15) a A</td>
</tr>
<tr>
<td>37</td>
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<td>C 5.80 (0.06) c B</td>
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<td>5 a 6.89 (0.16) a AB</td>
<td>B 6.46 (0.24) b BC</td>
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<tr>
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<td>10 a 6.91 (0.12) a A</td>
<td>A 6.86 (0.14) a A</td>
</tr>
</tbody>
</table>

*Standard deviations are shown in parentheses. Comparison of effect of lactoperoxidase: within the same storage temperature, values in the same column that are not followed by the same lower case letter are significantly different (p ≤ 0.05). Comparison of the effect of the effect of temperature: within the same lactoperoxidase concentration, values in the same column that are not preceded by the same lower case letter are significantly different (p ≤ 0.05). Comparison of the effect of time: values in the same row that are not followed by the same upper case letter are significantly different (p ≤ 0.05).*
CONCLUSIONS

Results of this study show that *E. sakazakii*, initially at a population of 0.03 CFU/ml, can grow to populations of $\geq 2.40$ log CFU/ml in reconstituted infant formula held at 30 or 37°C for 8 h, and to 0.60 log CFU/ml when held at 21°C for 12 h. In formula is treated with 10, 20, or 30 $\mu$g/ml LPO, inoculated at the same populations (0.03 CFU/ml), and stored at the same temperature, *E. sakazakii* was not detected by direct plating or enrichment for up to 12 h. *E. sakazakii* was not recovered by direct plating or enrichment of low-inoculum formula containing 10, 20, or 30 $\mu$g/ml LPO and stored at 37°C up to 24 h. The LPOS, when applied to reconstituted infant formula at concentrations of 10 – 30 $\mu$g/ml, inhibited growth of *E. sakazakii* initially at 0.03 CFU/ml ($\leq 25$ CFU/100 g of dry powder) to less than 1 CFU/ml for up to 24 h. This initial population was much higher than the average of 0.36 CFU/100 g (Nazarowec-White and Farber, 1997; Zink, 2003) reported to be present in powdered infant formula surveys. With the exception of one formula from the former Soviet Union, which contained 66 CFU/100 g, Muytjens et al. (1988) detected *E. sakazakii* at populations of $\leq 2.7$ CFU/100 g in powdered infant formula obtained in thirty-five countries. Our findings indicate that LPOS is effective in preventing the growth of *E. sakazakii* at populations exceeding those found in most commercially manufactured powdered infant formula when reconstituted and held for 24 h at temperatures up to 37°C.

Populations of *E. sakazakii* initially at 4.40 log CFU/ml in reconstituted infant formula containing 5 $\mu$g/ml LPO did not change significantly for up to 12 h when formulas were held at 21 and 30°C. The initially high population in reconstituted infant formula containing 10 $\mu$g/ml LPO decreased significantly or remained unchanged in formula stored at 21, 30, or 37°C up to 24, 8 and 8 h, respectively.
The presence of *E. sakazakii* in reconstituted infant formula poses a threat to neonates in hospitals and in the home. Our study indicates that use of LPOS in reconstituted infant formula inhibits the growth of *E. sakazakii*, thereby potentially reducing the risk of neonatal infections. Treatment of formula with LPOS may also have potential for reducing other gastrointestinal-acquired illnesses caused by microorganisms other than *E. sakazakii*. Treatment of reconstituted infant formula with LPOS clearly prevents the growth of *E. sakazakii* in temperature-abused formulas but should not be used to replace acceptable hygienic practices.
REFERENCES


Min, S., L.J. Harris, and J. Krochta. 2006. Inhibition of Salmonella enterica and Escherichia coli O157:H7 on roasted turkey by edible whey protein coatings incorporating the lactoperoxidase system. J. Food Prot. 69:784-793.


CHAPTER 6

SUMMARY AND CONCLUSIONS
The following is a summary of the results and conclusions drawn from the research reported in Chapters 2 – 5 of this dissertation.

1. The ability of tryptic soy agar supplemented with 0.1% pyruvate (TSAP) (control medium), two new fluorogenic agars developed by Leuschner, Baird, Donald, and Cox (LBDC) and Oh and Kang (OK), fecal coliform agar (FCA), Druggan-Forsythe-Iversen medium (DFI), VRBG agar, and Enterobacteriaceae enrichment agar (EE), to support colony development by healthy and heat-, freeze-, acid-, alkaline-, and desiccation-stressed cells of *Enterobacter sakazakii* was examined. With the exception of desiccation-stressed cells, suspensions of control and stressed cells were also plated on these media and on R & F *Enterobacter sakazakii* chromogenic plating medium (RF) using the ecometric technique.

- The general order of performance of media for recovering control and heat-, freeze-, acid-, and alkaline-stressed cells by spiral plating was TSAP > LBDC > FCA > OK > VRBG > DFI > EE.
- The general order of performance of media for recovering desciccated cells was TSAP > LBDC > FCA > OK > DFI > VRBG > EE.
- Using the ecometric technique, the general order of performance was TSAP > LBDC > FCA > RF > VRBG > OK > EE > DFI for growth indices of stressed cells.
- Results indicate that differential, selective media vary greatly in their ability to support resuscitation and colony formation by stressed cells of *E. sakazakii*.
- The general order of performance of media was similar using spiral plating and ecometric techniques, although results from spiral plating should be considered more conclusive.
2. The ability of *E. sakazakii* to survive in four commercially manufactured milk-based powdered infant formulas and two soy-based powdered infant formulas (aₜ 0.25 – 0.86) for up to 12 months at 4, 21, and 30°C was determined.

