AN *IN-VITRO* CO-CULTURE MODEL TO STUDY THE DISRUPTION OF THE BLOOD BRAIN BARRIER BY *CRONOBACTER SAKAZAKII* (FORMERLY *ENTEROBACTER SAKAZAKII*)

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An *in-vitro* Co-Culture Model to Study the Disruption of the Blood Brain Barrier

by *Cronobacter sakazakii* (formerly *Enterobacter sakazakii*)

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

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ABSTRACT

Koval, Erin Louise, MS, Program of Food Safety, College of Graduate and Interdisciplinary Studies, North Dakota State University, July 2010. An *In-vitro* Co-Culture Model to Study the Disruption of the Blood Brain Barrier by *Cronobacter sakazakii*. (formerly *Enterobacter sakazakii*). Major Professor: Dr. Penelope Gibbs.

Cronobacter spp. (formerly *Enterobacter sakazakii*) is an opportunistic pathogen associated with contaminated powdered infant formula. It causes necrotizing enterocolitis (NEC) and sepsis, which can develop into severe meningitis and brain abscess formation in infants. Very little is known about the specific pathogenic mechanisms of this organism. In this study, we propose a model to investigate the ability of *Cronobacter* spp. isolates to disrupt the blood-brain barrier. Biofilm formation of the *Cronobacter* spp. isolates used in the model was also assayed.

Secondary mouse endothelial and astrocyte cells were grown to confluency on polyethylene terephtalate (PET) membranes (1.0 μm pore size) in 24-well plate hanging culture inserts (Millipore ® Billerica, MA) to achieve a culture system similar to the physiological structure of the blood brain barrier *in vivo*. Initially, a monoculture system was tested containing only endothelial cells and then a co-culture system was developed with both cell types. Selected *Cronobacter* spp. isolates were added to the cell culture systems. *Escherichia coli* K1 and K12 strains were used as positive and negative controls, respectively. Some of the treatments did not have bacteria added to them to serve as cell controls, and membranes without cells were included as media control blanks. The transendothelial electrical resistance (TEER) was measured across the cells to determine if the cell barrier was disrupted. Initially, measurements were taken at 0, 6, 24, and 48 hours

after adding bacteria. Due to overall loss of cell integrity at 48 hours, a second experiment was performed where measurements were taken at 0, 6, and 24 hours after adding the bacteria. Biofilm formation was analyzed using a method described by O'Toole (72) and Skyberg (86).

The monoculture and co-culture systems worked based on TEER measurements. The positive control (*E. coli* K1) and *Cronobacter sakazakii* isolates disrupted the tight junctions between cells evidenced by significant decrease in TEER over time. The negative control (*E. coli* K12) did not have any significant effect on the cells.

The cell control and negative control (*E. coli* K12) maintained the highest resistance values in both the monoculture and co-culture experiments. The positive control (*E. coli* K1) and the *Cronobacter sakazakii* isolates caused the resistance across the cells to significantly decrease over time in both experiments.

According to the results of our biofilm assay, none of *Cronobacter* spp. isolates used in the culture models formed biofilms. Further testing would need to be done using other biofilm detection procedures to confirm this conclusion

Overall, we successfully constructed a co-culture model to depict the BBB. The selected *Cronobacter sakazakii* decreased resistance in this model of the BBB similar to the positive control K1 *E. coli* isolate. This assay can be used in future studies to test the potential pathogenicity of *Cronobacter* spp. bacteria as well as other bacteria involved with central nervous system diseases.

Key Words: *Cronobacter*, *Enterobacter sakazakii*, blood brain barrier models, TEER

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INTRODUCTION

Cronobacter spp. are opportunistic pathogens belonging to the family *Enterobacteriaceae*. This genus was previously classified as *Enterobacter sakazakii* until 2007 when its nomenclature was reorganized based on 16S rRNA sequences and other genetic differences (35, 36). *Cronobacter* spp. is recognized as an emerging opportunistic foodborne pathogen causing serious disease in infants including bacteremia, necrotizing enterocolitis (NEC), sepsis, and meningitis (54, 97). The case-fatality rate of *Cronobacter* infections in neonates ranges from 40-80% (33). Adult infections with *Cronobacter* are rare and may be underreported.

