USING THE NEONATAL MOUSE MODEL TO INVESTIGATE DOSE RESPONSE AND HOST SUSCEPTIBILITY TO *CRONOBACTER SAKAZAKII* INFECTIONS IN PREMATURE INFANTS

by

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(Under the Direction of Mary Alice Smith)

ABSTRACT

Cronobacter sakazakii (*C. sakazakii*) is an opportunistic pathogen that has been isolated from powdered infant formulas and can result in serious illnesses such as bacteremia, septicemia, meningitis and death in at-risk infants who are exposed to reconstituted powdered infant formulas. The purpose of our study was to describe whether neonatal mice are an appropriate animal model for *C. sakazakii* infection in premature infants and to evaluate the virulence and pathogenicity of *C. sakazakii*. The objectives were to 1) compare the susceptibilities of three mouse strains to *C. sakazakii*, 2) compare the virulence of three strains of *C. sakazakii* in neonatal mice, and 3) compare the susceptibilities of neonatal mice of different ages to *C. sakazakii* and identify biomarkers of infection. Neonatal mice were administered reconstituted powdered infant formula inoculated with *C. sakazakii* via oral gavage. On post-treatment day 7, mice were sacrificed, blood samples were collected, and brain, liver, and cecum tissues were excised and prepared for isolation of *C. sakazakii*. The CD-1 mouse strain was more susceptible than BALB/C or C57BL/6, with the lowest infectious dose and the lowest lethal dose (10^2 CFU). Two clinical strains (3290 and SK81) and one food isolate (MNW2) of *C. sakazakii* were tested

for virulence in the mouse model. *C. sakazakii* strain 3290 was significantly more invasive in brains (42.1% of mice) than were strains MNW2 (6.7%) and SK81 (15.9%). Mortality was observed for all strains of *C. sakazakii* tested, but with a much lower rate than infection. Age of the neonate affects susceptibility to *C. sakazakii* infection, as *C. sakazakii* was isolated from brains, livers, and ceca of neonatal mice treated at PND 1.5 and 5.5 but not from those of pups treated at PND 9.5. In conclusion, neonatal mice are susceptible to oral challenge with *C. sakazakii*. Our findings suggest that invasiveness does not necessarily correlate with mortality among different strains of *C. sakazakii*, and the clinical isolates (SK81 and 3290) are more virulent than the food isolate (MNW2). Neonatal mice also show a time-dependent susceptibility to *C. sakazakii* infection with resistance increasing with increasing age.

INDEX WORDS: *Cronobacter*; *E. sakazakii*; infant formula; infants; infection; mouse model; neonatal mice

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B.S., South Carolina State University, 2004

A Dissertation Submitted to the Graduate Faculty of the University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2010

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DEDICATION

This dissertation is dedicated to my loving and prayerful family. I am only continuing the legacy you began.

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CHAPTER 1

INTRODUCTION

Cronobacter sakazakii (*C. sakazakii*) is an emerging opportunistic bacterial pathogen that rarely infects healthy individuals, but can cause illness and death in at-risk infants. It is a ubiquitous organism with no known natural reservoir, and in recent years, it has been occasionally isolated from powdered infant formulas. *C. sakazakii* may contaminate powdered infant formulas during the manufacturing process, likely after pasteurization. Reported cases of *C. sakazakii* infection in human infants have led to powdered infant formula recalls. In cases of infection, *C. sakazakii* has been isolated from feces, cerebrospinal fluid, blood, urine, sputum, respiratory tracts, and bone marrow of infected infants.

C. sakazakii infection in susceptible human infants can result in serious illness and may lead to death. Infants who are considered at-risk are those born prematurely and/or have low birth weights. Premature and low birth weight infants are often solely fed reconstituted powdered infant formulas or are breast-fed and supplemented with reconstituted powdered infant formula in order to receive proper nutrition. Some of these infants are bottle-fed and some are fed via enteral feeding tubes. Most cases of *C. sakazakii* are sporadic; however, outbreaks of infection have occurred in neonatal intensive care units (NICUs) in different parts of the world.

In cases of *C. sakazakii* infection in neonates, powdered infant formula has been implicated as the source of the bacterium, being cultured from both opened and unopened cans of powdered infant formula used to feed infected infants and the surfaces on which the formulas were mixed and prepared for feedings (Muytjens et al., 1988, Nazarowec-White and Farber,

1997b, Biering et al., 1989, Simmons et al., 1989, Van Acker et al., 2001). *C. sakazakii* can survive long periods of dessication, such as during the long shelf-life of powdered infant formulas. The nonsterile powdered infant formula product and its association with *C. sakazakii* infection have prompted regulatory agencies such as the U.S. Food and Drug Administration (FDA) and the World Health Organization/Food and Agriculture Organization (WHO/FAO) to create guidelines for the safe preparation and handling of powdered infant formulas (WHO/FAO, 2007, FDA, 2002). Powdered infant formulas are not sterilized because the process decreases the nutritional value of the product, which is intended to provide essential nutrients to young infants. Proper hygiene and care should be considered when preparing infant formulas for consumption by at-risk infants. Basic measures such as regularly washing bottles, feeding tubes, and utensils, and refrigeration of rehydrated infant formulas are important in preventing any *C. sakazakii* that might be present in the formula from growing to infectious concentrations (WHO/FAO, 2007, WHO/FAO, 2008).

PATHOGENESIS OF *C. SAKAZAKII* INFECTION

The pathogenesis of *C. sakazakii* is not completely understood. *C. sakazakii* primarily infects humans via the oral route. After ingestion, it passes through the gastrointestinal tract, colonizes the intestines and translocates across the intestinal epithelium and enters systemic circulation. From the blood, *C. sakazakii* can travel and invade tissues throughout the body of the host, including the liver, and cross the blood-brain barrier to enter the central nervous system. *C. sakazakii* is an intracellular pathogen, allowing it to evade host immune responses.

Onset of *C. sakazakii* infection is generally acute. Initial symptoms of *C. sakazakii* infection in infants include irritability, respiratory distress, gastroenteritis/diarrhea, and fever. Infants who are severely ill often need ventilators to assist with respiration. As the infection

progresses, necrotizing enterocolitis, bacteremia, brain abscesses, meningitis, and septicemia may develop. When *C. sakazakii* reaches the central nervous system (CNS), infected infants may experience seizures. After *C. sakazakii* gains access to the CNS, patients have poor prognosis. Overall, *C. sakazakii* infection has a case-mortality rate of 40 – 80%.

Presently, treatment of *C. sakazakii* infection involves trial and error of a range of antibiotics. Series of single antibiotics or combinations are often administered to patients to clear the infection due to varying degrees of resistance and sensitivity. Because of this, successful treatment of *C. sakazakii* may take a number of days. Gentamicin, ceftazidine, amikacin, ciprofloxacin, chloramphenicol, and vancomycin are some of that have been administered intravenously to infants for the treatment of *C. sakazakii* infection.

PREVIOUS ANIMAL STUDY FOR *C. SAKAZAKII* INFECTION

There is currently no animal model for *C. sakazakii* infection in humans. Animal studies are needed to gain an understanding of how *C. sakazakii* causes infection in susceptible infants. The first *C. sakazakii* virulence study was conducted in 2003 (Pagotto et al., 2003). In this infectivity study, 3- to 4-day-old Swiss Webster mice were exposed to 18 *C. sakazakii* strains via oral gavage or intraperitoneal injection. *C. sakazakii* was administered at oral doses of 10^5, 10^7, and 10^8 CFU/neonatal mouse. Among orally treated mice, mortality was only observed among those administered *C. sakazakii* strains SK81 and MNW2 at 10^7 and 10^8 CFU/mouse. Building on the Pagotto et al. (2003) study, we wanted to further investigate which of three mouse strains would be most susceptible to infection as well as examine the ability of *C. sakazakii* to invade host tissues, identify sublethal endpoints, and identify biomarkers of infection. Our study results would provide basic information for the development of treatments and therapies for human infants with *C. sakazakii*.

The purpose of our study was to describe whether neonatal mice are an appropriate animal model for *C. sakazakii* infection in premature infants and to determine biological endpoints to evaluate the virulence and pathogenicity of *C. sakazakii*. The specific aims were to 1) determine whether or not there are differences in *C. sakazakii* susceptibility in three mouse strains using neonatal mice, 2) determine appropriate nonlethal endpoints for *C. sakazakii* infections in neonatal mice by examining changes in pro-inflammatory cytokines and intracranial pressure, and 3) determine the lowest infectious dose of *C. sakazakii* in neonatal mice.

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CHAPTER 2

LITERATURE REVIEW

Human infants that are fed *Cronobacter sakazakii* (*C. sakazakii*)-contaminated powdered infant formulas may become infected, fall severely ill, suffer life-long debilitating complications after recovering from infection, or the infection may result in death of the infant. There have been reported cases of *C. sakazakii* infection in older children and adults, however the majority of these occurred in persons with pre-existing conditions (i.e. surgical procedures, cancer therapies) (Lai, 2001b, Dennison and Morris, 2002). Outbreaks of *C. sakazakii* infection have occurred in neonatal intensive care units (NICUs) in several different countries (Arseni et al., 1987, Van Acker et al., 2001, Block et al., 2002, Muytjens et al., 1983). Although *C. sakazakii* infection is rare, the case-fatality rate ranges from 40-80% (Nazarowec-White and Farber, 1997a, Bowen and Braden, 2006, WHO/FAO, 2007, Willis and Robinson, 1988, Edelson-Mammel and Buchanan, 2004). Risk factors for *C. sakazakii* infection include premature birth, low birth weight, or other preexisting conditions.

C. sakazakii infection can have severe consequences. Necrotizing enterocolitis (Hunter et al., 2007, Van Acker et al., 2001), bacteremia (Monroe and Tift, 1979, Noriega et al., 1990), brain abscesses (Burdette and Santos, 2000b, Townsend et al., 2007c), meningitis (Biering et al., 1989, Muytjens et al., 1983, Willis and Robinson, 1988), and septicemia (Stoll et al., 2004), are some of the complications reported during *C. sakazakii* infection in an infant. Infants who survive the initial *C. sakazakii* infection may have life-long sequelae including hydrocephaly, mental retardation, or other neurological problems, including quadriplegia (Lai, 2001b, Rosset et al., 2007).

Very little is known about the mechanisms by which *C. sakazakii* causes severe disease in infants. Recently-published *C. sakazakii* studies using *in vivo* and *in vitro* experiments have provided limited information about the mechanisms by which *C. sakazakii* may infect a human

host and cause adverse effects (Townsend et al., 2007b, Townsend et al., 2007c, Pagotto et al., 2003, Kim and Loessner, 2008a). Although animals have been used to research *C. sakazakii* virulence, there currently is no accepted animal model to mimic *C. sakazakii* infection in human infants. The information gathered from examining the effects of *C. sakazakii* infection in animals can lead to advancements in treatments and therapies for human infants who become ill. Neonatal mice may serve as an appropriate model for *C. sakazakii* infection in premature human infants because their central nervous systems are underdeveloped at birth (Clancy et al., 2001).

We were invited to write a book chapter on intrauterine infections in 2008 for the "Comprehensive Toxicology" textbook published by Elsevier. In the text, we discussed various bacterial, viral, parasitic, and fungal infections, their mechanisms of infection, and adverse outcomes that occur in pregnant women, fetuses, and neonates after exposure to them. WHO/FAO (2008) stated that neonates and young infants have a greater risk of infection due to a temporary deficiency of the immune system (WHO/FAO, 2008). This immunodeficiency encompasses numerous factors which include decreased production of mucus, acid, and immunoglobulin, less gut motility, inadequate cytokine production, naive lymphocytes, and reduced macrophage activity (Niers et al., 2007, WHO/FAO, 2008). *C. sakazakii* was included in this book chapter because it can cause infections in susceptible neonates. This book chapter has been published and is cited as follows:

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CRONOBACTER SAKAZAKII INFECTION

SUSCEPTIBLE POPULATIONS

Infants, elderly, and immunocompromised persons are the populations that are most susceptible to *Cronobacter* infection. Whereas infants are usually exposed to *Cronobacter* spp. through ingestion of contaminated infant formulas, adults may become infected in post-operational periods as after abdominal or intestinal operations (Lai, 2001). Lai (2001) reported four cases of *C. sakazakii* infection in adult persons, three of whom were elderly. A 39-year-old male developed bronchopneumonia after undergoing a surgical procedure to remove a tonsillar carcinoma. *Cronobacter spp.* was isolated from his sputum. He was treated with antibiotics and fully recovered from the infection. All three elderly patients were reported to have died subsequent to the development of *Cronobacter* spp. infection.

EPIDEMIOLOGY

The first known cases of *Cronobacter* spp. infection were published by Urmenyi and Franklin in 1960. Two infants became ill and their health seemed improve; however, it subsequently declined rapidly and both cases resulted in death. Surfaces in the hospital were swabbed, including the incubators, etc. but the source of infection was not determined. The infants had no contact with each other, so one could not have contracted the infection from the other.

Epidemiological data on *Cronobacter* infection are very limited. Currently, there is no surveillance system for *Cronobacter* spp. infection; however, most countries possess a foodborne disease surveillance system and/or an outbreak reporting system that would include *Cronobacter* spp. infection (FAO/WHO, 2008). Between 120 and 150 cases of *Cronobacter* infection in infants ≤ 2 months old have been reported (Friedemann, 2009, WHO/FAO, 2008, Mullane et al.,

2007). In the United States it is estimated that *Cronobacter* infection occurs in is one out of every 100,000 infants. The incidence is over eight times greater for neonates with low birth weights (< 2500 g), with 8.7 per 100,000 becoming infected with *Cronobacter* spp. (CDC, 2002). Incidence of *Cronobacter* infection in neonates having very low birth weight (< 1500 g) is 9.4 per 100,000 (Stoll et al., 2004). Friedemann, (2009) reported statistical data on *Cronobacter* infection cases that were reported and published from 2000 – 2008. Of the 67 cases of neonatal *Cronobacter* infection with known outcomes, 26.9% ended in death. The case-fatality rate for infants developing meningitis as a result of *Cronobacter* infection within this time period was 41.9% ($P < 0.0001$).