- With the exception of two formulas at aₜ 0.25 – 0.30, initially high populations decreased significantly (p ≤ 0.05), although by less than 1 log CFU/g within 6 months at 4°C.
- Populations decreased significantly in all formulas at aₜ 0.25 – 0.50 during storage for 1 month at 21 or 30°C, and again between 1 and 6 months in most formulas; significant reductions occurred between 6 and 12 months in some formulas.
- At all storage temperatures, reductions in populations tended to be greater in formulas at aₜ 0.43 – 0.50 than in formulas at aₜ 0.25 – 0.30. *E. sakazakii*, initially at 0.80 log CFU/g, was detected in all formulas at one or more aₜ after storage for 6 months at 21 or 30°C, and in all formulas stored for 12 months at 4°C.
- Although death is more rapid as the aₜ and storage temperature are increased, survival of the pathogen in formulas (initial aₜ 0.25 – 0.50) stored for up to 12 months at 4, 21, and 30°C was generally unaffected by the composition of powdered infant formula. Exceptions were that populations decreased more rapidly at some aₜ/temperature/storage time combinations for two of the six formulas.
- *E. sakazakii* is resistant to desiccation in powdered infant formulas over a wide range of aₜ (0.25 – 0.86) and temperatures (4 – 30°C).
- *E. sakazakii* strain 2855 (mucoidal strain) survived in higher numbers in powdered infant formula C (initial aₜ 0.43 – 0.86), a soy-based formula, stored for 36 weeks at 4°C than in formula A, a milk-based formula.
• *E. sakazakii* strain 3396 (non-mucoidal) survived longer (24 weeks) than did strain 2855 in formulas at high initial $a_w$ (0.43 – 0.86) at all storage temperatures, suggesting that the ability of *E. sakazakii* to form extracellular mucoidal materials is not necessarily associated with protection against death caused by desiccation.

3. The effects of temperature (4, 12, 21, and 30°C) and infant formula composition (six formulas manufactured by four companies) on survival and growth of *E. sakazakii* upon reconstitution with water was studied.

• Growth did not occur in formulas stored at 4°C, although *E. sakazakii* was detected by enrichment of formulas 72 h after reconstitution.

• *E. sakazakii*, initially at a population of 0.02 CFU/ml, grew to populations of $\geq 1$ log CFU/ml in reconstituted infant formulas held at 12, 21, and 30°C for 48, 12, and 8 h, respectively.

• Initially at a population of 0.53 CFU/ml, the pathogen grew to populations of $\geq 1$ log CFU/ml in reconstituted infant formulas held at 12 and 21°C for 24 and 8 h, respectively, and to populations of $> 3$ log CFU/ml when held at 30°C for 8 h.

• Overall, growth of *E. sakazakii* did not appear to be greatly influenced by the composition of formula.

• Populations of *E. sakazakii* initially at 0.02 and 0.53 CFU/ml (equates to ca. 13 CFU/100 g and ca. 409 CFU/100 g of powdered formula, respectively) in all reconstituted formulas increased to 0.25 and 0.40 log CFU/ml, respectively, when held at 30°C for 4 h.

• The U.S. Food and Drug Administration recommendation that reconstituted infant formula should be held for no longer than 4 h at room temperature after preparation
in neonatal intensive care units in an attempt to reduce the risk of infections is supported by observations made in this study showing that *E. sakazakii* is able to rapidly grow in reconstituted infant formula held at 21 and 30°C.

4. A study was done to determine the effects of the lactoperoxidase system (LPOS) (0, 5, 10, 20, and 30 µg LPO/ml) and storage temperature (21, 30, and 37°C) on survival of *E. sakazakii* (0.03 or 4.40 log CFU/ml) in a milk-based powdered infant formula reconstituted with water.

- *E. sakazakii*, initially at a population of 0.03 CFU/ml, grew to populations of ≥2.40 log CFU/ml in reconstituted infant formula held at 30 or 37°C for 8 h, and to 0.60 log CFU/ml when held at 21°C for 12 h.

- When formula was treated with 10, 20, or 30 µg/ml LPO, inoculated at the same populations (0.03 CFU/ml), and stored at the same temperatures, *E. sakazakii* was not detected by direct plating or enrichment for up to 12 h.

- *E. sakazakii* was not recovered by direct plating or enrichment of low-inoculum formula containing 10, 20, or 30 µg/ml LPO and stored at 37°C up to 24 h.

- The LPOS, when applied to reconstituted infant formula using LPO concentrations of 10 – 30 µg/ml, inhibited growth of *E. sakazakii* initially at 0.03 CFU/ml (≤25 CFU/100 g of dry powder) to less than 1 CFU/ml for up to 24 h at 21 – 37°C. This compares to 4.04, 8.46, and 8.89 log CFU/ml in formula at 21, 30, and 37°C, respectively, not treated with LPOS.

- Populations of *E. sakazakii* initially at 4.40 log CFU/ml of reconstituted infant formula containing 5 µg/ml LPO did not increase for up to 12 h when formulas were held at 21 and 30°C.
• Initially high populations in reconstituted infant formula containing 10 µg/ml LPO decreased significantly or remained statistically unchanged in formulas stored at 21, 30, or 37°C up to 24, 8 and 8 h, respectively.

• Results indicate that the LPOS is effective in preventing the growth of *E. sakazakii* initially at populations exceeding those found in commercially manufactured powdered infant formula when reconstituted and held for 24 h at temperatures up to 37°C.

• Treatment of reconstituted infant formula with LPOS clearly prevents the growth of *E. sakazakii* in temperature-abused formulas but should not be used to replace acceptable hygienic practices.