

## **ENTEROBACTER SAKAZAKII**

### **EFFECTIVENESS OF DISINFECTANTS IN KILLING *ENTEROBACTER SAKAZAKII* IN SUSPENSION, DRIED ON THE SURFACE OF STAINLESS STEEL, AND IN BIOFILM**

(H. Kim, J.-H. Ryu, and L. R. Beuchat)

The presence of *E. sakazakii* on the surface of utensils and equipment used for infant formula preparation has been reported to occur in clinical settings where neonatal infections have been documented. The ability of bacteria to form biofilms on abiotic surfaces raises the possibility that infections may occur following cross-contamination of freshly prepared infant formulas upon contact with soiled surfaces in formula preparation areas in hospitals, day-care centers, food service kitchens, and the home. Surface disinfection is routinely carried out in formula preparation areas by applying liquid chemical disinfectants to food contact and non-food contact surfaces. The microbicidal activity of commercial surface cleaners and disinfectants is largely based on quaternary ammonium compounds, phenolic compounds, organic acids, alcohols, chlorine, and iodophors. During infant formula preparation and feeding, reconstituted formula containing *E. sakazakii* may contaminate abiotic surfaces. These surfaces may be treated with disinfectants immediately after contamination occurs, after the formula remains on the surface and dries, or after growth of *E. sakazakii* and the formation of biofilm. The efficacy of commercial disinfectants used in formula preparation areas in hospitals and child day-care centers in killing *E. sakazakii* in dried infant formula and biofilm has not been described. We undertook studies to determine the effectiveness of thirteen disinfectants in killing *E. sakazakii* in suspension, dried on the surface of stainless steel, and embedded in biofilm on stainless steel. Quaternary ammonium and phenolic disinfectants commonly used in infant formula preparation areas, laboratories, and hospital, food service, and child day-care settings were evaluated. The effects of time elapsed after drying cells on stainless steel as well as the age of biofilms on resistance of cells to disinfectants were determined. *E. sakazakii* exhibited various levels of resistance to the disinfectants, depending on the composition of the disinfectants, amount and type of organic matrix surrounding cells, and exposure time. Populations of planktonic cells suspended in water (7.22 - 7.40 log CFU/ml) decreased to undetectable levels (< 0.30 log CFU/ml) within 1 - 5 min upon treatment with disinfectants, while numbers of cells in reconstituted infant formula were reduced by only 0.02 - 3.69 log CFU/ml after the treatment for 10 min. The presence of infant formula also enhanced the resistance of cells dried on the surface of stainless steel to the disinfectants. The resistance of cells in 6-day-old and 12-day-old biofilms on the surface of stainless steel to disinfectants was not significantly different. The overall order of efficacy of disinfectants in killing *E. sakazakii* was planktonic cells > cells inoculated and dried on stainless steel > cells in biofilms on stainless steel. Findings show that disinfectants routinely used in hospital, day-care, and food service kitchen settings are ineffective in killing some cells of *E. sakazakii* embedded in organic matrices.

### **CONTROL OF *ENTEROBACTER SAKAZAKII* IN RECONSTITUTED INFANT FORMULA USING THE LACTOPEROXIDASE SYSTEM**

(J.B. Gurtler and L.R. Beuchat)

Neonatal bacteremia and meningitis caused by *Enterobacter sakazakii* have been associated with consumption of reconstituted powdered infant formula. Reconstituted formula is an excellent substrate for growth of *E. sakazakii*, thus raising a need for elimination or control interventions. The lactoperoxidase system (LPOS) has been shown to prevent the growth of *Escherichia coli*, *Campylobacter jejuni*, *Salmonella*, *Shigella*, and other foodborne pathogens in various types of foods. We undertook a study to determine if LPOS can be used to control 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. Initially at 0.04 CFU/ml, *E. sakazakii* grew to 2.40 - 2.74 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 for 12 h at 21°C. The pathogen was not detected (less than 1 CFU/227 ml) by enrichment of formula treated with 10 - 30 µg/ml lipoperoxidase (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 of 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 ( $p \leq 0.05$ ) or were unchanged in formula supplemented with 10 µg/ml LPO and stored at 21, 30, or 37°C for up to 24, 8, and 8 h, respectively. Results indicate that LPOS can be used to control

the growth of *E. sakazakii* in reconstituted infant formula, thereby potentially reducing the risk of neonatal infections resulting from consumption of formula that may be contaminated with the pathogen.