The true reservoir for *Cronobacter* is unknown, but the organism has been isolated from various environmental and clinical sources. Powdered infant formula (PIF) has been identified as a significant source of most *Cronobacter* infections (8, 49, 88, and 97). Besides PIF, *Cronobacter* has also been isolated from food products (fruits, vegetables, meat products, tofu), drinking water systems, clinical specimens (cerebrospinal fluid, blood, wounds, and digestive tracts of humans), and animal feces (21, 22, 25, 52, 67). Some studies have suggested that the intestinal tract or manure of domestic animals may be the reservoir of *Cronobacter* spp., but this remains to be determined (26, 60). Recently, plants have been documented as a potential reservoir for these bacteria (83).

The specific virulence factors and pathogenic mechanisms of *Cronobacter* spp. infections have not been thoroughly defined in the literature. Initial studies on pathogenesis found evident differences in virulence mechanisms between the various *Cronobacter* species (73). The organism, being a Gram-negative rod, does possess the lipid A core endotoxin in the lipopolysaccharide (LPS) layer of its cell wall. The lipid A

endotoxin has been found to be heat stable at 100°C and can remain biologically active in heat-treated PIF (94). Further, the LPS has been found to have pleiotropic effects by increasing blood-brain barrier (BBB) penetration in rat pups and translocation of the bacteria across this barrier (94). *Cronobacter* has also been found to attach to and invade microvascular endothelial cells, which is the mechanism it employs to breech the BBB and enter the CNS. A previous study reported that *Cronobacter* strains form two distinct adhesion patterns: a diffuse pattern and a localized pattern with cluster of bacteria present on the cell surface (57).

Infantile meningitis caused by *Cronobacter* spp. is of particular concern due to its severity. Currently, there is no *in-vitro* diagnostic/screening tool available to determine which species of *Cronobacter* have the potential to cause meningitis. An *in-vivo* newborn rat model has been used to study meningitis caused by *Cronobacter* (93). *In-vitro* BBB models have been used in previous studies to assay the effect of certain drugs and pathogens (mainly *E. coli* K1 strains) on barrier integrity (54, 90). We propose an *in-vitro* co-culture model consisting of endothelial cells and astrocytes to determine the effect of *Cronobacter* isolates on the BBB. Because biofilm formation has been linked to pathogenesis, we also screened *Cronobacter* isolates for biofilm formation and compare these results with those obtained using the co-culture BBB model.

OBJECTIVES

The objectives of this study include:

1) To test a monoculture system consisting of endothelial cells to model the BBB using selected *Cronobacter* spp. isolates by measuring transendothelial electrical resistance (TEER)
2) To develop an *in-vitro* co-culture system consisting of endothelial cells and astrocytes to model the BBB.
3) To determine the effect selected *Cronobacter* spp. isolates have on the co-culture model of the blood brain barrier using TEER measurements.
4) Using the *in-vitro* co-culture system and crystal violet biofilm assay to determine if biofilm formation by the *Cronobacter* isolates correlates with pathogenesis in the culture model.

LITERATURE REVIEW

Cronobacter spp. (formerly *Enterobacter sakazakii*)

Cronobacter spp. are motile, facultatively anaerobic, Gram-negative rods belonging to the family *Enterobacteriaceae* (67). When the organism was first characterized in the 1960s it was referred to as "yellow pigmented *Enterobacter cloacae*" (95). In 1980, it was renamed *E. sakazakii* after the Japanese bacteriologist Richii Sakazakii (25). This reclassification was proposed when differences were discovered between *E. sakazakii* and *E. cloacae* in DNA relatedness, biochemical reactions, and yellow pigment production (19). As research with this organism progressed, the serotypes of *E. sakazakii* were shown to be more genetically diverse than originally thought. So, in 2007, it was proposed that *E. sakazakii* be reclassified as the novel genus *Cronobacter* based upon polyphasic taxonomic analysis (35, 36). Currently, the genus *Cronobacter* is comprised of 6 species: *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter turicensis*, *Cronobacter muytjensii*, *Cronobacter dublinensis*, and *Cronobacter genomospecies* 1 (35, 36).

Growth, thermotolerance, and stress resistance

The presence of *Cronobacter* spp. in powdered infant formula has been attributed to their growth profiles (30). They are known to achieve optimal growth at 37°C-43°C, but can grow at room temperature (25°C) as well (33).