MORPHOLOGY AND DESCRIPTION

Cronobacter spp. are motile, peritrichous, non-sporeforming, gram-negative bacteria. They are opportunistic pathogens and do not usually cause infection in healthy individuals. *Cronobacter* spp. can survive long periods of desiccation, making it able to survive the potentially long shelf-life of powdered infant formulas (Mullane et al., 2006). *Cronobacter* spp. can grow at temperatures ranging from 6 to 47°C (Lehner and Stephan, 2004, Iversen and Forsythe, 2003).

NATURAL RESERVOIR AND PLACES FOUND

Cronobacter spp. are ubiquitous bacteria with no known natural reservoir. It is speculated that plant materials, water, and soil are natural sources of *Cronobacter* spp. (Iversen et al., 2004, Iversen and Forsythe, 2003). It has been isolated from production-line environments within food production factories and household vacuum cleaner bags (Kandhai et al., 2004). *Cronobacter* spp. have been isolated from foods including lettuce, other vegetables, minced beef, sausage meat (Leclercq, 2002), spices, etc. Animal sources in which *Cronobacter* spp. have

been found include stable flies (Hamilton et al., 2003), Mexican fruit flies (Kuzina et al., 2001), and rats (Gakuya et al., 2001).

Proper hygiene and care should be considered when preparing infant formulas. Refrigeration of rehydrated infant formulas is important. *Cronobacter* spp. are able to form biofilms, allowing for survival on surfaces where food is processed or prepared (Palcich et al., 2009, Dancer et al., 2009). If contaminated powdered infant formula comes in contact with a surface, attachment and subsequent biofilm formation of *C. sakazakii* is possible (Kim et al., 2006). Cross-contamination may occur when preparing reconstituted infant formulas for infant feedings if utensils and appliances used to mix a contaminated batch are not cleaned following initial use and are then used to prepare uncontaminated powdered formula batches.

C. sakazakii may grow to high concentrations if left at room temperature for extended time periods. In a study conducted by Nazarowec-White and Farber (1997), *Cronobacter* spp. was found to have a generation time of 0.67 hours when grown in reconstituted powdered infant formula at 23°C and 4.98 hours at 10°C (Nazarowec-White and Farber, 1997b). For hospitalized infants who are administered reconstituted powdered infant formulas via enteral feeding tubes, hygiene is critical. *C. sakazakii* may grow to high concentrations in feeding tubes that are not adequately cleaned and are used in the inadvertent feedings of at-risk infants with *C. sakazakii*-contaminated formulas (Kim et al., 2006). Hurrel et al. (2009) isolated *C. sakazakii* from enteral feeding tubes collected from two NICUs and tested for the presence of *Enterobacteriaceae* (Hurrell et al., 2009). Mature biofilms that colonize enteral feeding tubes can break off and contaminate fresh reconstituted powdered infant formula passing through the tube (Hurrell et al., 2009). *C. sakazakii* can multiply to higher concentrations by feeding on the available nutrients

in the fresh formula, thereby increasing the risk of *C. sakazakii* infection in the intubated infant (Hurrell et al., 2009).

PERSISTENCE IN THE ENVIRONMENT

Cronobacter spp. possess structural and functional characteristics that aid in its survival in the environment. These traits include the production of a yellow pigment to guard against ultraviolet damage, an ability to produce capsules and fimbriae for adhesion to inert and cellular surfaces, and the ability to survive extended periods of dessication (Mullane et al., 2006). *Cronobacter* spp. can also survive other environmental stresses such as osmotic stress and heat. *Cronobacter* spp. have been known to produce capsules, another structure that may contribute to survival of environmental stresses (Iversen and Forsythe, 2003).

BIOFILM FORMATION

Cronobacter spp. can adhere to and form biofilms on surfaces such as silicon, latex, stainless steel, glass, polycarbonate, and polyvinyl chloride (PVC) (Iversen et al., 2004, Kim et al., 2006, Lehner et al., 2005). Nutrient availability, temperature, pH, surface characteristics (living and nonliving), and other factors affect attachment and biofilm formation (Kim et al., 2006). Biofilms provide physical protection of microorganisms from ultraviolet light, osmotic stress, heat, starvation, acids, antibiotics, and other possible environmental stresses (Lehner et al., 2005). *Cronobacter* spp. strains were discovered to produce cellulose within the extracellular matrix of their biofilms (Zogaj et al., 2003, Grimm et al., 2008) and pellicles at an air/liquid medium interface (Zogaj et al., 2003). Sanitizers and disinfectants are not always effective in killing *C. sakazakii* in areas where powdered infant formulas are reconstituted and prepared. Biofilm matrices produced after colonization of a surface and hidden surfaces can protect the bacterial cells against exposure to these microbicidal substances (Kim et al., 2006).

In a study by Kim et al. (2006), *Cronobacter* spp. that was attached to stainless steel surfaces and enteral feeding tubes and then submerged in infant formula broth was shown to form biofilms at a temperature of 25°C, which is room temperature (Kim et al., 2006). Kim et al. also conducted a study in 2007, in which *C. sakazakii* was suspended in hard water, phosphate buffered saline, and reconstituted powdered infant formula, then dried and embedded in biofilm on a stainless steel surface. The *C. sakazakii* strains used were shown to have a range of resistance when exposed to thirteen quaternary ammonium and phenolic disinfectants that are commonly used in laboratories, food processing facilities, food-service kitchens, and areas where infant formula is prepared for consumption such as at day-care facilities and hospitals (Kim et al., 2007).

INFANT FORMULA CONTAMINATION

Cronobacter contamination is a significant hurdle for the powdered infant formula manufacturers in terms of product safety and public health impact on neonates and at-risk infants (Arku et al., 2008). Infant formulas are made to mimic the nutrient content of human mothers' milk (Breeuwer et al., 2003, Arku et al., 2008). Dried powdered infant formulas that are sold in stores are not sterilized as are their liquid counterparts (Iversen and Forsythe, 2003). Heating powdered infant formulas to high temperatures that are proven to kill bacteria after all ingredients have been added is not recommended because this will degrade the nutrients necessary for the health of infants (Giovannini et al., 2008). It is important that powdered infant formulas are prepared according to the manufacturers' instructions. A study was conducted by Muytjens et al. (1988), in which *Cronobacter* spp. were isolated from 20/141 (14%) powdered infant formula samples manufactured in 35 countries. The amount of *Cronobacter* spp. in the positive samples ranged from 0.36 to 66 CFU/100g in 20 samples from 13 countries. However, the combination of mass production, worldwide distribution, and low incidence of *Cronobacter*

infection in healthy infants shows that these formulas are not a significant health risk when they are prepared properly (Arku et al., 2008).

Cronobacter spp. can contaminate reconstituted infant formula intrinsically and/or extrinsically (Mullane et al., 2006). Intrinsic contamination occurs during the addition of contaminated dry ingredients after the pasteurization and drying processes or from exposure to a contaminated food processing environment. Extrinsic contamination takes place during formula preparation and reconstitution (Mullane et al., 2006). In an infant formula processing facility, the raw materials and the production environment must be monitored continuously in order to diminish the likelihood of contamination (Drudy et al., 2006).

The degree of microbial control in the manufacturing process is a major factor in contamination of powdered infant formulas. *C. sakazakii* has been isolated from infant formula manufacturing facilities, allowing for contamination of the product. There are three ways in which infant formula can be manufactured: wet-mix, dry-mix, and a combination of the two (WHO/FAO, 2008, Mullane et al., 2006). Microbial contamination may occur via air vents inside the facilities in which powdered infant formulas are processed, or through other routes. Air filters within a processing facility may not be correctly fitted, properly installed and/or maintained. Non-sporeforming microorganisms can attach to dust particles (becoming airborne) and water droplets and become aerosolized (Mullane et al., 2008). The purpose of environmental air filters in a food production facility is to provide clean air to a controlled area by removing dust particles. External air should not be allowed entrance inside the air system. Therefore, it is of utmost importance that air-handling systems be controlled in order to prevent product contamination (Mullane et al., 2008). Temperature control is also imperative in infant

formula processing facilities so as to prevent the promotion of *C. sakazakii* attachment and biofilm formation on surfaces (Kim et al., 2006).

Arku et al. (2008) examined the survival of four *Cronobacter* spp. during the spray-drying process used in the manufacturing of powdered infant formula. Each *Cronobacter* spp. strain was able to survive the process at all sampling times at high and low initial inoculation concentrations. All *Cronobacter* spp. strains were also detectable in stored powdered infant formula after a period of 12 weeks; however, storage survival at low initial inoculation levels varied.

C. sakazakii cannot survive pasteurization temperatures; therefore, the infant formula must be contaminated in later production steps such as spray-drying (Gurtler et al., 2005, Nazarowec-White and Farber, 1997b, Nazarowec-White and Farber, 1997c). *Cronobacter* spp. are typically present in dry powdered infant formulas at a concentration of less than or equal to 1 CFU/100g (Edelson-Mammel and Buchanan, 2004). *C. sakazakii* present in powdered infant formula is able to grow once reconstituted, especially when the rehydrated formula is allowed to sit at room temperature for extended periods of time in the household or NICU environment, prior to infant feeding (FAO-WHO, 2004). To prevent this, adequate heating during mixing and preparation is the recommended method to deactivate *Cronobacter* spp. in rehydrated powdered infant formula.

Infant milk formula is regularly tested for coliform bacteria, which are indicators of poor hygiene. Currently, *Salmonella* is the only genus in the family *Enterobacteriaceae* to have a maximum limit for presence in infant formula (< 1 cell per 25g, with multiple samples collected from each batch) (Mullane et al., 2006). Infant milk formulas that have tested within the

acceptable limits of Salmonella and coliform bacteria have tested positive for *Cronobacter* spp. (Iversen and Forsythe, 2004).

PUBLISHED ANIMAL MODELS

Pagotto et al. (2003) conducted the first *C. sakazakii* virulence study, in which neonatal mice were challenged via oral gavage or intraperitoneal injection (Pagotto et al., 2003). There have been other published *C. sakazakii* models since the study by Pagotto et al. (2003), both *in vivo* and *in vitro*. Townsend et al. (2007) observed that *Cronobacter* spp. were able to invade rat capillary endothelial brain cells (rBCEC4). *Cronobacter* spp. were used in a gentamicin protection assay to demonstrate BBB invasion (Townsend et al., 2007). Three of six *Cronobacter* strains were not significantly different from three of four positive controls (*E. coli* K1, *Citrobacter freundii*, and *Citrobacter koseri*) (Townsend et al., 2007). The same study investigated the persistence of *Cronobacter* spp. after being phagocytosed by human macrophages. All *Cronobacter* strains tested were able to survive inside the macrophages for a maximum of 96 hours (Townsend et al., 2007). Townsend et al. (2007) also conducted a study in which infant rats were administered injections of *Cronobacter* spp. intracranially as to analyze histology and inflammatory response in brains. Numerous points of inflammation, microabscesses, and macrophages were found in the infant rat brains at post-treatment day 9 (Townsend et al., 2007).

To determine how *Cronobacter* spp. associates with and invades host cells, an *in vitro* experiment using Caco-2 cells was conducted by Kim and Loessner (2008). The authors observed that if Caco-2 cell tight junctions were disrupted by EGTA, subsequent *Cronobacter* spp. invasion increased. Increased host cell invasion by *Cronobacter* spp. after tight junction

disruption may be a key factor in understanding the development of infection in human infants (Kim and Loessner, 2008).

Some *Cronobacter* spp. possess a virulence factor called outer membrane protein A (OmpA). Two studies have proven that *Cronobacter* cells expressing this protein more readily invade brain cells (human brain microvascular endothelial cells (HBMEC) and rat brain cells). $OmpA^+$ *Cronobacter* orally administered to rats passed through the intestinal wall, proliferated in systemic circulation, and crossed the BBB (Mittal et al., 2009). The $OmpA^-$ cells did not have such an effect; however, ability to infect the host was reestablished with the insertion of the *ompA* gene (Mittal et al., 2009). OmpA is an integral membrane protein that connects the outer membrane to the underlying peptidoglycan layer, thereby maintaining structure of the cell surface (Koebnik et al., 2000). OmpA assists a bacterial cell in attaching to the surface of a host cell. Singamsetty et al. (2008) reported that *Cronobacter* spp. expressing ompA was able to enter HBMEC *in vitro* and that ompA expression was necessary for its invasiveness. Mohan Nair et al. (2009) found that an $OmpA^+$ *Cronobacter* mutant was significantly more invasive in human brain microvascular endothelial cells (HBMEC) than a wildtype *Cronobacter* strain. Mittal et al. (2009) conducted *in vivo* and *in vitro* studies on the ability *Cronobacter* spp. to invade and cause meningitis in the rat brain. $OmpA^+$ *Cronobacter* spp. was significantly more invasive in animal tissues and significantly more lethal than $OmpA^-$ *Cronobacter* spp. Furthermore, $OmpA^-$ *Cronobacter* cells inserted with ompA plasmids ($pOmpA^+$) regained the ability to invade host cells and cause death (Mittal et al., 2009).

Mittal et. al (2009) also conducted an *in vivo* study in which 2-day-old rats were orally challenged with $OmpA^+$, $OmpA^-$ (10^4 or 10^5 CFU), and $pOmpA^+$ (10^4 CFU) *Cronobacter* spp. At 48 h post-treatment, the blood titer of count of *Cronobacter* cells was significantly lower

(3.25 ± 0.16 $\log_{10}$ CFU/ml, $P < 0.001$) in animals treated with the OmpA$^-$ *Cronobacter* than in those administered OmpA$^+$ (9.31 ± 0.41) and pOmpA$^+$ (9.20 ± 0.38) *Cronobacter* cells. *Cronobacter* spp. invasion occurred in brains of all rat pups treated with the OmpA$^+$ mutant and in none of the pups administered the OmpA$^-$ *Cronobacter* mutant. *Cronobacter* spp. were cultured from cerebrospinal fluid of all pups challenged with the OmpA$^+$ or pOmpA$^+$ *Cronobacter* cells, whereas no *Cronobacter* was isolated from CSF of animals given the OmpA$^-$ mutant. Treatment of neonatal rats with the OmpA$^+$ mutant caused apoptosis in enterocytes, whereas treatment with the OmpA$^-$ cells did not. The OmpA$^+$ mutant proved lethal by 16 h post-treatment for 100% of pups given the 10^5 CFU dose and by 48 h post-treatment for 100% of those administered 10^4 CFU. No mortality was observed in neonatal rats treated with *Cronobacter* spp. lacking the *ompA* gene.