### **SURVIVAL OF *ENTEROBACTER SAKAZAKII* IN POWDERED INFANT FORMULA AS AFFECTED BY COMPOSITION, WATER ACTIVITY, AND TEMPERATURE**

(J. B. Gurtler and L. R. Beuchat)

Infant *Enterobacter sakazakii* infection was linked to cross-contamination from a blender used to prepare reconstituted powdered infant formula, but the pathogen was not isolated from the powdered formula. Although the blender was washed in a commercial dish-washing machine daily, the authors surmised that *E. sakazakii* may have adhered to, undergone desiccation, 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. Other reports suggest that reconstituted infant formula that has subsequently dried on abiotic surfaces may provide a suitable harbor whereby *E. sakazakii* survives and poses a risk of cross-contamination. Conditions to which powdered 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. We did a study to determine the ability of *E. sakazakii* to survive in six commercially manufactured milk-based and soy-based powdered infant formulas. A ten-strain mixture of *E. sakazakii* was inoculated into the six infant formulas at three  $a_w$  ranges ( $a_w$  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. At an initial population of 0.80 log CFU/g, *E. sakazakii* was detected by enrichment in 6 of 6, 4 of 6, and 1 of 6 formulas stored for 12 months at 4, 21, and 30°C, respectively. In 4 of 6 formulas at  $a_w$  0.25 – 0.30, initially high populations decreased significantly ( $p \leq 0.05$ ), although by less than 1 log CFU/g, within 6 months at 4°C. 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 – 0.50 than in formulas at  $a_w$  0.25 – 0.30. The rate of inactivation of *E. sakazakii* in formulas was not markedly influenced by formula composition. Cells from mucoid and non-mucoid colonies formed by two strains on violet red bile glucose agar supplemented with pyruvate were inoculated into a milk-based powdered infant formula and a soybean-based powdered infant formula at  $a_w$  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  $a_w$  and storage temperature. The presence of mucoidal extracellular materials on the surface of *E. sakazakii* cells was not associated with protection against death. This study shows that the retention of viability of *E. sakazakii* in powdered infant formula is affected by  $a_w$  and temperature. Increases in both parameters cause an increase in the rate of death.

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

(J. B. Gurtler and L. R. Beuchat)

The Food and Agricultural Organization and the World Health Organization 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 confirmed to be a vehicle of infection. At least 76 cases of *E. sakazakii* infections and 19 deaths of infants and children have been documented. The first outbreak of *E. sakazakii* infection linked to powdered infant formula obtained from a previously unopened can occurred in 2001. In another outbreak, powdered formula tested negative for *E. sakazakii* but the blender used to prepare the reconstituted formula was positive for the pathogen. This suggests that contamination of the blender could have resulted from contact with powdered, reconstituted infant formula, or some other source containing the pathogen. Because powdered infant formula contains an abundance of nutrients to potentially support the growth of *E. sakazakii*, appropriate temperature control of reconstituted formula is critical to inhibiting multiplication and minimizing the risk of illness. It is not known if differences in infant formula composition affect the rate of growth at various storage temperatures.

We undertook a study to determine survival and growth characteristics of *E. sakazakii*, inoculated at populations of 0.02 and 0.53 CFU/ml (ca. 13 CFU/log and 40% CFU/100 g of powdered formula, respectively), as

affected by composition of six powdered 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 of all formulas 72 h after reconstitution. Initially at a population of 0.02 CFU/ml, *E. sakazakii* grew to populations  $\geq 1$  log CFU/ml of reconstituted formulas 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  $\geq 1$  log CFU/ml in reconstituted infant formula held at 12 and 21°C for 24 and 8 h, respectively, and to populations  $> 3$  log CFU/ml when held at 30°C for 8 h. Populations initially at 0.02 and 0.53 CFU/ml of reconstituted formula increased to  $\leq 0.25$  and 0.40 log CFU/ml, respectively, when formulas were held at 30°C for 4 h. Growth was not greatly influenced by the composition of formulas. Results of our study 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. Portions of reconstituted infant formula not fed to infants should be stored at  $\leq 4^\circ\text{C}$ , a temperature at which *E. sakazakii* will not grow.