Cronobacter spp. are viable at temperatures as low as 3.4°C to 5.7°C meaning it can persist during refrigeration (6, 33, and 69). However, at refrigeration temperature it is unlikely the bacteria can multiply to a sufficient level to cause an infection (33). The thermotolerance of *Cronobacter* spp. has been extensively studied. Edelson-Mammel and Buchanan (17) used a submerged coil apparatus to determine the ability of 12 *Cronobacter*

spp. isolates to survive heating at 58°C in rehydrated infant formula. They observed two heat resistance phenotypes and determined the decimal reduction time (D-values) ranged from 30.5 to 591.9 s. The D-value is the time required to kill 90% of the *Cronobacter* isolates at 58°C. The z-value for the most heat resistance strain they tested was 5.6°C. In the other thermotolerance studies conducted with *Cronobacter* spp. there has been significant variability among the reported D- values from 0.4 min (6), to 2.6 min (33), and 4.2 min (68). There is general agreement *Cronobacter* spp. are indeed thermotolerant organisms.

Cronobacter spp. cells are remarkably resistant to osmotic and desiccation stress, especially when compared to the other species within *Enterobacteriaceae*. It has been suggested this may contribute to their ability to survive in PIF (11). Breeuwer et al. (6) observed a significant accumulation of trehalose in stationary phase *Cronobacter* cells unlike the stationary phase *E. coli*. It has been suggested this molecule may play a role protecting cells from dessication stress by stabilizing the phospholipid membranes and proteins, giving *Cronobacter* spp. a competitive advantage in dry environments (38, 39). It has been further reported in the literature that the bacteria may produce a heteropolysaccharide capsule which could assist its survival for a long period of time in PIF as well as contribute to biofilm formation (24, 30, and 41).

Diseases

Cronobacter spp. are important opportunistic foodborne pathogens. They have been associated with rare, sporadic cases of life-threatening illness in infants such as necrotizing enterocolitis (NEC), septicemia, and meningitis (46, 97). Seizures have also

been reported to occur in about one-third of cases (99). Disease in adults is quite rare (or may be underreported) and has only occurred among the immunocompromised (46).

Necrotizing enterocolitis (NEC)

NEC is one of the most severe gastrointestinal diseases in the newborn primarily affecting premature infants. There is a positive correlation between NEC caused by *Cronobacter* spp. and oral PIF feeding (57). The incidence rate of NEC is 2-5% in premature infants and a mortality rate of 10-55% (97). NEC is characterized by inflammation of the intestines which ultimately causes necrosis (tissue death) of part or the entire organ. Further, pneumatosis intestinalis (gas formation in the walls of the bowel) can also occur in NEC cases (25, 67). NEC can lead to septicemia (bacteremia) and eventually meningitis in infected infants.

Meningitis

Infantile meningitis is the usually direct result of septicemia (presence of large numbers of pathogenic bacteria in the blood). It is an inflammation of the meninges (the membranes surrounding the brain). The first two known cases of neonatal meningitis caused by *Cronobacter* spp. were reported by Urmenyi and Franklin in England in 1961 (95). At that time, *Cronobacter* spp. was still referred to as "yellow pigmented *E. cloacae*." Although the number of reported infections is low, it is a very serious disease. The case fatality rate among infants has been reported to be as high as 80% (25, 67, and 77). The pathogenesis of infantile meningitis involves invasion of the BBB (93). This can result in ventriculitis (inflammation of the brain ventricles), brain abscesses (pus formation in the brain caused by inflammation and collection of infected material from a local infection), infarctions (damage and death of the brain tissues due to interrupted flow of

oxygen to the brain), and cyst formation (accumulation of fluid or semisolid material in the brain) (22, 46). Prognosis of this form of meningitis is poor and survivors of the infection often suffer from severe neurological disorders (deafness, blindness, and learning disabilities). In a case study conducted by the CDC, 74% of meningitis survivors experienced an adverse neurological outcome and other sources have cited adverse outcomes in 20-78% of survivors (5, 9, 28, and 88).

Treatment

Antibiotic therapy for *Cronobacter* spp. infections has traditionally been a combination of ampicillin and gentamycin (11, 46). However, the emergence of strains resistant to ampicillin has led to the consideration of using cephalosporins in treatment regimens (49). Overall, antibiotic therapy does help reduce the bacterial load, but it is best to avoid neonatal exposure to this bacterium altogether (93).