SUMMARY

In summary, *C. sakazakii* infections are newly emerging and not well understood. Many infections can be prevented by diligent and proper hygiene measures. However, the ubiquitous occurrence of *C. sakazakii* will make it difficult to completely remove from our food supply. As our medical knowledge and technology increases, it is likely there will be an increase in susceptible populations. With this increase, there may be more cases resulting from *C. sakazakii* infection, particularly in premature infants and the elderly. Developing animal models for susceptible populations and learning more about the mechanisms by which *C. sakazakii* causes morbidity and mortality will be important to the health of susceptible populations. The following chapters describe our work with *C. sakazakii*. Chapter 3 is a manuscript published in the *Journal of Food Protection* in November 2009 and describes the differences in susceptibility to *C. sakazakii* between three strains of neonatal mice. Chapter 4 was accepted for publication in

January 2010 by the *Journal of Food Protection* and describes the virulence of three strains of *C. sakazakii*. Chapter 5 is a manuscript in preparation describing the effects of age on the susceptibility of neonatal mice to *C. sakazakii* infection. These chapters are followed by a Summary and Conclusion section.

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CHAPTER 3

NEONATAL MICE AS MODELS FOR *CRONOBACTER SAKAZAKII* INFECTION IN INFANTS[1]

[1] **Richardson, A. N.**, S. Lambert, and M. A. Smith. 2009. *Journal of Food Protection*. 72:2363-2367. Reprinted here with permission of publisher.

ABSTRACT

Cronobacter sakazakii is an opportunistic pathogen that has been isolated from powdered infant formulas. *C. sakazakii* infection can result in serious illnesses such as bacteremia, septicemia, meningitis and death in at-risk infants who are orally fed contaminated reconstituted powdered infant formulas. The objective of this study was to compare the susceptibilities of BALB/C, C57BL/6, and CD-1 mice to *C. sakazakii* strain MNW2. We acquired timed-pregnant CD-1 mice and allowed them to give birth naturally. On postnatal day 3.5, each pup was administered a total dose of approximately 10^2 to 10^{11} CFU *C. sakazakii* strain MNW2 in reconstituted powdered infant formula. Mice were observed twice daily for morbidity and mortality. At postnatal day (PND) 10.5, remaining pups were euthanized and brain, liver, and cecum were excised and analyzed for the presence of *C. sakazakii*. *C. sakazakii* was isolated from brains, livers, and ceca in all three mouse strains. The CD-1 mouse strain was the most susceptible of the three, with the lowest infectious dose (10^2 CFU) and the lowest lethal dose (also 10^2 CFU).

Human infants, particularly neonates, low-birth-weight (< 2500 g), premature (< 38 weeks), and/or immunocompromised infants, inadvertently fed reconstituted powdered infant formulas contaminated with *C. sakazakii*, are at-risk for bacteremia (Noriega et al., 1990), septicemia (Lai, 2001b), necrotizing enterocolitis (Van Acker et al., 2001), and meningitis (Burdette and Santos, 2000b, Gallagher and Ball, 1991, Kleiman et al., 1981, Lai, 2001b). Although risk factors for *C. sakazakii* infection have not been identified, infants with low birth weight appear to have greater adverse outcomes (Lai, 2001b). Severe neurological complications are common when meningitis results from infection and the case fatality rate has been estimated to be as high as 50% (Biering et al., 1989, Lai, 2001b). Neonates who recover from infection may endure subsequent morbidities such as hydrocephalus, mental retardation, and developmental delays (Lai, 2001b, Mullane et al., 2006). The bacterium has been cultured and isolated from the cerebrospinal fluid (CSF), blood, and stool of infected newborns (Block et al., 2002, Kleiman et al., 1981, Lai, 2001b, Stoll et al., 2004). Mortality rates from infection are greater among infants who are very young, were born prematurely, or have very low birth weights. It is assumed that this is because these infants have compromised or underdeveloped immune systems. *C. sakazakii* is an opportunistic gram-negative bacillus that has been associated with outbreaks in neonatal intensive care units (NICUs). *C. sakazakii* rarely infects healthy individuals.

Cronobacter was proposed as an alternative classification for *Enterobacter sakazakii* in order to clarify nomenclature for *Enterobacter* spp. (Iversen et al., 2008). *Cronobacter* spp. is found in many places but its reservoir in the natural environment is not known. It has been detected in diverse locations ranging from the gut of stable fly larvae (Hamilton et al., 2003a), to soil (Khan et al., 1998), and to household vacuum-cleaner bags (Kandhai et al., 2004).

Cronobacter spp. has also been isolated from various foods such as vegetables, cheese, minced beef, and sausage meat (Leclercq et al., 2002).

Cronobacter spp. has been been detected in food production environments, suggesting that because it cannot survive pasteurization, it could be introduced to powdered infant formula after the pasteurization process (Kandhai et al., 2004, Lehner and Stephan, 2004a, Nazarowec-White and Farber, 1997c). *Cronobacter* spp. may proliferate in reconstituted powdered infant formula if left at room temperature for extended periods of time (Weir, 2002, Iversen and Forsythe, 2003b). *Cronobacter* spp. was cultured and identified from a sample taken from a blender that was used to mix infant formula (Block et al., 2002). Thus, proper refrigeration, adequate cleaning, and sterilization of equipment and utensils that are used in the preparation of reconstituted powdered infant formula are preventative measures of infection (Bar-Oz et al., 2001).

The risk of *C. sakazakii* infection to human neonates is unknown. Human studies to examine *C. sakazakii* infection are unethical because mortality is a possible outcome for susceptible individuals. Animal surrogate studies are essential for extrapolation of *C. sakazakii* infection in humans. The design of an animal model for *C. sakazakii* infection is fundamental in gaining knowledge of why premature and immunocompromised human infants are at greater risk of infection. Furthermore, an animal model will allow us to test different strains of *C. sakazakii* for virulence.

There are very few reports of animal models for *C. sakazakii* infection. In a study by Pagotto, et al. (Pagotto et al., 2003), 3-4 day-old Swiss Webster mouse pups were exposed to 18 *C. sakazakii* strains orally and intraperitoneally to test bacterial virulence. They found that two of the bacterial strains (MNW2 and SK81) were lethal to mice when administered by oral

gavage. We have previously used animal models for *Listeria monocytogenes* infection using mice (Takeuchi et al., 2006), guinea pigs (Irvin et al., 2008, Williams et al., 2007b) and nonhuman primates (Smith et al., 2008). Because of our previous experience in working with animal models and because the central nervous system in mice is underdeveloped at birth (Clancy et al., 2001) when compared to humans, we tested neonatal mice as an animal model for *C. sakazakii* infection in humans. The objectives of this study were to determine the infectivity and lethality of *C. sakazakii* strain MNW2 in three different strains of neonatal mice after oral exposure and to determine whether neonatal mice could be used as an animal model for *C. sakazakii* infection in premature infants.

MATERIALS AND METHODS

Animals. Timed-pregnant mice of the BALB/C, C57BL/6, and CD-1 strains were acquired at gestation day (GD) 15 from Charles River Laboratories (Wilmington, Massachusetts). Mice were housed individually in shoebox cages in a room with a 12h:12h light/dark cycle. Dams were acclimatized and allowed to give birth naturally (GD 19 or 20). Neonates were treated orally by gavage on postnatal day (PND) 3.5 using a 24 x 1'' W/1-1¼ stainless steel animal feeding needle (Popper & Sons, Inc., New Hyde Park, New York) attached to a 1 ml syringe.

Serial dilutions of reconstituted powdered infant formula inoculated with various concentrations of *C. sakazakii* strain MNW2 were prepared. All neonates received a total volume of 0.1 ml of the inocula or vehicle control (powdered infant formula). Confirmed administered doses per experimental animal were 10^2 to 10^{11} CFU. Suckling mice were observed twice daily for morbidity or mortality. We defined morbidity as noticeable lethargy and/or change of skin color from pink to blue or gray. Neonates were sacrificed at PND 10.5 and

weights and lengths were recorded. Liver, brain, and cecum tissues were excised and cultured for *C. sakazakii* from the neonates.

All animal work was done in full compliance with federal regulations including the Animal Welfare Act and was approved by the Institutional Animal Care and Use Committee (IACUC). The University of Georgia is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Culture preparation. Stock cultures of *C. sakazakii* strain MNW2 were stored on ceramic beads at -80°C. Bacteria were grown to high concentrations in tryptic soy broth (Oxoid, LTD, Basingstoke, Hampshire, England). Cells were transferred twice in the four-day period. The inoculated medium was incubated for 18-24 hours at 37°C. Cell suspensions were concentrated on day 4 to obtain the high concentrations needed for treatments. Bacteria were centrifuged at 2500 to 3600 × *g*, washed with sterile deionized water, and resuspended during serial dilution in reconstituted powdered infant formula. The powdered infant formula used was milk-based and iron-fortified. We reconstituted the powdered infant formula in the appropriate volume of sterile deionized water according to the manufacturer's instructions for preparation. Serial dilutions were continued in deionized water and 0.1 ml of each dilution was spread plated in duplicate onto tryptic soy agar (TSA) (Oxoid) for dose confirmation. Plates were incubated for 18-24 hours at 37°C and administered *C. sakazakii* concentrations were confirmed by colony enumeration. Bacterial concentrations were averaged within 2 log CFU (Tables 1-5).

Tissue preparation and analysis. After excision, the tissues of three to five pups were pooled into groups within litters because of the small tissue size and to increase the likelihood of *C. sakazakii* isolation from the samples. The results in Tables 1-3 are reported for each litter based on whether *C. sakazakii* was isolated from the pooled tissue. The pooled tissues were

homogenized and macerated in *Enterobacteriaceae* enrichment (EE) broth (Oxoid). Homogenized tissues were incubated at 37°C for 18-24h. Tissues were streaked onto Violet Red Bile Glucose (VRBG) agar plates in duplicate for *Enterobacter* spp. isolation. Plates were incubated for 18-24h at 37°C. Growths on VRBG plates were streaked onto TSA and incubated overnight. RapID One Identification System (Remel, Inc., Lenexa, Kansas, USA) was utilized for positive identification of *C. sakazakii*.

Statistics. All statistical analysis in this study was done using SAS version 9 (SAS Institute, Cary, NC). One-way analysis of variance (ANOVA) tests using Dunnett's *t*-test were conducted to determine which treatment groups were significantly different from the control groups ($P < 0.05$). A Duncan's *F*-test was done in SAS to determine significant differences in *C. sakazakii*-related deaths between the three mouse strains.

RESULTS

C. sakazakii crossed the intestinal barrier and entered other tissues in all three mouse strains tested. Seven days after treating 3.5 day old neonatal mice by oral gavage, *C. sakazakii* was isolated from liver, brain and cecum of BALB/C, C57BL/6 and CD-1 mouse strains. In addition to these three mouse strains, we initially tested mice of the A/J strain. Timed-pregnant A/J mice were difficult to obtain from our supplier in sufficient numbers to carry out the experiment, and our preliminary data suggested that this mouse strain was no more susceptible than the other three strains. Therefore, we did not continue our investigation into the susceptibility of the A/J mouse strain.

Animal weights and lengths. When weights and lengths of neonatal pups were compared after treatment with *C. sakazakii,* there were some differences between treated and control groups. However, these differences were not dose dependent except for weights of

BALB/C pups (Table 1). In the CD-1 mouse strain, all significant differences in body weights and lengths occurred at doses in which *C. sakazakii* was also isolated from brain, liver, and cecum.

Isolation of *C. sakazakii* from brain. Because meningitis is one possible outcome of neonatal *C. sakazakii* infection in humans, we investigated the invasion of *C. sakazakii* in brain tissues of neonatal mice. There were no overt signs of meningitis in the mouse pups. *C. sakazakii* was isolated from the brains of CD-1 mouse pups at every administered dose level and was the only strain from which *C. sakazakii* was isolated after treatment with $10^{2.3}$ CFU/animal *C. sakazakii* (Table 2). The lowest dose at which *C. sakazakii* was isolated from BALB/C brains was $10^{8.5}$ CFU/animal, whereas the lowest infectious dose of C57BL/6 was $10^{4.2}$ CFU/animal. *C. sakazakii* was isolated in brain tissues from a maximum of 75% of litters in all three mouse strains, occurring at $10^{10.7}$ CFU/animal in BALB/C and CD-1 neonates and at $10^{8.5}$ CFU/animal in C57BL/6 neonates. There was no *C. sakazakii* isolated from any of the brain tissues from the control animals.

Isolation of *C. sakazakii* from liver. *C. sakazakii* was isolated from liver of all three strains of mice at treatment doses greater than $10^{2.3}$ CFU/animal. However, isolation of *C. sakazakii* was not always dose-dependent. Treatments for BALB/C and C57BL/6 mice were not significantly different from their controls but this may have resulted from the small number of litters examined (Table 3). *C. sakazakii* was isolated from 100% (4/4) of the CD-1 litters administered $10^{10.7}$ CFU/animal. *C. sakazakii* was isolated from livers of experimental BALB/C and CD-1 mice at all doses administered.

Isolation of *C. sakazakii* from cecum. To determine whether *C. sakazakii* colonized the intestine of neonatal mice, we isolated *C. sakazakii* from the cecum. *C. sakazakii* was only

isolated from ceca of BALB/C neonates at $10^{10.7}$ CFU/animal (Table 4). Because there was no evident dose-dependency, we added all doses together to compare isolation of *C. sakazakii* regardless of dose. In all three mouse strains, *C. sakazakii* was isolated from ceca of 16-17% of litters.