**SURVIVAL OF *ENTEROBACTER SAKAZAKII* IN INFANT CEREAL  
AS AFFECTED BY COMPOSITION, WATER ACTIVITY, AND TEMPERATURE**  
(L.-C. Lin, and L. R. Beuchat)

Outbreaks of infections have implicated powdered milk substitute infant formulas as vehicles of *E. sakazakii*. Studies have shown that *E. sakazakii* is more thermotolerant than some Enterobacteriaceae in milk products, e.g., in infant milk formula; however, standard pasteurization practices are thought to be effective for inactivation of the bacterium. Post-pasteurization contamination of powdered infant formulas before packaging may occur in some commercial operations, as evidenced by its isolation from previously unopened cans of formula. *E. sakazakii* has been isolated from various dry foods, dry food processing plants, and the environment. The pathogen has been detected in rice seed, rice starch and flour, brown rice, and dry infant cereals. Survival characteristics of *E. sakazakii* in infant cereals as affected by the type of grain component,  $a_w$ , and storage temperatures under which they are held during distribution or in hospital, day-care center, and home settings have not been reported. We did a study to determine the survival characteristics of *E. sakazakii* initially at populations of 0.31 and 5.03 log CFU/g of infant rice cereal ( $a_w$  0.30, 0.45 - 0.46, and 0.68 - 0.69). Cereal was stored at 4, 21, and 30°C and populations were monitored for up to 12 months. Survival of the pathogen in infant rice, barley, oatmeal, and mixed grain cereals ( $a_w$  0.63 - 0.66, 0.76, or 0.82 - 0.83) initially containing a population of 4.93 - 5.64 log CFU/g and held at 4, 21, and 30°C up to 24 weeks was determined. Populations decreased significantly ( $p \leq 0.05$ ) in all cereals stored at 21 and 30°C, regardless of  $a_w$ . Increases in  $a_w$  or storage temperature accelerated the rate of death of *E. sakazakii* in dry infant cereals. However, at an initial population of 0.31 log CFU/g, *E. sakazakii* survived in rice cereal ( $a_w$  0.30 - 0.69) for up to 12 months at all storage temperatures. Survival of *E. sakazakii* was not affected by the composition of dry infant rice, barley, mixed grain, and oatmeal cereals (initial  $a_w$  0.63 - 0.83) stored for up to 24 weeks at 4, 21, or 30°C. This study reveals that *E. sakazakii* can survive for up to 12 months in infant cereals having a wide range of  $a_w$  when storage is at temperatures simulating those to which they may be exposed during distribution, at retail, and in the home.

**SURVIVAL AND GROWTH OF *ENTEROBACTER SAKAZAKII* IN INFANT CEREAL  
AS AFFECTED BY COMPOSITION, RECONSTITUTION LIQUID, AND STORAGE TEMPERATURE**  
(L.-C. Lin and L. R. Beuchat)

During the period of 1958 - 2005, documented *Enterobacter sakazakii* infections were associated largely with neonates and infants less than 2 months of age. Infections in children as old as 4 years and in adults also have been reported. *E. sakazakii* has been shown to have a high tolerance to desiccation. It has been isolated from various dry foods, dry food processing plants, and the environment. The pathogen has been detected in rice starch and flour, dried infant cereals, dried infant foods, and environmental samples from 8 of 9 food factories, including a cereal factory. Studies have shown that some bacterial pathogens can survive and grow in reconstituted infant cereal. However, little is known about the behavior of *E. sakazakii* in infant cereals as affected by composition of cereal, composition of reconstitution liquid, and temperature at which they may be prepared and stored in hospital, day-care center, and home settings. We did a study to determine the survival and growth characteristics of *E. sakazakii* in infant cereals reconstituted with various liquids as influenced by storage temperature. Survival and growth characteristics of *E. sakazakii* initially at populations of 0.005 and 0.52 CFU/ml of infant rice cereal, oatmeal cereal, or rice with mixed fruit cereal reconstituted with water, milk, or apple juice were determined.