Pathogenesis and Virulence Factors

Much research conducted with *Cronobacter* spp. has focused on characterization and detection, but not so much on pathogenesis. In fact, the specific pathogenic/virulence mechanisms implemented by this organism during infection are virtually unknown. Pagotto et al. (73) were the first to conduct a study investigating the pathogenicity and dose-response relationship in *Cronobacter* spp. infection. They used the suckling mouse assay to test isolates of *Cronobacter* spp. for enterotoxin production by injecting the strains into mice both orally and intraperitoneally. Out of 19 strains tested (9 food strains, 9 clinical strains, and 1 type/control strain (ATCC# 29544)), 22% tested positive for enterotoxin production. Out of these positive enterotoxin strains of *Cronobacter* spp., only one was isolated from food. Furthermore, two strains not able to produce the enterotoxin

were found to still cause death in the mice via the peroral route. All strains inoculated into the mice via the intraperitoneal route were lethal. The overall findings in this study overall suggest there are evident differences between the virulence mechanisms used by various *Cronobacter* spp. isolates.

Since *Cronobacter* spp. are Gram-negative, these bacteria are known to contain a lipopolysaccharide (LPS) layer of their cell wall (81, 93). The LPS is composed of core lipid A endotoxin (a complex lipid made up of fatty acids and sugars), a conserved core oligosaccharide, and an O-antigen (composed of variable polysaccharide side chains) (62). The lipid A is the core structure that anchors the core oligosaccharide and O-antigen to the outer membrane. Mullane et al. (62) characterized the O-antigen gene cluster (*rfb* locus) and identified two serotypes of *Cronobacter* spp. (O:1 and O:2). The PCR assay they implemented in their study would be useful to identify these two *E. sakazakii/Cronobacter* spp. serotypes from environmental, food, and clinical sources (62). Townsend et al. (94) suggest that the LPS heat stable endotoxin can enhance the translocation of *Cronobacter* spp. from the blood to the cerebrospinal fluid (CSF). This would facilitate the systemic spread of the bacteria and sepsis.

In mammalian cell and tissue culture, *Cronobacter* spp. can attach to intestinal epithelial and brain endothelial cells. Mange et al. (57) were the first to study the ability of *Cronobacter* spp. strains to adhere to HEp-2 and Caco-2 epithelial cells. They also used human brain microvascular endothelial cells (HBMEC) to investigate the ability of *Cronobacter* spp. to breech the blood-brain barrier (BBB) and invade the central nervous system (CNS). The results of their study showed two adhesion patterns displayed by the *Cronobacter* spp. strains: a diffuse adhesion and the formation of localized clusters of

bacteria on the cell surface. *Cronobacter* spp. isolates were found to display the cluster adhesion pattern with the addition of blood to the bacterial growth media. Further, adherence was maximal during late exponential phase and increased with high multiplicity of infection (MOI). Their findings suggest there are diverse adhesion patterns displayed within different *Cronobacter* spp. strains. Also, they found no association between the adhesive capacity of *Cronobacter* spp. onto cells and the production of specific fimbriae. Kim et al. (42) showed *Cronobacter* spp. can invade human intestinal Caco-2 cells. This invasion is dependent on the presence of microtubules and actin filaments. They also observed *Cronobacter* spp. invasion was enhanced with the disruption of the tight junctions between the Caco-2 cells.

The specific adhesions involved in bacterial attachment remain to be determined. Adeghola et al. (2) identified a mannose-sensitive hemagglutinin associated type 1 fimbriae in *Cronobacter* strains which they suggested may play a role in adhesion. Kothary et al. (45) isolated and characterized a cell-bound metalloprotease that is unique to all the 135 *Cronobacter* isolates tested. The researchers found this enzyme was active against azocasein and caused rounding of Chinese hamster ovarian cells due to break down of the extracellular proteins. They suggest it may allow the organism to cross the BBB and cause cell damage in neonates. Townsend et al. investigated the ability of the bacterium to invade HBMECs *in-vitro* (93). They also determined *Cronobacter* spp. can survive within macrophages. This could also facilitate the ability of *Cronobacter* spp. to cross the BBB.