***C. sakazakii*-related deaths.** There was no significant difference between CD-1, BALB/C, and C57BL/6 mice in *C. sakazakii*-related deaths among individual neonates (Table 5). CD-1 mice had the lowest dose at which mortality occurred and the lowest infectious dose for all tissues of all the mouse strains with $10^{2.3}$ CFU/animal. This cell concentration is 5 log CFU below the lowest lethal dose given to neonatal mice by oral gavage in the study by Pagotto et al. (Pagotto et al., 2003). $10^{4.2}$ CFU/animal was the lowest infectious dose (LID) administered that we were able to isolate *C. sakazakii* from all tissues and the lowest lethal dose in the C57BL/6 mouse strain. BALB/C mice were the least susceptible to *C. sakazakii*, with an LID of $10^{8.5}$ CFU/animal in brains and livers and $10^{10.7}$ CFU/animal in ceca, and a lowest lethal dose of $10^{6.3}$ CFU/animal.

DISCUSSION

C. sakazakii infection in premature infants is rare but often fatal. When the infection is not fatal, it can result in severe morbidity and lifetime disabilities such as meningitis, hydrocephaly, and mental retardation (Lai, 2001b, Biering et al., 1989). Developing an animal model as a surrogate for human prematurity is an important step forward, enabling subsequent research into understanding the mechanisms of infection, morbidity, prevention, and treatments. There are only two published papers testing *C. sakazakii* animal models (Townsend et al., 2007a, Pagotto et al., 2003). We decided to test neonatal mice as surrogates for *C. sakazakii* infection in

premature infants because the nervous system is much less mature at birth when compared to humans (Clancy et al., 2001).

Lowest infectious dose, mortality and isolation of *C. sakazakii* from examined tissues were all factors in determining the susceptibilities of the three mouse strains in this study. From our observations, the CD-1 mouse strain was the most susceptible of the three mouse strains to *C. sakazakii* infection based on the lowest infectious dose for tissues and the lowest dose to result in mortality. The lowest dose that resulted in our isolation of *C. sakazakii* from brains, livers, and ceca in this mouse strain was $10^{2.3}$ CFU/animal. The lowest dose at which death resulted in the CD-1 mice was also $10^{2.3}$ CFU/animal. In the C57BL/6 mouse strain, $10^{4.2}$ CFU/animal was the lowest infectious dose for all tissues and the lowest dose at which *C. sakazakii*-related death occurred. The lowest infectious dose for the BALB/C neonates was $10^{8.5}$ CFU/animal in brains and livers and $10^{10.7}$ CFU/animal in ceca. The lowest lethal dose for the BALB/C mice was $10^{6.3}$ CFU/animal. Although body weights and lengths were not indicators of infection, weights and lengths of experimental animals that differed significantly from the controls all occurred at doses at which *C. sakazakii* was isolated from all three tissues (brain, liver, and cecum) in the CD-1 mouse strain.

It is interesting that we isolated *C. sakazakii* from fewer ceca than other tissues examined. This may have been the result of the time of tissue collection that was 7 days post treatment. *C. sakazakii* could have been present in higher concentrations early in the post-treatment phase and the cells could have died over time. The immune responses of the neonatal mice could have killed the *C. sakazakii* or the *C. sakazakii* could have been competitively excluded by other bacterial species that colonized the neonatal gut during the post-treatment period.

There was not a clear dose-dependent response after treatment with *C. sakazakii*. There are several possible explanations for this. For example, these are neonates that are housed with littermates and with their birth mother. It is possible that through contact with the mother and with other littermates, they may have been exposed to additional amounts of *C. sakazakii* (through licking and coprophagy). The endpoint with the clearest dose-dependent effect was mortality. Although the rate of mortality was only 35% at the highest dose tested.

Our objective for this study was to determine which mouse strain was most susceptible to *C. sakazakii* infection. Our results indicate that the CD-1 mouse strain was the most susceptible to *C. sakazakii* infection compared to BALB/C and C57BL/6, with the lowest infectious dose in brain, liver, and cecum, and the lowest lethal dose at $10^{2.3}$ CFU/animal. Our studies also show that *C. sakazakii* can invade the brain tissues of neonatal mice after oral exposure. This is important because meningitis, hydrocephaly, and other neurological sequelae are known to occur in human infants as a result of *C. sakazakii* infection. In conclusion, our findings show that *C. sakazakii* can reach the brains of mice, and this animal species may serve as an appropriate model for *C. sakazakii* infection in humans.

ACKNOWLEDGEMENTS

Funding for this research project was provided by the International Life Sciences Institute of North America (ILSI-NA). The Alfred P. Sloan Foundation and the Interdisciplinary Toxicology Program are acknowledged for providing a graduate stipend (ANR) and the College of Public Health and the College of Agricultural and Environmental Sciences for salary support (MAS). We would also like to thank Dr. Larry Beuchat for providing the *C. sakazakii* strain MNW2.

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Table 3.1: Individual neonatal weights (g) and lengths (cm) after oral exposure to **C. sakazakii**

Confirmed Dose (CFU/animal)	Weights (g)			Lengths (cm)		
	BALB/C [a] Mean ± SD (N=number of neonates)	**C57BL/6 [a] Mean ± SD (N=number of neonates)**	**CD-1 [a] Mean ± SD (N=number of neonates)**	**BALB/C [a] Mean ± SD (N=number of neonates)**	**C57BL/6 [a] Mean ± SD (N=number of neonates)**	**CD-1 [a] Mean ± SD (N=number of neonates)**
Control	6.10 ± 0.63 A (N=12)	5.15 ± 0.89 A (N=33)	5.72 ± 0.94 A (N=75)	3.29 ± 0.39 A (N=12)	2.92 ± 0.25 A (N=33)	5.89 ± 0.38 A (N=75)
$10^{2.3}$	ND	4.18 ± 0.94 B (N=18)	6.05 ± 0.82 B (N=50)	ND	2.85 ± 0.24 B (N=18)	6.01 ± 0.32 A (N=50)
$10^{4.2}$	6.49 ± 0.54 A (N=16)	4.94 ± 0.49 A (N=27)	5.83 ± 0.67 A (N=73)	3.84 ± 0.15 B (N=16)	3.04 ± 0.14 A (N=27)	5.71 ± 0.53 B (N=73)
$10^{6.3}$	6.03 ± 0.38 A (N=17)	4.13 ± 0.54 B (N=20)	5.53 ± 0.85 A (N=81)	3.47 ± 0.28 A (N=17)	2.27 ± 0.18 B (N=20)	5.79 ± 0.43 A (N=81)
$10^{8.5}$	5.41 ± 0.46 B (N=25)	5.47 ± 0.46 A (N=25)	6.08 ± 0.75 B (N=139)	3.10 ± 0.20 A (N=25)	2.73 ± 0.30 A (N=25)	5.94 ± 0.35 A (N=139)
$10^{10.7}$	5.31 ± 0.55 B (N=24)	4.93 ± 0.50 A (N=32)	5.52 ± 0.20 A (N=24)	2.95 ± 0.18 B (N=24)	3.07 ± 0.31 A (N=32)	5.88 ± 0.22 A (N=24)

[a]Groups with different letters are significantly different within columns ($P < 0.05$).

ND: not done

Table 3.2: Isolation of C. sakazakii from neonatal mouse brain 7 days after oral exposure to C. sakazakii-contaminated infant formula

Confirmed Dose (CFU/animal)	BALB/C % *C. sakazakii* positive[a, b]	C57BL/6 % *C. sakazakii* positive [a, b]	CD-1 % *C. sakazakii* positive [a, b]
Control	0% (0/3)	0% (0/5)	0% (0/8)
$10^{2.3}$	ND	0% (0/3)	37% (3/8)
$10^{4.2}$	0% (0/3)	40% (2/5)	71% (5/7)*
$10^{6.3}$	0% (0/4)	50% (1/2)	20% (2/10)
$10^{8.5}$	40% (2/5)	75% (3/4)	43% (6/14)
$10^{10.7}$	75% (3/4)*	25% (1/4)	75% (3/4)
Total Isolation	26% (5/19)	30% (7/23)	37% (19/51)

[a] Denotes percentage of litters out of the total number of litters that were positive for *C. sakazakii*.

[b] Experimental groups with an asterisk (*) are significantly different from the control group of the same mouse strain ($P < 0.05$).

ND: not done

***Table 3.3: Isolation of* C. sakazakii *from neonatal mouse liver 7 days after oral exposure to C. sakazakii-*contaminated infant formula**

Confirmed Dose (CFU/animal)	BALB/C % *C. sakazakii* positive [a, b]	C57BL/6 % *C. sakazakii* positive [a, b]	CD-1 % *C. sakazakii* positive [a, b]
Control	0% (0/3)	0% (0/5)	0% (0/8)
$\mathbf{10^{2.3}}$	ND	0% (0/3)	37% (3/8)
$\mathbf{10^{4.2}}$	33% (1/3)	20% (1/5)	43% (3/7)
$\mathbf{10^{6.3}}$	25% (1/4)	50% (1/2)	20% (2/10)
$\mathbf{10^{8.5}}$	40% (2/5)	75% (3/4)	36% (5/14)
$\mathbf{10^{10.7}}$	50% (2/4)	50% (2/4)	100% (4/4)*
Total Isolation	31% (6/19)	30% (7/23)	33% (17/51)

[a] Denotes percentage of litters out of the total number of litters positive for *C. sakazakii*.

[b] Experimental groups with an asterisk (*) are significantly different from the control group of the same mouse strain ($P < 0.05$).

ND: not done

Table 3.4: Isolation of* C. sakazakii *from neonatal mouse cecum 7 days after oral exposure to C. sakazakii-contaminated infant formula

Confirmed Dose (CFU/animal)	BALB/C % *C. sakazakii* positive [a, b]	C57BL/6 % *C. sakazakii* positive [a, b]	CD-1 % *C. sakazakii* positive [a, b]
Control	0% (0/3)	0% (0/5)	0% (0/8)
$\mathbf{10^{2.3}}$	ND	0% (0/3)	37% (3/8)
$\mathbf{10^{4.2}}$	0% (0/3)	20% (1/5)	29% (2/7)
$\mathbf{10^{6.3}}$	0% (0/4)	50% (1/2)	0% (0/10)
$\mathbf{10^{8.5}}$	0% (0/5)	50% (2/4)	14% (2/14)
$\mathbf{10^{10.7}}$	75% (3/4)*	0% (0/4)	25% (1/4)
Total Isolation	16% (3/19)	17% (4/23)	16% (8/51)

[a] Denotes percentage of litters out of the total number of litters with at least one positive for *C. sakazakii*.

[b] Experimental groups with an asterisk (*) are significantly different from the control group of the same mouse strain ($P < 0.05$).

ND: not done

Table 3.5: Percentages of **C. sakazakii**-*related deaths among individual neonates*

Confirmed Dose (CFU/animal)	BALB/C (# deaths/total # neonates)	C57BL/6 (# deaths/total # neonates)	CD-1 (# deaths/total # neonates)
Control	0% (0/12)	0% (0/33)	0% (0/75)
$10^{2.3}$	ND	0% (0/18)	2% (1/51)
$10^{4.2}$	0% (0/16)	4% (1/28)	1% (1/74)
$10^{6.3}$	19% (4/21)	0% (0/20)	2% (2/83)
$10^{8.5}$	7% (2/27)	0% (0/25)	7% (10/149)
$10^{10.7}$	4% (1/25)	0% (0/32)	35% (15/43)
Total Death	7% (7/101)	0.6% (1/156)	6.1% (29/475)

ND: not done

CHAPTER 4

COMPARISON OF VIRULENCE OF THREE STRAINS OF *CRONOBACTER SAKAZAKII* IN NEONATAL CD-1 MICE[2]

[2] **Richardson, A. N.**, L. R. Beuchat, S. Lambert, D. Williams, and M. A. Smith. Accepted by *Journal of Food Protection.* Reprinted here with permission of publisher, 3/8/2010.

ABSTRACT

Cronobacter sakazakii (*Enterobacter sakazakii)* is an emerging pathogen that has been isolated from powdered infant formula and associated with outbreaks of infection in infants in neonatal intensive care units. In a previous study, we observed that neonatal CD-1 mice are susceptible to *C. sakazakii* infection and that the pathogen invades brain, liver, and cecum tissues. The study objective was to compare the virulence of three strains of *C. sakazakii* in neonatal CD-1 mice. The strains tested were MNW2 (a food isolate), SK81 (a clinical isolate), and 3290 (a clinical isolate). Timed-pregnant CD-1 mice were allowed to give birth on gestation day 19 or 20. Neonatal mice were sexed and culled to 10 per litter, each having five males and five females. Neonates were orally gavaged with *C. sakazakii* strains MNW2, SK81, or 3290 at doses ranging from $10^{2.8}$ – $10^{10.5}$ CFU on postnatal day (PND) 3.5. Pups surviving to PND 10.5 were sacrificed and brain, liver, and cecum tissues were excised. *C. sakazakii* was isolated from all three tissues in mice treated with *C. sakazakii*, regardless of strain. *C. sakazakii* strain 3290 was significantly more invasive in brains (42.1% of mice) than were strains MNW2 (6.7%) and SK81 (15.9%). Mortality was observed for all strains of *C. sakazakii* tested, with SK81 being significantly more lethal (5.6%) than MNW2 (1.2%) or 3290 (0.6%). Our findings suggest that invasiveness does not necessarily correlate with mortality among different strains of *C. sakazakii*, and the clinical isolates are more virulent than the food isolate.