Reconstituted cereals were stored at 4, 12, 21, and 30°C and populations were monitored for up to 72 h. Growth did not occur in reconstituted cereals stored at 4°C or in cereals reconstituted with apple juice and stored at 12°C. Populations ( $\geq 1$  CFU/ml) were detected in cereals reconstituted with water or milk and stored at 12, 21, and 30°C for 24, 8, and 4 h, respectively. The composition of infant cereals did not markedly affect the survival or growth of *E. sakazakii* in reconstituted cereals. Populations of *E. sakazakii* in reconstituted cereal decreased with increases in populations of mesophilic aerobic microflora up to 8 - 9 log CFU/ml, which was concurrent with decreases in pH. *E. sakazakii*, initially at 2.62 log CFU/ml of rice cereal reconstituted with apple juice (pH 4.32), survived at 4°C for at least 14 days. The pathogen was not detected ( $< 1$  CFU/10 ml) in cereal stored at 21°C for 5 days or 30°C for 4 days. Initially at 7.32 log CFU/ml, *E. sakazakii* was detected in rice cereal stored at 4°C for 50 days. It is recommended that reconstituted infant cereals stored at 21°C or 30°C be discarded within 4 h after preparation or stored at  $\leq 4^\circ\text{C}$ , temperatures at which it will not grow.

#### **ATTACHMENT AND BIOFILM FORMATION BY *ENTEROBACTER SAKAZAKII* ON STAINLESS STEEL AND ENTERAL FEEDING TUBES**

(H. Kim, J.-H. Ryu, and L. R. Beuchat)

*Enterobacter sakazakii* is a foodborne pathogen capable of causing meningitis, sepsis, bacteremia, and necrotizing enterocolitis in preterm neonates and immunocompromised adults. Powdered infant formula and milk powder have been implicated as vehicles in outbreaks of *E. sakazakii* infections. However, the pathogen also has been isolated from various clinical sources, food processing plants, the environment, lettuce, alfalfa sprouts, tomatoes, and other vegetables, cheese, minced beef, and sausage. Its presence in fresh produce raises the possibility of this food group serving as a vehicle of the pathogen for infections in immunocompromised adults, particularly patients in hospitals and elderly adult assisted-care facilities. *E. sakazakii* has been reported to be able to attach to and form biofilms on silicon, latex, polycarbonate, stainless steel, glass, and polyvinyl chloride. Foods such as powdered infant formula and fresh produce represent potential vehicles of *E. sakazakii* infections in infants and immunocompromised adults, respectively. Contact of these and other foods containing the pathogen with abiotic or biotic surfaces could result in attachment and biofilm formation. Removal or inactivation of pathogens on inert surfaces in infant formula preparation areas and produce processing environments by washing with water or treating with disinfectants or sanitizers is not always achieved, possibly because cells are enmeshed in biofilms or otherwise protected against exposure to antimicrobials. Attachment and biofilm formation by *E. sakazakii* as affected by temperature and nutrient availability have been given only meager research attention.

We did a study to determine the effects of temperature and nutrient availability on attachment and biofilm formation by *E. sakazakii* on stainless steel and enteral feeding tubes. Five strains grown to stationary phase in tryptic soy broth (TSB), infant formula broth (IFB), and lettuce juice broth (LJB) at 12°C and 25°C were examined for the extent to which they attach to these materials. Higher populations attached at 25°C than at 12°C. Stainless steel coupons and enteral feeding tubes were immersed for 24 h at 4°C in phosphate-buffered saline suspensions (7 log CFU/ml) to facilitate attachment of 5.33 - 5.51 and 5.03 - 5.12 log CFU/cm<sup>2</sup>, respectively, before immersing in TSB, IFB, or LJB and incubating at 12°C or 25°C for up to 10 days. Biofilms were not produced at 12°C. The number of cells of test strains increased by 1.42 - 1.67 log CFU/cm<sup>2</sup> and 1.16 - 1.31 log CFU/cm<sup>2</sup> in biofilms formed on stainless steel and feeding tubes, respectively, immersed in IFB at 25°C; biofilms were not formed on TSB and LJB at 25°C, indicating that nutrient availability plays a major role in processes leading to the accumulation of biometrics on the surfaces of these inert materials. These observations emphasize the importance of temperature control in reconstituted infant formula preparation and storage areas in preventing attachment and biofilm formation by *E. sakazakii*.