Outer membrane protein A (OmpA) is known to be involved in *E. coli* K1 invasion of HBMECs (40, 76, and 90). *E. coli* K1 strain possesses the K1 capsular polysaccharide and is one of the most common causes of neonatal meningitis (40, 90). Since *Cronobacter*

spp. bacteria also cause infant meningitis, research has been done focusing on the role of OmpA protein in *Cronobacter* spp. pathogenesis. Singamesetty et al. (85) conducted a study investigating the role of OmpA in *Cronobacter* spp. pathogenesis. They found that OmpA expression is required for *Cronobacter* spp. invasion of HBMECs. This is similar to the invasion characteristics of *E. coli* K1 strains, but it is believed that different epitopes are involved in *Cronobacter* spp. invasion. Furthermore, Singamesetty et al. (85) found *Cronobacter* spp. invasion was dependent on microtubule condensation/reorganization in host cells (influenced by 1-phosphatidylinositol 3- kinase and protein kinase C-α). Kim et al. (43) identified OmpX in *Cronobacter sakazakii* strains and found this protein along with OmpA was essential for apical and basolateral invasion of Caco-2 epithelial cells. OmpX has previously been shown to be involved in invasion of HBMECs by *Enterobacter cloacae* and *Yersinia pestis* (14, 44).

Food and Environmental Sources

Cronobacter spp. has been isolated from various clinical and environmental samples, but its true reservoir remains undetermined. Some food sources to contain *Cronobacter* spp. include: cereals, legume products (tofu, alfalfa sprouts, and bean sprouts), herbs and spices, fruits, vegetables, meat products, egg products, and, less frequently, fish (21). *Cronobacter* spp. has also been cultured from biofilms in drinking water systems (21). Interestingly, *Cronobacter* spp. is reported to have been isolated from the Mexican fruit fly *Anastrepha ludens* and from the midgut of the stable fly *Stomoxys calcitrans* (25, 26, and 60). Two studies conducted by Hamilton et al. (26) and Mramba et al. (60) were the first to isolate *Cronobacter* spp. from the gut of *S. calcitrans*, but the low prevalence of the organism in the flies suggests they are not an important vector for this

pathogen. *Cronobacter* spp. strains have been isolated from many clinical sources including bone marrow, blood, CSF, sputum, urine, intestinal tract, respiratory tract, the eye, and the ear (22, 52). Despite the ubiquitous nature of *Cronobacter* spp. in foods and the environment, the majority of neonatal infections have been associated with the consumption of PIF (49, 97). PIF is a nonsterile product and contamination with *Cronobacter* spp. can occur intrinsically (during manufacture) or extrinsically (during reconstitution due to use of contaminated utensils). Several investigations into the presence of *Cronobacter* spp. in PIF have been performed. Mutyjens et al. (63) screened for the presence of *Cronobacter* spp. in 141 different breast milk substitute powders from 35 different countries including the United States. They found that 52.2% of the samples tested contained members of *Enterobacteriaceae* and *Cronobacter* spp. was isolated from 20 samples at levels ranging from 0.36 to 66.0 CFU per 100 grams. Iversen and Forsythe (34) surveyed 83 PIF powder products purchased from retailers across the United Kingdom and two tested positive for *Cronobacter* spp. contamination. Mullane et al. (61) hypothesized the manufacturing environment is a primary route for sporadic contamination of PIF by using pulsed field gel electrophoresis (PFGE) to trace the prevalence of *Cronobacter* in a formula processing facility. In terms of control measures for *Cronobacter* spp. in PIF, recommendations have been made to promote breast feeding and inclusion of warnings on PIF and other breast milk substitutes (3).

In 2004, the FAO/WHO classified microorganisms of concern in PIF into three groups: A, B, and C (18). Group A are considered to be organisms with "clear evidence of causality" meaning they are a well-established cause of disease in infants and are found in PIF. Group B organisms are described as "causality plausible, but not yet demonstrated"

meaning they are not well-established causes of disease in infants. Group C pathogens are organisms that have not been identified in PIF, but do cause illness in infants. *Cronobacter* spp. and *Salmonella* were classified into Group A.

In a recently published study, samples collected from a PIF processing plant were analyzed for *Cronobacter* spp. prevalence (77). They found a higher prevalence of the bacteria in powders obtained from vacuum cleaner bags with 28% of positive samples collected from a single location. These findings support the conclusion that PIF is a nonsterile product and require effective control measures in the manufacturing plant as well as during reconstitution in hospitals or homes.