Cronobacter sakazakii (*Enterobacter sakazakii)* is an opportunistic pathogen associated largely with outbreaks of infection in infants (Bowen and Braden, 2006, Iversen and Forsythe, 2003b, Lehner and Stephan, 2004a, Townsend et al., 2008, Van Acker et al., 2001, WHO/FAO, 2007, WHO/FAO, 2008), although it also can cause illness in adults (Dennison and Morris, 2002, Iversen and Forsythe, 2003b, Lai, 2001b). It is a ubiquitous organism that is known to contaminate powdered infant formula and other foods (Beuchat et al., 2009, Friedemann, 2007, Gurtler et al., 2005, Iversen and Forsythe, 2003b). Improper handling of reconstituted powdered infant formula has been attributed to causing cases of *C. sakazakii* infection (Noriega et al., 1990, WHO/FAO, 2007). *E. sakazakii* has been recovered from various foods including minced beef, sausage meat, lettuce (Soriano et al., 2001) and other vegetables (Leclercq et al., 2002), herbs and spices (Leclercq et al., 2002, Iversen and Forsythe, 2004), and cheese (Leclercq et al., 2002). Whether all of these isolates are actually *C. sakazakii* using current taxonomic schemes (Healy et al., 2009, Iversen et al., 2008, Kuhnert et al., 2009) is not known. *C. sakazakii* has been detected in food production facilities that manufacture powdered infant formula (Mullane et al., 2008, Kandhai et al., 2004, Shaker et al., 2007), but the point at which powdered infant formula may become contaminated is not known. It is assumed that contamination occurs after dry production processes because *C. sakazakii* cannot survive these processes (Nazarowec-White and Farber, 1997c). The potential for contamination in wet production processes would appear to be greater.

C. sakazakii infection rarely occurs but can have severe, life-threatening outcomes. Infants who have predisposed conditions such as prematurity, low-birth weight, or infants exposed to poor hygienic practices in neonatal intensive care units are at increased risk for *C. sakazakii* infection. Outbreaks have occurred in hospital neonatal intensive care units in several

countries (Caubilla-Barron et al., 2007, Arseni et al., 1987, Van Acker et al., 2001). Infants who are fed rehydrated *C. sakazakii*-contaminated formula via bottles or enteral feeding tubes can develop illnesses such as necrotizing enterocolitis (Van Acker et al., 2001), bacteremia (Noriega et al., 1990), meningitis (Kleiman et al., 1981, Gallagher and Ball, 1991, Burdette and Santos, 2000b, Lai, 2001b), and septicemia (Lai, 2001b). The prognosis for survival of infected human neonates is poor (Mullane et al., 2006), with a case fatality rate ranging from 40 to 80% (Bowen and Braden, 2006, Nazarowec-White and Farber, 1997a, WHO/FAO, 2007, Willis and Robinson, 1988). Infants who survive the infection frequently suffer developmental delays, hydrocephaly, mental retardation, and other neurological sequelae (Drudy et al., 2006a, Lai, 2001b).

The mechanism by which *C. sakazakii* infects human infants is not clearly understood. Animal models using neonatal mice could provide a means to better understand how *C. sakazakii* causes illness. Previous studies have shown that neonatal mice are susceptible to *C. sakazakii* infection through oral and intraperitoneal routes. A study was conducted by Pagotto et al. (Pagotto et al., 2003) in which 18 strains of *C. sakazakii* were administered to 3- to 4-day-old Swiss Webster mice via oral gavage. Pups were observed for mortality following treatment with the pathogen at populations of 10^5, 10^7, and 10^8 CFU. Two strains of *C. sakazakii*, MNW2 and SK81, were lethal at populations of 10^7 and 10^8 CFU (Pagotto et al., 2003). In a previous study, we examined the susceptibilities of CD-1, C57BL/6, and BALB/C neonatal mice to *C. sakazakii* strain MNW2 infection administered by oral gavage (Richardson et al., 2009). Pups were treated with *C. sakazakii* at populations of 10^2 - 10^{12} CFU at 3.5 day of age. The CD-1 mouse strain was the most susceptible; therefore, we used the CD-1 mouse strain for subsequent experiments.

A comparison of different strains of *C. sakazakii* isolated from different sources would be helpful to determine which are more virulent and more likely to cause morbidity and/or mortality

in premature human infants. In our present study, a range of populations of *C. sakazakii* was administered to neonatal mice via the oral route. The objective was to compare the virulence of *C. sakazakii* strains MNW2 (food isolate), SK81 (clinical isolate), and 3290 (clinical isolate) upon oral gavage of 3.5-day-old CD-1 mice. The results of these studies provide more information on the acceptability of neonatal mice as models for *C. sakazakii* infection in infants, and could lead to the development of therapies for treatment of infections and mitigation of subsequent health complications in human infants.

MATERIALS AND METHODS

Source and maintenance of mice. We acquired timed-pregnant mice of the CD-1 strain from Charles River Laboratories (Wilmington, Mass.) at gestation day (GD) 15. Dams were housed individually in shoebox cages and kept in a room with a 12 h:12 h light/dark cycle. Mice received drinking water and rodent chow *ad libitum*, and were acclimatized and permitted to give birth naturally (GD 19 or 20). Newborn pups were sexed and randomly assigned to foster mothers. Each litter of 10 pups consisted of five males and five females.

Source and preparation of inocula. *C. sakazakii* strains MNW2 (HPB 2871; isolated from a commercially manufactured powdered infant formula [*31*]), SK81 (HPB 2855; clinical isolate [*31*], and 3290 (clinical isolate from infected infant) were used. At the initiation of our study, these strains were classified as *E. sakazakii*; however, all three strains have been subsequently designated as *C. sakazakii* using current reclassification schemes (Iversen et al., 2008). Strains MNW2 and SK81 have been reported to cause death of mice by the peroral route (Pagotto and Farber, 2009, Pagotto et al., 2003). The level of virulence of strain 3290 is not known. Cultures were stored on ceramic beads at -80°C. To prepare inoculum, *C. sakazakii* was grown in tryptic soy broth (TSB; Oxoid, Ltd., Basingstoke, Hampshire, England). Cells were

transferred into TSB on days 2 and 3 of growth. The inoculated TSB was incubated for 18 - 24 h at 37°C. Cells were collected by centrifugation (2,500 - 3,600 x *g* for 10 - 15 min until there was a firm pellet) on day 4, washed with sterile deionized water, and resuspended in a commercially manufactured milk-based, iron-fortified powdered infant formula which was reconstituted in sterile deionized water. The number of *C. sakazakii* in the inoculated formula was determined by serially diluting the formula in sterile deionized water and spread plating duplicate 0.1-ml samples onto tryptic soy agar (TSA) (Oxoid). Plates were incubated for 18 - 24 h at 37°C and colonies formed by *C. sakazakii* were counted. To compare a range of treatment doses, inoculated reconstituted formula was diluted in formula to result in *C. sakazakii* populations at approximately 2-log CFU/ml increments in the range of approximately 10^4 - 10^{12} CFU/ml.

Treatment of mice. On the day of treatment, the nose (snout) of each neonate and mother was swabbed with vanilla flavoring (The Kroger Co., Cincinnatti, Ohio) in order to mask scents and create olfactory confusion, thereby gaining neonatal acceptance by the foster mother. Neonates were treated by oral gavage on postnatal day (PND) 3.5 using a 24 x 1'' (25.4 mm) W/1-1¼ stainless steel animal feeding needle (Popper & Sons, Inc., New Hyde Park, N.Y.) attached to 1-ml syringe.

A suspension of *C. sakazakii* prepared as described above was serially diluted in reconstituted powdered infant formula to obtain the needed populations for treatment of mice. Each neonate was administered 0.1 ml of inoculated reconstituted powdered infant formula or reconstituted powdered infant formula containing no *C. sakazakii* as a vehicle control. The confirmed administered doses ranged from approximately 10^3 to 10^{11} CFU per experimental animal. Pups were observed for mortality at 12-h intervals. On PND 10.5, viable pups were

euthanized and necropsied, and tissues were collected. Pup weights and lengths were recorded and liver, brain, and cecum tissues were excised and analyzed for *C. sakazakii*. For mice succumbing before sacrifice day, tissues were not examined for presence of *C. sakazakii*.

All mouse work was done in full compliance with federal regulations including the Animal Welfare Act and was approved by the Institutional Animal Care and Use Committee (IACUC). The University of Georgia is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Tissue preparation and analysis. Tissue analyses were previously described (Richardson et al., 2009). Briefly, individual tissues were homogenized and macerated in Enterobacteriaceae enrichment (EE) broth (Oxoid). Tissue suspensions were incubated at 37°C for 18 - 24 h, then streaked onto violet red bile glucose (VRBG) agar (Oxoid). VRBG plates were incubated at 37°C for 18 – 24 h. Cells from presumptive-positive colonies of *C. sakazakii* were subcultured on TSA and incubated 18 - 24 h at 37°C. The RapID One Identification System (Remel, Inc., Lenexa, Kansas) was used to confirm *C. sakazakii* by biochemical reactions.

Statistical analyses. All statistical analyses were done using SAS version 9.1 (SAS Institute, Cary, N.C.). Scheffe's tests were used to determine significant differences ($P \leq 0.05$) in values among the three *C. sakazakii* strains in treated mice. One-way analysis of variance (ANOVA) tests were done using Dunnett's *t*-test to determine if treatment groups were significantly ($P \leq 0.05$) different among the control group and groups administered each *C. sakazakii* strain.

RESULTS

We examined the invasion of *C. sakazakii* in brain and liver tissues in inoculated neonatal mice to compare the level of virulence among strains of *C. sakazakii* isolated from one food and two clinical samples. Cecum tissues were examined to determine if *C. sakazakii* colonized the gastrointestinal tract, particularly in mice showing systemic invasion of other tissues. Because there was no detectable dose dependency among treated groups, the data from all treatment groups were combined and compared to the control group. Mortality was noted for any mouse dying before the day of scheduled sacrifice.

Isolation of *C. sakazakii* from brain tissue. Among the three strains tested, *C. sakazakii* strain 3290 was the most invasive strain. Regardless of dose, 42% of brains from mice treated with this strain were positive for the pathogen as compared to 6.7% for strain MNW2 and 15.9% for strain SK81 (Table 1). Strain 3290 was the only strain that was isolated from more than 21% of brains; 55-61% of brains from mice administered $10^{4.8}$-$10^{8.5}$ CFU of strain 3290 were positive for the pathogen. When comparing neonates treated with the three strains of *C. sakazakii*, there was no significant difference ($P > 0.05$) between the mean values for total isolations (%) from brains of mice dosed with strains MNW2 and SK81 (Table 1). However, isolation of *C. sakazakii* from the brains of pups administered strain 3290 was significantly greater than isolations from mice given strains MNW2 or SK81 (Table 1).

The three *C. sakazakii* strains varied in their ability to invade the brain of the neonatal mice. Of the pups treated with strain MNW2, the percent of pups from which *C. sakazakii* was isolated from brain tissue was low (≤ 10%) and there were no significant differences between any of the treatment groups and the control. In contrast, mice administered strain SK81 at $10^{4.8}$ or $10^{8.5}$ CFU showed significantly greater percentages (21 and 15%, respectively) of brains

containing *C. sakazakii* as compared to mice in the control and other treatment groups. For neonatal mice treated with strain 3290, the only treatment group that did not have a significantly higher percentage of mice with brains infected with *C. sakazakii*, compared to the control, was the low-dose group ($10^{2.8}$ CFU/mouse).

Isolation of *C. sakazakii* from liver tissue. When comparing values for total isolation of *C. sakazakii* from liver tissues, there was a significantly greater ($P \leq 0.05$) recovery from mice administered strain 3290 (39.6%) than from mice treated with strains MNW2 (6.1%) or SK81 (7.9%) (Table 2). Strain 3290 was recovered from at least 50% of the liver samples from mice treated with $10^{4.8}$ - $10^{8.5}$ CFU.

Among pups treated with *C. sakazakii* strain MNW2, there was no significant difference ($P > 0.05$) between treatments and the control in the percent of liver tissues positive for the pathogen (Table 2). The highest percentage (16%) of tissues from which *C. sakazakii* strain SK81 was recovered was in mice inoculated $10^{4.8}$ CFU. Isolation of *C. sakazakii* from liver tissues of neonatal mice treated with strain 3290 was significantly greater ($P \leq 0.05$) than from the control group at all doses except the lowest dose tested ($10^{2.8}$ CFU/mouse).

Isolation of *C. sakazakii* from cecum tissue. As for the brain and liver tissues, the total isolation of *C. sakazakii* strain 3290 from the cecum was significantly ($P \leq 0.05$) greater than that of strains MNW2 and SK81 (Table 3). There were no significant differences ($P > 0.05$) in the percentage of cecum tissue samples from the control group and any treatment groups for mice that were administered strains MNW2 or SK81. Of the mouse pups treated with strain 3290, those administered $10^{2.8}$ CFU were the only experimental group in which the percent of positive samples was not significantly higher than that of the control.

Mortality. Although mortality was not an experimental endpoint, all mortalities occurring before the scheduled sacrifice were noted. The highest mortality (5.6%) occurred in neonatal mice administered *C. sakazakii* strain SK81, which was significantly higher ($P \leq 0.05$) than that of pups treated with strains MNW2 (1.2%) or 3290 (0.6%) (Table 4). Mortality was not significantly affected by dose. Invasion of brain, liver, or cecum tissues did not appear to correlate with mortality for any of the test strains of *C. sakazakii*. However, to investigate the possibility that invasion data were underestimated due to mortality, the number of mice affected (as determined by mortality or infection) in both Tables 1 and 4 were combined. There were still more infected mice after inoculation with strain 3290 (68/160) as compared to strain SK81 (33/160), despite the higher mortality rate caused by SK81.

DISCUSSION

The objective for this study was to compare the virulence of *C. sakazakii* strains MNW2, SK81, and 3290 when administered by oral gavage to neonatal CD-1 mice. All three strains of *C. sakazakii* were able to invade neonatal mouse brain, liver, and cecum tissues. We observed that *C. sakazakii* strains MNW2, SK81, and 3290 also caused some mortality in the pups. In comparing the presence of *C. sakazakii* in excised tissues and percent mortality, our data suggest that *C. sakazakii* invasiveness in host tissues of neonatal CD-1 mice does not always result in death.

It is interesting that neonates given *C. sakazakii* strain SK81 had the highest total percent mortality among all the groups of treated mice. Mortality did not exceed 0.6% and 1.2% for mice administered strains 3290 and MNW2, respectively. Total mortality was low (5.6%) in mice given strain SK81, with the highest mortality (13%) in the group receiving the highest test dose ($10^{8.5}$ CFU/mouse). Strain 3290 was consistently the most invasive of the three strains for

brain, liver, and cecum tissues. It is tempting to conclude that the invasiveness and lethality of *C. sakazakii* strains SK81 and 3290 in our neonatal mouse model may be correlated to their source of isolation, i.e., they were isolates from infected patients; however, further testing is needed to verify this correlation.