#### **DOSE RESPONSE OF *ENTEROBACTER SAKAZAKII* INFECTIONS IN CD-1 NEONATAL MICE**

(M.A. Smith)

*Enterobacter sakazakii* (*E. sakazakii*) has been associated with nosocomial infections in premature and very low birth weight human infants. The affected infants were exposed to *E. sakazakii* when fed with contaminated reconstituted powdered infant formula. In this study, experimental CD-1 suckling mice were orally challenged with a single dose of 0.1 ml reconstituted powdered infant formula inoculated with 10<sup>2</sup> to 10<sup>11</sup> CFU *E. sakazakii* strain MNW2 on postnatal day 3. Deaths occurring immediately after or less than 15 hours post-treatment were suspected to result from gavage technique and were not included in the analysis. Twenty-six deaths occurred at least 15 hours post-treatment and were assumed to result from *E. sakazakii* infection. The surviving mice were euthanized

and weighed on postnatal day 10. Brains, ceca, and livers were excised and pooled into groups within each litter for culturing. *E. sakazakii* was isolated from brain, liver, and cecum tissues in animals treated with  $10^{11}$  CFU as compared to only brain and liver tissues in neonates administered  $10^9$  CFU. *E. sakazakii* was not found in control tissues. Three out of six litters at  $10^9$  CFU had neonatal deaths, whereas all litters (4/4) treated with  $10^{11}$  CFU had at least three neonatal deaths. There was 14.5% lethality among pups administered  $10^9$  CFU and 34.8% lethality among pups given  $10^{11}$  CFU as compared to no deaths among control pups. *E. sakazakii* infection in neonatal mice may be similar to that in premature human neonates because of their underdeveloped CNS at full-term birth. Thus neonatal mice may potentially serve as a model for *E. sakazakii* infection in premature and very low birth weight human infants.

**NEONATAL MICE AS MODELS FOR PREMATURE INFANTS**  
**FED *ENTEROBACTER SAKAZAKII*-CONTAMINATED INFANT FORMULA**  
(A.N. Richardson, S. Massengill, and M.A. Smith)

Premature or very-low-birth-weight human infants exposed to *Enterobacter sakazakii* in reconstituted powdered infant formula may develop infections resulting in septicemia, necrotizing enterocolitis, meningitis, hydrocephalus, or death. Animal models are needed to estimate and understand the infectivity of *E. sakazakii* in human infants. Due to their underdeveloped central nervous system at birth, the infection of neonatal mice with *E. sakazakii* may mimic that of premature human infants. Our objective was to compare the susceptibilities of three mouse strains to *E. sakazakii* strain MNW2 by observing mortality and infectivity. Timed-pregnant dams of the CD-1, BALB/C and C57BL/6 strains were obtained, acclimatized, and allowed to give birth naturally. At postnatal day (PND) 3 or 4, the pups were orally gavaged with a single dose of vehicle or  $10^4$  -  $10^{12}$  colony-forming units (CFU) *E. sakazakii* strain MNW2 per ml reconstituted powdered infant formula. All pups surviving to PND 10 or 11 were sacrificed and brains, livers, and ceca excised and analyzed for the presence of *E. sakazakii*. *E. sakazakii* was isolated from 66.7%, 38.5% and 36.4% of brains, 60.0%, 30.8% and 45.4% of livers, and 26.7%, 0% and 18.2% of ceca from treated CD-1, BALB/C and C57BL/6 litters, respectively. No deaths occurred in any of the control groups for any mouse strain. Among the three strains, CD-1 appears to be the most sensitive demonstrating a dose-dependent response in mortality ( $10^{11}$  CFU resulted in 34.8% mortality). In C57BL/6 mice, mortality occurred only at the highest dose administered (4.2% at  $10^{12}$  CFU). Although BALB/C mice had 19% mortality at  $10^7$  CFU, it was not dose-dependent. The results of this experiment suggest that the CD-1 mouse strain is the most susceptible to *E. sakazakii* infection and may serve as a potential animal model for the infection in human infants. (Funded by ILSI North America Technical Committee on Food Microbiology to MAS).