Methods of Identification

Cultural and biochemical methods

The primary means of biochemical identification and isolation of *Cronobacter* spp. has been based on the Food and Drug Administration (FDA) standard isolation method for *Enterobacteriaceae.* This method consists of enrichment of samples in Enterobacteriaceae Enrichment (EE) broth, subculturing on Violet Red Bile Glucose Agar (VRBGA), a further subculturing of selected colonies onto Trypticase Soy Agar (TSA), and subsequent biochemical identification is performed using the API 20E biochemical confirmation system (51, 57, and 96). Overall this procedure can take up to five days to complete and does not specifically select for *Cronobacter* spp. isolates, so there is a strong need for more precise and simplified methods of isolation/identification (32, 51, and 61). This has been the focus of the majority of research in biochemical identification.

Most of the chromogenic agar media developed is based on the enzyme α-glucosidase produced by *Cronobacter* spp. which hydrolyzes maltose to glucose by

cleaving the α-1, 4-glycosidic linkage in the maltose structure. Enzymatic profiling identified this enzyme in 129 *Cronobacter* spp. isolates and it was not detected in 97 other *Enterobacter* species (64.). This has lead to the development of highly selective and differential media to detect *Cronobacter* spp. in mixed cultures by using different substrates targeted by this enzyme. Leuschner et al. (52) developed a media for the presumptive identification of the organism consists of standard nutrient agar supplemented with 4-methyl-umbelliferyl α-D-glucoside (α-MUG). The α-MUG serves as a substrate metabolized by the α-glucosidase enzyme to produce a UV fluorescent product. *Cronobacter* spp. colonies will grow yellow on this media and fluoresce upon exposure to UV light. Druggan-Forsythe-Iversen (DFI) agar utilizes the chromogenic substrate 5-bromo-4-chloro-3-indolyl-α, D-glucopyranoside (XαGlc) to detect α-glucosidase production (31). XαGlc is hydrolyzed by the organism to an indigo pigment producing blue-green colonies on the agar (32). Yet another chromogenic media was developed by Oh and Kang (71) utilizing 4-methylumbelliferyl-α-D –glucoside as the fluorogenic substrate of α-glucosidase. *Cronobacter* spp. isolates on this media will fluoresce when exposed to UV light. It should be noted that α-glucosidase is not an enzyme limited to *Cronobacter* spp. and has been reported in some other members of *Enterobacteriaceae* (32).

E. sakazakii plating medium (ESPM) and *E. sakazakii* screening media (ESSM) have also been developed to assist in the detection of the organism in mixed cultures by Restaino et al. (78). ESPM contains two chromogenic substrates of α-glucosidase (5-bromo-4-chloro-3-idoxyl-alpha-D-glucopyranoside and 5-bromo-4-chloro-3-indoxyl-beta-D-cellobioside), three sugars (D-arabitol, sorbitol, and adonitol), and inhibitors (bile salts,

vancomycin, and cefsulodin). ESSM contains two sugars (melibiose and sucrose) which are metabolized by *Cronobacter* spp. to produce acid yielding a yellow color on the media. The three sugars present in ESPM are fermented by the organism to produce blue-black/blue-gray colonies on the plate. ESPM and ESSM are often used together to identify *Cronobacter* spp. in environmental and food samples. The advantage of this system is the inclusion of two substrates which minimize the possibility of missing certain *Cronobacter* spp. isolates that are only weakly positive for a single substrate. In their study, Restaino et al. (78) recovered a greater number of *Cronobacter* spp. isolated using EPSM-ESSM than by the standard FDA procedure and there were fewer false-positive and false-negative results.

Recently, a differential enrichment broth, "*Cronobacter*" screening broth (CSB), was developed by Iversen et al. (37). This broth can be used in complement with any chromogenic agar medium and allows samples to be screened for *Cronobacter* spp. without using selective agents that may minimize the recovery of sensitive strains. Detection of *Cronobacter* spp. is achieved by the inclusion of sucrose and bromocresol blue pH indicator. The broth will change color from purple to yellow when sucrose is fermented by *Cronobacter* spp. strains (37).