Pagotto et al. (2003) tested the virulence of *C. sakazakii* at oral doses of 10^5, 10^7, and 10^8 CFU/ neonatal mouse. Mortality occurred at 10^7 and 10^8 CFU/mouse administered strains SK81 and MNW2 (Pagotto et al., 2003). The level of virulence of strain 3290 is not known. In our study, we wanted to test invasiveness in mouse pups treated with lower numbers of *C. sakazakii* via oral gavage. We observed mortality in mouse pups treated with as low as $10^{2.8}$ CFU of strains MNW2 and SK81, suggesting that ingestion of low doses of *C. sakazakii* by mice can in fact lead to infection and subsequent adverse outcomes. It is somewhat a paradox that invasion of brain and liver tissues at ≥50% did not necessarily predict a high mortality rate at that dose.

Newborns and infants with preexisting conditions, such as prematurity at birth, are at increased risk for *C. sakazakii* infection (Gurtler et al., 2005, Iversen and Forsythe, 2003b, Lai, 2001b). *C. sakazakii* infection is a rare occurrence but can lead to serious illnesses such as bacteremia, meningitis, and septicemia, and cause mortality. Individuals who survive *C. sakazakii* infection may develop a number of morbidities including hydrocephaly, neurological disorders, and mental retardation (Drudy et al., 2006a, Iversen and Forsythe, 2003b, Lai, 2001b). Currently, there is limited information available about the mechanisms *C. sakazakii* uses to invade and infect host cells. However, recent findings suggest that the ability of *C. sakazakii* to invade host tissues is dependent on the presence of the outer membrane protein A (ompA) gene (Singamsetty et al., 2008, Mohan Nair et al., 2009, Mittal et al., 2009). We do not know if the strains of *C. sakazakii* used in our study have the ompA gene. A recent study showed that a

specific sequence type may represent a particularly virulent grouping of *C. sakazakii* (Baldwin et al., 2009). Sequence typing of the three strains of *C. sakazakii* used in our study may provide valuable insights to enable more meaningful comparisons of virulence and mortality versus source of isolation.

In summary, our data show that *C. sakazakii* strains MNW2, SK81, and 3290, when administered via oral gravage, are able to invade brain, liver, and cecum tissues of neonatal mice, and can be recovered from these tissues 7 days post-treatment. Overall, *C. sakazakii* was isolated at higher frequencies from brain and liver tissues than from cecum tissues. All test strains were able to cause death in the mouse pups; however, treatment with *C. sakazakii* strain SK81 led to significantly higher mortality than did treatment with strains MNW2 or 3290.

Only a few animal models testing the virulence and mortality of *C. sakazakii* have been published (Townsend et al., 2007b, Townsend et al., 2007c, Pagotto et al., 2003). It is imperative that more studies be conducted in order to better understand the development of *C. sakazakii* infection and the adverse affects it can have on human infants. Neonatal mice show promise for use as surrogates for *C. sakazakii* infection in at-risk infants because they are susceptible to *C. sakazakii* invasion and their immune and nervous systems are not as developed at birth as those of humans (Clancy et al., 2001). Animal models provide the opportunity to develop and test medicines, and therapies can be developed as knowledge concerning mechanisms of *C. sakazakii* invasion and infection is advanced.

ACKNOWLEDGEMENTS

We would like to thank the International Life Sciences Institute of North America (ILSI-NA) Technical Committee on Food Microbiology and the University of Georgia Center for Food Safety for funding. The Alfred P. Sloan Foundation and the Interdisciplinary Toxicology

Program are acknowledged for providing graduate stipends (ANR) and the College of Public Health and the College of Agricultural and Environmental Sciences for salary support (MAS).

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***Table 4.1: Isolation of* C. sakazakii *from neonatal mouse brain 7 days after oral exposure to C. sakazakii-*contaminated infant formula**

Confirmed dose (CFU/mouse)	Strain[a]		
	MNW2	**SK81**	**3290**
Control	0 (0/24) A	0 (0/30) A	0 (0/25) A
$10^{2.8}$	8 (3/38) A	4 (2/49) A	8 (4/50) A
$10^{4.8}$	10 (3/30) A	21 (8/38) B	57 (17/30) B
$10^{6.7}$	2 (1/40) A	16 (6/38) A	55 (22/40) B
$10^{8.5}$	0 (0/29) A	15 (4/26) B	61 (24/39) B
$10^{10.5}$	4 (1/26) A	ND[b]	ND
Total isolation[c]	A 6.7 (11/163)	A 15.9 (24/151)	B 42.1 (67/159)

[a] Brain tissue positive (%) for *C. sakazakii* and (number of mice with brain tissue positive for *C. sakazakii* out of the number of mice analyzed). Within each column, values for mice receiving inocula containing 0 (control) - $10^{10.5}$ CFU of *C. sakazakii* that are not followed by the same letter are significantly different ($P \leq 0.05$).

[b] Not determined.

[c] Values for strains MNW2, SK81, and 3290 that are not preceded by the same letter are significantly different ($P \leq 0.05$).

***Table 4.2: Isolation of* C. sakazakii *from neonatal mouse liver 7 days after oral exposure to C. sakazakii-*contaminated infant formula**

Confirmed dose (CFU/mouse)	Strain[a]		
	MNW2	**SK81**	**3290**
Control	0 (0/24) A	0 (0/30) A	0 (0/25) A
$10^{2.8}$	10 (4/38) A	4 (2/49) A	6 (3/50) A
$10^{4.8}$	7 (2/30) A	16 (6/38) B	67 (20/30) B
$10^{6.7}$	7 (3/40) A	8 (3/38) A	50 (20/40) B
$10^{8.5}$	0 (0/29) A	4 (1/26) A	51 (20/39) B
$10^{10.5}$	4 (1/26) A	ND[b]	ND
Total isolation[c]	A 6.1 (10/163)	A 7.9 (12/151)	B 39.6 (63/159)

[a] Percent of mice with liver tissue positive for *C. sakazakii* and (number of mice with liver tissue positive for *C. sakazakii* out of the number of mice analyzed). Within each column, values for mice receiving inocula containing 0 (control) - $10^{10.5}$ CFU of *C. sakazakii* that are not followed by the same letter are significantly different ($P \leq 0.05$).

[b] Not determined.

[c] Values for strains MNW2, SK81, and 3290 that are not preceded by the same letter are significantly different ($P \leq 0.05$).

***Table 4.3: Isolation of* C. sakazakii *from neonatal mouse cecum 7 days after oral exposure to C. sakazakii-*contaminated infant formula**

Confirmed dose (CFU/mouse)	Strain[a]		
	MNW2	**SK81**	**3290**
Control	0 (0/24) A	0 (0/30) A	0 (0/25) A
$10^{2.8}$	3 (1/38) A	4 (2/49) A	6 (3/50) A
$10^{4.8}$	10 (3/30) A	3 (1/38) A	27 (8/30) B
$10^{6.7}$	0 (0/40) A	8 (3/38) A	37 (15/40) B
$10^{8.5}$	7 (2/29) A	4 (1/26) A	28 (11/39) B
$10^{10.5}$	0 (0/26) A	ND[b]	ND
Total isolation[c]	A 4.3 (7/163)	A 4.6 (7/151)	B 23.3 (37/159)

[a] Percent of mice with cecum tissue positive for *C. sakazakii* and (number of mice with cecum tissue positive for *C. sakazakii* out of the number of mice analyzed). Within each column, values for mice receiving inocula containing 0 (control) - $10^{10.5}$ CFU of *C. sakazakii* that are not followed by the same letter are significantly different ($P \leq 0.05$).

[b] Not determined.

[c] Values for strains MNW2, SK81, and 3290 that are not preceded by the same letter are significantly different ($P \leq 0.05$).

Table 4.4: Neonatal mouse mortality 7 days after oral exposure to* C. sakazakii*-contaminated infant formula

Confirmed dose	**Strain**[a]		
(CFU/mouse)	**MNW2**	**SK81**	**3290**
Control	0 (0/24) A	0 (0/30) A	0 (0/25) A
$\mathbf{10^{2.8}}$	3 (1/39) A	2 (1/50) A	0 (0/50) A
$\mathbf{10^{4.8}}$	0 (0/30) A	5 (2/40) A	0 (0/30) A
$\mathbf{10^{6.7}}$	0 (0/40) A	5 (2/40) A	0 (0/40) A
$\mathbf{10^{8.5}}$	3 (1/30) A	13 (4/30) A	2 (1/40) A
$\mathbf{10^{10.5}}$	0 (0/26) A	ND[b]	ND
Total mortality[c]	A 1.2 (2/165)	B 5.6 (9/160)	A 0.6 (1/160)

[a] Mortality (%) in neonatal mice 7 days after exposure to *C. sakazakii* and (number of mice that died out of the number of mice exposed to *C. sakazakii*). Within each column, values for mice receiving inocula containing 0 (control) - $10^{10.5}$ CFU of *C. sakazakii* that are not followed by the same letter are significantly different ($P \leq 0.05$).

[b] Not determined.

[c] Values for strains MNW2, SK81, and 3290 that are not preceded by the same letter are significantly different ($P \leq 0.05$).

CHAPTER 5

SUSCEPTIBILITY TO *CRONOBACTER SAKAZAKII* CHANGES WITH INCREASING AGE IN NEONATAL CD-1 MICE[3]

[3] **Richardson, A. N.**, E. A. Pollak, D. Williams, A. K. Agyekum, and M. A. Smith. To be submitted to *Journal of Food Protection*.

ABSTRACT

Neonatal, premature, or very-low-birth-weight infants orally fed reconstituted powdered infant formula contaminated with *Cronobacter sakazakii* may develop infections resulting in severe outcomes such as septicemia, necrotizing enterocolitis, meningitis, or death. Infants who recover from infection may have morbidities such as hydrocephalus, mental retardation, or developmental delays. Although increasing age appears to reduce susceptibility, it is not known at what age these infants become less susceptible to *C. sakazakii* infection. Our study objectives were to compare the susceptibilities of neonatal mice of different ages to *C. sakazakii* infection and to identify biomarkers of infection. Timed-pregnant CD-1 mice were obtained and allowed to give birth naturally. Neonatal mice were orally gavaged at postnatal day (PND) 1.5, 5.5, and 9.5 with a single dose of vehicle or 10^4, 10^8, or 10^{11} colony-forming units (CFU) *C. sakazakii* strain MNW2 per ml reconstituted powdered infant formula. Pups were sacrificed on post-treatment day 7. Blood was collected and brains, livers, and ceca were excised and analyzed for *C. sakazakii* invasion. *C. sakazakii* was isolated from brains, livers, and ceca of neonatal mice treated at PND 1.5 and 5.5 but not from those of pups treated at PND 9.5. *C. sakazakii* was more invasive in brains than in livers and ceca with total isolations of 25.3%, 21.2%, and 19.7%, respectively. Mortality was only observed in neonates treated at PND 1.5. Serum amyloid A was detected at 0.9992 ng/ml in only one neonatal mouse, which was treated at PND 1.5. In conclusion, neonatal mice show a time-dependent susceptibility to *C. sakazakii* infection with resistance increasing with increasing age, similarly to the trend seen in humans.

Cronobacter sakazakii is an emerging pathogen that has been known to contaminate powdered infant formulas. Outbreaks of *C. sakazakii* infection have been reported in neonatal intensive care units in several countries/nations (Arseni et al., 1987, Van Acker et al., 2001, Block et al., 2002, Urmenyi and White Franklin, 1961). *C. sakazakii* infection is rare, but can be life-threatening to neonatal, premature, or low-birth weight infants. Possible illnesses occurring in infants following *C. sakazakii* exposure include necrotizing enterocolitis (Hunter et al., 2007, Van Acker et al., 2001), brain abscesses (Burdette and Santos, 2000), cerebral infarction (Gallagher and Ball, 1991), bacteremia (Noriega et al., 1990), meningitis (Burdette and Santos, 2000, Gallagher and Ball, 1991, Kleiman et al., 1981, Lai, 2001), and septicemia (Lai, 2001). *C. sakazakii* infection may be lethal if the primary infection progresses and the case-fatality rate ranges from 40 to 80% (Bowen and Braden, 2006, Nazarowec-White and Farber, 1997a, WHO/FAO, 2007, Willis and Robinson, 1988). Infants who recover from *C. sakazakii* infection may suffer morbidities such as hydrocephaly, developmental delays, mental retardation, or other permanent neurological disorders (Lai, 2001, Drudy et al., 2006).

C. sakazakii was formerly classified as *Enterobacter sakazakii* until a name change was accepted in 2008 (Iversen et al., 2008). The reservoir for *C. sakazakii* is not known; however, it has been detected in soil (Khan et al., 1998), insects (Hamilton et al., 2003, Kuzina et al., 2001), household vacuum bags (Kandhai et al., 2004), and foods such as vegetables, cheese, minced beef (Leclercq et al., 2002), chocolate, cereal products (Kandhai et al., 2004), and brown rice (Jung and Park, 2006). *C. sakazakii* has been isolated from facilities in which foods, including powdered infant formulas, are processed (Kandhai et al., 2004, Shaker et al., 2007).

Animal studies are needed to better understand the pathogenicity of *C. sakazakii*. However, there are few animal studies reported in the literature. Pagotto et al. (2003) conducted

a study in which 3-4-day-old Swiss Webster mice were orally gavaged with 18 different *C. sakazakii* strains at relatively high doses (10^5, 10^7, or 10^8 CFU).

In our previous studies, we have investigated the susceptibility of three mouse strains to *C. sakazakii* (Richardson et al., 2009) and virulence differences of three *C. sakazakii* strains in CD-1 neonatal mice (Richardson et al., 2010). In our first study, we compared the susceptibilities of three strains of neonatal mice (BALB/C, CD-1, and C57BL/6). Neonatal CD-1 mice were the most susceptible to *C. sakazakii* infection, having a lowest infectious dose and lowest lethal dose of 10^2 CFU after orally challenge. The second study examined the differences in virulence between *C. sakazakii* strains MNW2 (food isolate), SK81 (clinical isolate), and 3290 (clinical isolate). The two clinical isolates were more virulent than the food isolate, with 3290 being significantly more invasive in brain, liver, and cecum tissues and SK81 causing significantly more deaths.

Further animal studies on *C. sakazakii* exposure are needed to understand its mechanisms of pathogenicity in human infants. We have previously shown that neonatal mice are susceptible to *C. sakazakii* infection via the oral route (Richardson et al., 2009). The objective of this study was to examine whether there are differences in susceptibility to *C. sakazakii* infection among juvenile CD-1 mice of different ages. It is not known for certain whether postnatal age is a major factor in the susceptibility of the human infant to *C. sakazakii* infection.

MATERIALS AND METHODS

Animals. Timed-pregnant CD-1 mice were obtained from Charles River Laboratories (Wilmington, Massachusetts) at gestation day (GD 15). Animals were maintained in an animal room with a 12 h: 12 h light/dark cycle. Rodent chow and drinking water were available *ad libitum*. Dams were housed individually and allowed to give birth naturally at GD 19 or 20.

Neonatal mice were sexed and randomly assigned to foster mothers to result in 5 males and 5 females per litter.

Culture preparation. Stock cultures of *C. sakazakii* strain MNW2 were frozen on ceramic beads at -80°C. *C. sakazakii* was grown to high concentrations in tryptic soy broth (Oxoid, LTD, Basingstoke, Hampshire, England). Bacterial cells were transferred into fresh broth on days 2 and 3 of growth. Inoculated medium was incubated at 37°C for 18-24 h. Bacterial suspensions were concentrated by centrifugation on day 4 to obtain the highest cell concentration needed for treatment doses. *C. sakazakii* was centrifuged, washed with sterile deionized water, and resuspended in reconstituted powdered infant formula. Powdered infant formula was mixed with sterile deionized water for reconstitution, per the manufacturer's instructions. Cells were serially diluted in sterile deionized water and 0.1 ml of each dilution was spread plated onto tryptic soy agar (TSA) (Oxoid) for dose confirmation. TSA plates were incubated for 18-24 h at 37°C. *C. sakazakii* concentrations were confirmed by colony enumeration.

Treatment of mice. Methods for the treatment of neonatal mice were previously published (Richardson et al., 2010). Pups were treated on postnatal day (PND) 1.5, 5.5, or 9.5 via oral gavage using a 24 x 1'' (25.4 mm) W/1-1¼ stainless steel animal feeding needle (Popper & Sons, Inc., New Hyde Park, N.Y.) attached to a 1 ml syringe. Prior to litter assignment, vanilla flavoring (The Kroger Co., Cincinnati, O.H.) was applied onto the nose (snout) of each dam to mask animal scents and create olfactory confusion. This was done to increase acceptance of the pups by the foster mothers.

Serial dilutions of reconstituted powdered infant formula inoculated with various concentrations of *C. sakazakii* strain MNW2 were prepared. Each pup received a volume of 0.1

ml or inoculated reconstituted powdered infant formula or the vehicle control. Experimental mouse pups received confirmed *C. sakazakii* doses of 10^3, 10^7, and 10^{10} CFU. Neonates were observed for mortality twice a day during the post-treatment period. All pups viable at post-treatment day (PTD) 7 were euthanized. Brain, liver, and cecum tissues were excised during necropsy and prepared for *C. sakazakii* isolation.

All mouse work was done in full compliance with federal regulations including the Animal Welfare Act and was approved by the Institutional Animal Care and Use Committee. The University of Georgia is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Tissue preparation and analysis for detection of *C. sakazakii*. Individual tissues were homogenized and macerated in *Enterobacteriaceae* enrichment (EE) broth (Oxoid). Tissue suspensions were incubated at 37°C for 18-24 h and streak plated onto violet red bile glucose (VRBG) agar in duplicate for selective growth of *Enterobacter* spp. VRBG plates were incubated at 37°C for 18-24 h. Growths were subcultured on TSA and incubated overnight at 37°C. RapID One Identification System (Remel, Inc., Lenexa, K.S., USA) was used for positive biochemical identification of *C. sakazakii*.

Detection of potential biomarkers of *C. sakazakii* infection. Blood samples were collected from neonatal mice on day of sacrifice. Samples were centrifuged and serum supernatants were pipetted and stored at -20°C. An enzyme-linked immunosorbent assay (ELISA) was used for the detection of serum amyloid A 2 (SAA2) (Life Diagnostics, Inc., West Chester, P.A.) in mouse serum samples. Serum samples were diluted 50-fold. Sample absorbances were measured at a wavelength of 450 nm in a spectrophotometer. Standard curves

were fitted and SAA concentrations were calculated using KC4 computer software (BioTek Instruments, Inc, Winooski, V.T.).

Brain samples from 3.5-day-old mice were analyzed for expression of vascular endothelial growth factor (VEG-F). After necropsy, brain tissues were stored in RNAlater (Sigma-Aldrich, St. Louis, M.O.) and frozen at -20°C. Total RNA was extracted and purified using the RNeasy Mini Kit (Qiagen, Inc., Valencia, C.A.). VEG-F and GAPDH primer pairs and positive controls were obtained (R&D Systems, Minneapolis, M.N.). Samples were prepared for reverse-transcription polymerase chain reaction (PCR) using a One-Step RT-PCR Kit (Qiagen). Gel electrophoresis of PCR products was performed on a 1% agarose DNA gel stained with ethidium bromide. VEG-F expression was visualized under ultraviolet light.

Statistical analyses. Statistical analyses for *C. sakazakii* infectivity and mortality data were done using SAS version 9.1 (SAS Institute, Cary, N.C.) and Microsoft Excel (Microsoft Corporation, Redmond, W.A.). Significant differences ($P \leq 0.05$) in values comparing the ages of treated animals were determined using Scheffe's test and Excel *t*-test. One-way analysis of variance (ANOVA) tests were done using Dunnett's *t*-test and Excel *t*-test to determine significant differences between treatment groups and the control group ($P \leq 0.05$) for each mouse age.

RESULTS

Previously, we tested the infectivity and lethality of *C. sakazakii* exposure via oral gavage on neonatal BALB/C, C57BL/6, and CD-1 mice (Richardson et al., 2009). The results of the study showed that CD-1 neonates were the most susceptible to *C. sakazakii* infection of the mouse strains tested. Next, we examined whether there were virulence differences between three *C. sakazakii* strains (one food isolate and two clinical isolates) in CD-1 neonates treated via the

oral route (Richardson et al., 2010). We found that *C. sakazakii* strain 3290 (clinical isolate) was the most invasive in the neonatal mouse tissues, whereas SK81 (clinical isolate) was the most lethal. *C. sakazakii* infection as it relates to the age of human infants at the onset of infection is not yet understood. In the current study, we examined whether there were differences in susceptibility of juvenile mice at three different ages to oral challenge with various doses of *C. sakazakii* in reconstituted powdered infant formula.

Isolation of *C. sakazakii* from cecum tissue. Of the three age groups, 1.5-day-old pups had the highest percentages of *C. sakazakii* isolation from cecum tissue at all doses (Table 1). *C. sakazakii* was isolated from the cecum tissue at all doses in 1.5-day-old mice, as compared to only one dose (10^3 CFU) in 5.5-day-old pups, and *C. sakazakii* was not isolated from any cecum tissue of 9.5-day-old pups. Pups treated at PND 1.5 also had significantly higher total *C. sakazakii* isolation from cecum tissue compared to 5.5- and 9.5-day-old pups (Table 1).

Isolation of *C. sakazakii* from liver tissue. *C. sakazakii* was isolated in liver tissues from at least one mouse at all treatments in both 1.5- and 5.5-day-old pups; however, it was not isolated from the liver tissue of 9.5-day-old pups at any dose administered (Table 2). Among 1.5-day-old pups, *C. sakazakii* isolation from liver tissue of the 10^7 CFU treatment group was significantly higher ($P \leq 0.05$) than the age-matched control. The lowest infectious dose was 10^3 CFU in 1.5- and 5.5-day-old mice.

Isolation of *C. sakazakii* from brain tissue. The highest percentage of *C. sakazakii* isolation from brain tissue for any dose administered occurred in 1.5-day-old mice administered 10^7 CFU (Table 3). *C. sakazakii* was detected in brain tissue at all treatments in 1.5-day-old pups, at two doses in 5.5-day-old mice, but was not isolated from 9.5-day-old mice. The lowest infectious dose in brain tissue was 10^3 CFU in 1.5-day-old mice as compared to 10^7 CFU in pups

treated at 5.5 days of age. Young age was a significant predictor of isolation of *C. sakazakii* from brain tissue of treated animals ($P \leq 0.05$).

Mortality. Mortality was only observed in mice treated on PND 1.5 (Table 4). One death occurred at the lowest administered *C. sakazakii* dose. Although not significant, percent mortality increased with *C. sakazakii* concentration with 17% (5/30) mortality in 1.5-day-old pups administered 10^{10} CFU.

Detection of potential biomarkers of infection. SAA2 was detected at a level of 0.9992 ng/ml in the blood serum sample of only one mouse, at 1.5 days of age, which was administered 10^{10} CFU *C. sakazakii* (data not shown). All other blood serum samples from animals treated at older ages were below the detection limit for SAA2.

We analyzed 7 brain tissue samples from mice treated with *C. sakazakii* at PND 3.5 for VEG-F expression. One sample was from a control neonatal mouse with no *C. sakazakii* isolated from the brain, three were from treated neonates with *C. sakazakii*-positive brains, and three from treated animals with *C. sakazakii*-negative brains. All samples analyzed expressed VEG-F (Figure 5.1).

DISCUSSION

The objective of this study was to test whether CD-1 mouse pups at three different ages displayed differences in susceptibility to *C. sakazakii* following oral gavage. It is not known at what age human infants become significantly less susceptible to *C. sakazakii* infection but the incidence of morbidity and mortality decrease with age. However, our study indicates that CD-1 neonatal mice display an age-dependent susceptibility to *C. sakazakii*. In this study, 9.5-day-old mice were not susceptible to *C. sakazakii* infection by oral gavage, based on the lack of *C. sakazakii* isolation from the three tissues sampled and no observed mortality.

SAA is a major acute–phase biomarker of infection that is found in humans and most other mammals (Uhlar and Whitehead, 1999, Pizzini et al., 2000, Hari-Dass et al., 2005). It is a protein that is synthesized by the liver and it possesses anti-inflammatory properties (Kluve-Beckerman et al., 1997, De Beer et al., 1991). SAA levels are known to increase more than 1000-fold in a host within 24 h after infection (Pizzini et al., 2000, Uhlar and Whitehead, 1999). Mouse and human SAA genes and their proteins are closely related and similar in structure and induction characteristics (Kluve-Beckerman et al., 1997). Hari-Dass et al. (2005) demonstrated that SAA can bind to OmpA integral protein found on the cell surface of several species of gram-negative bacteria tested. In our study, we investigated levels of SAA in neonatal mice 7 days after treatment with *C. sakazakii*. SAA was detected in the blood serum of one mouse treated at PND 1.5. Although we do not know why SAA did not show a significant increase in our animals, it may be that 7 days post-treatment is too late to see any acute response to *C. sakazakii* infection. *C. sakazakii* was isolated from the brain tissue of the mouse with the detectable SAA2, but not from its liver or cecum tissues (data not shown). SAA levels may increase during *C. sakazakii* infection; however, studies are needed to examine SAA within a few days after treatment to detect any acute elevation.

VEG-F is a protein that plays a major role in the regulation of angiogenesis and vascular permeability, and is also implicated in the development of brain edema (Lafuente et al., 2006, Yancopoulos et al., 2000, Ferrara, 2004). In this study, all samples analyzed expressed VEG-F, regardless of whether each animal was administered *C. sakazakii* or whether *C. sakazakii* was isolated from the brain (Figure 5.1). It may be worthwhile to use a quantitative method such as real-time-PCR to detect the actual levels of VEG-F expression in brain tissue samples and determine whether VEG-F could be used as a biomarker of *C. sakazakii* infection.

In comparing our data from the previous study with neonatal mice treated with *C. sakazakii* strain MNW2 on PND 3.5 at equivalent *C. sakazakii* concentrations (Richardson et al., 2010), we found that *C. sakazakii* infection appears to be age-dependent (data not shown). *C. sakazakii* was isolated from 3% (1/38) of cecum tissues from 3.5-day-old pups administered $10^{2.8}$ CFU *C. sakazakii*. The only age group with *C. sakazakii* isolation from cecum tissues at 10^7 and 10^{10} CFU was the 1.5-day-old pups. The 3% *C. sakazakii* isolation in 3.5-day-old mice is a decrease from 21% (4/19) isolation in 1.5-day-old pups given 10^3 CFU *C. sakazakii* , although not significant ($P > 0.05$), and it is not significantly different from the 5% (1/20) *C. sakazakii* isolation from cecum tissue of pups treated at PND 5.5. When compared to 3.5-day-old mice from our previous study, 1.5-day-old mice had significantly higher ($P \leq 0.05$) total *C. sakazakii* isolation from cecum tissue (data not shown) (Richardson et al., 2010).

C. sakazakii was isolated from liver tissue of pups treated at PND 1.5 and 5.5 at all administered treatments, as in the 3.5-day-old pups from the previous study. There was no significant difference ($P > 0.05$) in total *C. sakazakii* isolation from liver tissue between mice treated at 1.5- or 3.5- days-old (data not shown). Total isolation of *C. sakazakii* from liver tissue of 1.5- and 5.5-day-old mouse pups (14% and 7%, respectively) were significantly higher ($P \leq 0.05$) than that of 9.5-day-old mice (0%).

C. sakazakii invasion in brain tissue was also age-dependent with the highest total isolation occurring in 1.5-day-old mice, followed by 5.5-day-old mice, and no isolation from the brains of 9.5-day-old mice. The total *C. sakazakii* isolation from brain tissue for pups treated at PND 1.5 (22%) and 5.5 (4%) are both significantly higher than those of 5.5- and 9.5-day-old (3% and 0%, respectively) mice. *C. sakazakii* was detected in all treatment brains in 1.5-day-old mice and 3.5-day-old mice from the previous study. Total *C. sakazakii* isolation from brain

tissue in pups treated at PND 1.5 was not significantly different ($P > 0.05$) from total *C. sakazakii* isolation from brain tissues of 3.5-day-old mice (data not shown).

Interestingly, invasion of the neonatal mouse brain did not necessarily predict mortality, as there was a higher percentage of brains with isolated *C. sakazakii* (22%) compared to mortality (11%) in 1.5-day-old neonates. For the 1.5-day-old pups, total isolation of *C. sakazakii* was highest in the brains than in liver and cecum tissues with 14% and 18%, respectively. This suggests that even in this susceptible age group, there is some defense against infection in the neonatal brain. Total *C. sakazakii* isolation for 1.5-day-old mice was 11% (9/80), which was significantly higher ($P \leq 0.05$) than that of 3.5-day-old mice previously used (data not shown).

The results of this study show that 1.5-day-old CD-1 mice are the most susceptible to *C. sakazakii* infection, followed by 5.5- and 9.5-day-old mice, respectively. This observation is in agreeance with the fact that younger human infants, specifically neonates, are more susceptible to *C. sakazakii* infection than their older counterparts. The current study suggests that neonatal mice can be used as surrogates for *C. sakazakii* infection in human infants. Further research using animal models is needed to examine the mechanisms by which *C. sakazakii* infects and causes adverse outcomes in human infants.

ACKNOWLEDGEMENTS

Funding for this research project was provided by the International Life Sciences Institute of North America (ILSI-NA) Technical Committee on Food Microbiology and the University of Georgia (UGA) Center for Food Safety. The Alfred P. Sloan Foundation and the UGA Interdisciplinary Toxicology Program are acknowledged for providing graduate stipends (ANR) and the UGA College of Public Health and the College of Agricultural and Environmental

Sciences for salary support (MAS). We would also like to thank Dr. Larry Beuchat for providing the *C. sakazakii* strain MNW2.

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TABLE 5.1: Isolation of* C. sakazakii *from mouse cecum 7 days after oral exposure to* C. sakazakii*-contaminated infant formula

	Age (days)[a]		
Confirmed Dose (CFU/mouse)	**1.5**	**5.5**	**9.5**
Control	0% (0/10) $_{A}$	0% (0/10) $_{A}$	0% (0/10) $_{A}$
10^3	21% (4/19) $_{B}$	5% (1/20) $_{A}$	0% (0/20) $_{A}$
10^7	12% (2/17) $_{A}$	0% (0/20) $_{A}$	0% (0/30) $_{A}$
10^{10}	28% (7/25) $_{B}$	0% (0/20) $_{A}$	0% (0/30) $_{A}$
Total isolation[b]	$_{A}$ 18% (13/71)	$_{B}$ 1% (1/70)	$_{B}$ 0% (0/90)

[a] Percent of mice with cecum tissue positive for *C. sakazakii* and (number of mice with cecum tissue positive for *C. sakazakii* total number of mice analyzed). Within each column, values for mice receiving inocula containing 0 (control) - 10^{10} CFU of *C. sakazakii* that are not followed by the same letter are significantly different ($P \leq 0.05$).

[b] Values for 1.5, 5.5, and 9.5 days of age that are not preceded by the same letter are significantly different ($P \leq 0.05$).

TABLE 5.2: Isolation of* C. sakazakii *from mouse liver 7 days after oral exposure to* C. sakazakii*-contaminated infant formula

Confirmed dose (CFU/mouse)	Age (days)[a] 1.5	5.5	9.5
Control	0% (0/10) $_{A}$	0% (0/10) $_{A}$	0% (0/10) $_{A}$
10^3	16% (3/19) $_{A}$	15% (3/20) $_{A}$	0% (0/20) $_{A}$
10^7	29% (5/17) $_{B}$	5% (1/20) $_{A}$	0% (0/30) $_{A}$
10^{10}	8% (2/25) $_{A}$	5% (1/20) $_{A}$	0% (0/30) $_{A}$
Total isolation[b]	$_{A}$ 14% (10/71)	$_{A}$ 7% (5/70)	$_{B}$ 0% (0/90)

[a] Percent of mice with liver tissue positive for *C. sakazakii* and (number of mice with liver tissue positive for *C. sakazakii* total number of mice analyzed). Within each column, values for mice receiving inocula containing 0 (control) - 10^{10} CFU of *C. sakazakii* that are not followed by the same letter are significantly different ($P \leq 0.05$).

[b] Values for 1.5, 5.5, and 9.5 days of age that are not preceded by the same letter are significantly different ($P \leq 0.05$).

TABLE 5.3: Isolation of* C. sakazakii *from mouse brain 7 days after oral exposure to* C. sakazakii*-contaminated infant formula

Confirmed dose (CFU/mouse)	Age (days)[a] 1.5	5.5	9.5
Control	0% (0/10) $_{A}$	0% (0/10) $_{A}$	0% (0/10) $_{A}$
10^3	16% (3/19) $_{A}$	0% (0/20) $_{A}$	0% (0/20) $_{A}$
10^7	35% (6/17) $_{B}$	5% (1/20) $_{A}$	0% (0/30) $_{A}$
10^{10}	28% (7/25) $_{B}$	5% (1/20) $_{A}$	0% (0/30) $_{A}$
Total isolation[b]	$_{A}$ 22% (16/71)[a]	$_{B}$ 3% (2/70)	$_{B}$ 0% (0/90)

[a] Percent of mice with brain tissue positive for *C. sakazakii* and (number of mice with brain tissue positive for *C. sakazakii* total number of mice analyzed). Within each column, values for mice receiving inocula containing 0 (control) - 10^{10} CFU of *C. sakazakii* that are not followed by the same letter are significantly different ($P \leq 0.05$).

[b] Values for 1.5, 5.5, and 9.5 days of age that are not preceded by the same letter are significantly different ($P \leq 0.05$).

TABLE 5.4: Mouse mortality 7 days after oral exposure to **C. sakazakii**-*contaminated infant formula*

Confirmed dose (CFU/mouse)	Age (days)[a] 1.5	5.5	9.5
Control	0% (0/10) $_{A}$	0% (0/10) $_{A}$	0% (0/10) $_{A}$
10^3	5% (1/20) $_{A}$	0% (0/20) $_{A}$	0% (0/20) $_{A}$
10^7	15% (3/20) $_{A}$	0% (0/20) $_{A}$	0% (0/30) $_{A}$
10^{10}	17% (5/30) $_{B}$	0% (0/20) $_{A}$	0% (0/30) $_{A}$
Total isolation[b]	$_{A}$ 11% (9/80)	$_{B}$ 0% (0/70)	$_{B}$ 0% (0/90)

[a] Mortality (%) in neonatal mice 7 days after exposure to *C. sakazakii* and (number of mice that died out of the number of mice exposed to *C. sakazakii*). Within each column, values for mice receiving inocula containing 0 (control) - 10^{10} CFU of *C. sakazakii* that are not followed by the same letter are significantly different ($P \leq 0.05$).

[b] Values for 1.5, 5.5, and 9.5 days of age that are not preceded by the same letter are significantly different ($P \leq 0.05$).

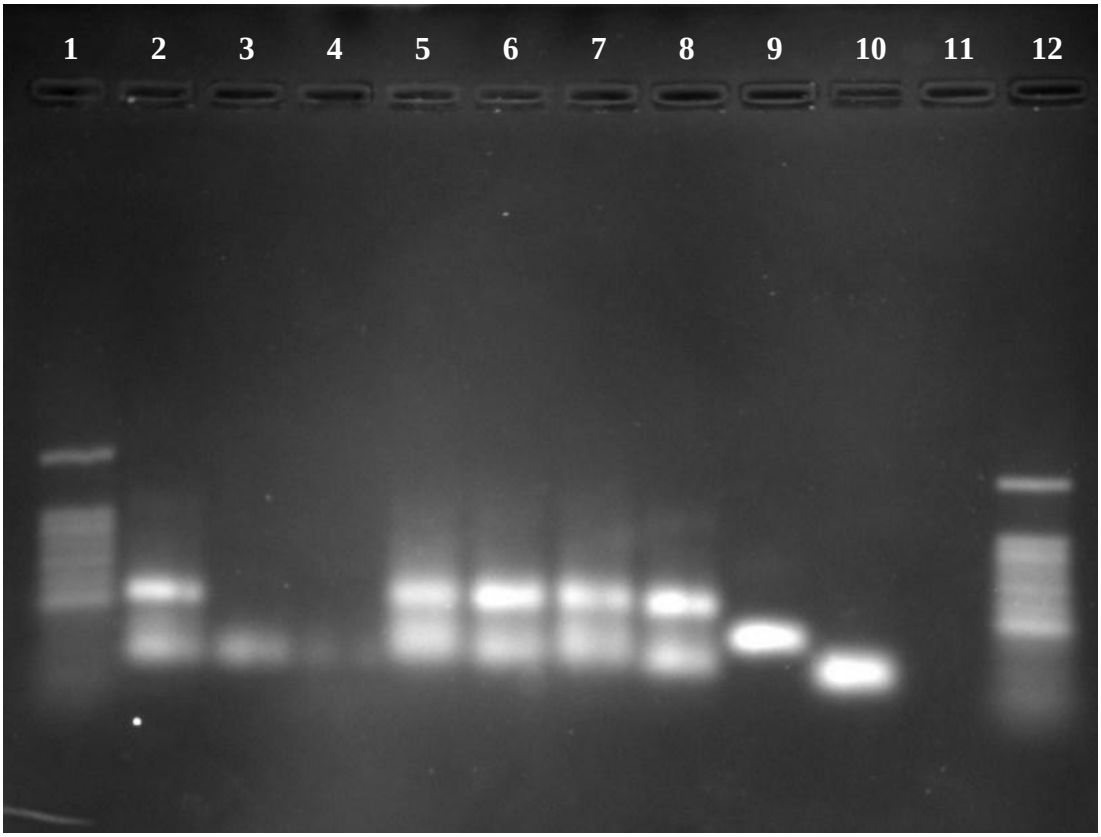

FIGURE 5.1: Gel electrophoresis analysis of brain samples of 3.5-day-old mice for VEG-F expression. Lanes 1 and 12 contain the DNA ladder. The control animal is represented in lane 2. Lanes 3, 6, and 8 contain *C. sakazakii*-negative brain samples from treated animals. Lanes 4, 5, and 7 represent *C. sakazakii*-positive brain samples from treated animals. Lanes 9 and 10 contain the GAPDH and VEG-F positive controls, respectively, and lane 11 is empty.

CHAPTER 6

SUMMARY AND CONCLUSIONS

C. sakazakii infection in neonatal and at-risk infants is a rare occurrence, but can have life-threatening consequences. Infection in infants has been linked to *C. sakazakii*-contaminated powdered infant formulas with which seriously ill infants were fed. Contamination of the powdered infant formula is assumed to occur within the food production environment during the manufacturing process, and *C. sakazakii* has been isolated from surfaces within food production facilities (Kandhai et al., 2004, Shaker et al., 2007). Infants who become infected with *C. sakazakii* may develop necrotizing enterocolitis, bacteremia, meningitis, sepsis, which may ultimately result in death. The objective of our research was to determine whether neonatal mice can be used as surrogates for *C. sakazakii* infection in susceptible human infants and to examine the potency and mechanism(s) of infection of *C. sakazakii*.

We determined differences in susceptibility to oral challenge with *C. sakazakii* among three strains of neonatal mice. Our results indicated that the CD-1 mouse strain was the most susceptible to *C. sakazakii* infection compared to BALB/C and C57BL/6, with CD-1 mice having the lowest infectious dose in brain, liver, and cecum, and the lowest lethal dose at $10^{2.3}$ CFU/animal. Our studies also show that *C. sakazakii* can invade the brain tissues of neonatal mice after oral exposure. This is important because meningitis, hydrocephaly, and other neurological sequelae are known to occur in human infants as a result of *C. sakazakii* infection. In conclusion, our findings show that *C. sakazakii* can reach the brains of neonatal mice, and this animal species appears to be an appropriate model for *C. sakazakii* infection in humans.

In our second experiment, we compared the virulence of three *C. sakazakii* strains by observing mortality and analyzing invasion of tissues in neonatal CD-1 mice. *C. sakazakii* strains MNW2 (food isolate), SK81 (clinical isolate) and 3290 (clinical isolate) invaded brain, liver, and cecum tissues of the treated mouse pups. *C. sakazakii* was isolated from more brain and liver tissues than from cecum tissues. All three *C. sakazakii* strains were able to infect neonatal mice at a low administered dose of $10^{2.8}$ CFU. Mortality was observed in the neonatal mice after treatment with either *C. sakazakii* strain but SK81 resulted in significantly more deaths with 5.6% total mortality.

Thirdly, the susceptibilities of 1.5-, 5.5-, and 9.5-days-old neonatal CD-1 mice to *C. sakazakii* infection were investigated. We observed that *C. sakazakii* infection in neonatal mice decreases with increasing age, with 1.5-day-old mice being the most susceptible followed by 5.5- and 9.5-day-old mice, respectively. *C. sakazakii* was isolated from brain, liver, and cecum tissues of neonatal mice treated at PND 1.5 and 5.5 but not from the same tissues of pups treated at PND 9.5. *C. sakazakii* was isolated more readily from brain tissues than liver and cecum tissues. Deaths only occurred among neonates treated at 1.5 days of age. The results of this study support the hypothesis that younger or less developed human infants are at a greater risk of developing *C. sakazakii* infection than their older or more developed counterparts.

Little is known and understood about the mechanisms of *C. sakazakii* infection. Our research has shown that neonatal mice are susceptible to *C. sakazakii* infection with invasion of tissues relevant to those affected in premature human infants. Additionally, livers and brains of treated neonatal mice are indicators of infection and can be used to differentiate pathogenicity of *C. sakazakii* strains. *C. sakazakii* infection in neonatal mice, as in human infants, appears to be age-dependent. In conclusion, our findings suggest that neonatal mice can serve as surrogates

for human infants in *C. sakazakii* infection studies, and age-related differences can provide a means to investigate changes resulting in a resistance to *C. sakazakii* infection.

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