A study conducted by Mullane et al. (61) found that cationic-magnetic-bead capture techniques can be used in conjunction with the chromogenic media to improve the sensitivity of detection in PIF. In the study, they were able to detect between 1 and 5 CFU in 500 grams of PIF in less than 24 hours.

Molecular methods of identification

Molecular methods have also been developed to detect and characterize *Cronobacter* spp. These are more rapid and specific than traditional biochemical culture methods (56, 84). Malorny and Wagner (84) developed a real-time (RT) PCR method for the specific detection of *Cronobacter* spp. that targeted the 16S rRNA gene. Seo et al. (56) developed a more specific RT PCR method by targeting a specific sequence within the macromolecular synthesis (MMS) operon. The MMS operon consists of 3 genes: *rpsU*, *dnaG*, and *rpoD*. These genes are involved in protein, DNA, and RNA synthesis. The *dnaG- rpoD* intergenic sequence has been found to differ in length and primary sequence between different species (98). Using this RT PCR method they were able to discriminate *Cronobacter* spp. among 10 other *Enterobacter* and 55 non-*Enterobacter/Cronobacter* bacteria, detect approximately 100 colony forming units (CFU)/ml of *Cronobacter* spp. in PBS and powdered infant milk with enrichment procedure, and detect low numbers of the bacteria (0.6 CFU/g) in PIF with only a single enrichment performed.

Random amplification of polymorphic DNA (RAPD) is a type of PCR reaction found to be a very discriminatory typing method for *Cronobacter* spp. (66). RAPD allows random amplification of the DNA segments using short primers (8-12 nucleotides) with no previous sequence knowledge needed. This method has been used in a number of studies to type *Cronobacter* spp. strains (30, 66). Further, pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), ribotyping, and multilocus enzyme electrophoresis tests have also been used to characterize *Cronobacter* spp. isolates (25). Stoop et al (89) developed various PCR systems, based on the *rpoB* gene, that are able to differentiate between the different species within the genus *Cronobacter* (89).

Recently, the FDA has developed a new method to detect *Cronobacter* spp. in PIF.

This method involves enrichment in media made with buffered peptone water, followed by PCR for screening, and two different chromogenic agars for obtaining isolated colonies of *Cronobacter*. Then, PCR and RAPID ID 32E (bioMèrieux, Durham, NC) biochemical tests are performed to confirm culture identification obtained from the agars (47). This procedure is undergoing collaborative validation study for final adoption into the FDA *Bacteriological Analytical Manual.*

Biofilm Formation

Cronobacter spp. is one of the many bacterial species able to form biofilms under certain conditions. Biofilms are sessile communities of microbial organisms attached to a surface or each other via polymeric substances produced by the bacteria (41). These biofilms provide a physical barrier to protect cells from various environmental stresses such as UV light, detergents, antibiotics, heat, and antibodies (12). The ability of *Cronobacter* spp. to form these biofilms is temperature and nutrient dependent. Kim et al. (41) conducted a study investigating the effects of temperature and nutrient availability on the attachment and biofilm formation of *Cronobacter* spp. They found more bacteria attached to the tested materials (stainless steel and enteral feeding tubes) at 25°C than at 12°C. In fact, they found no biofilm formation occurring at 12°C. Iversen et al. (33) found encapsulated *Cronobacter* spp. cells grown in reconstituted PIF formed biofilms on silicon, latex, polycarbonate, and to a lesser extent stainless steel. These isolates contained the capsule and formed biofilms to a greater extent than the non-encapsulated strains tested. These materials are commonly used for infant feeding equipment and in formula preparation utensils. Scheepe-Leberkühne and Wagner (82) described the production of an exopolysaccharide capsular material which may enhance attachment and adherence of

Cronobacter spp. to these types of surfaces. Lehner et al. (50) investigated the ability of *Cronobacter* isolates from various sources to form biofilms, express cellulose-like fibrils as part of the surface pellicles, and produce extracellular polysaccharide (EPS). Pellicle and flock formation was more pronounced on strains grown on Brain Heart Infusion (BHI) agar. 24.8% of the isolates tested produced EPS, which may contribute to the tolerance of *Cronobacter* spp. to high osmotic pressure and dry conditions. They also found the *Cronobacter* spp. bacteria possessed the genes for cellulose biosynthesis. Bacterial cellulose has a structural role in other bacteria species. Specifically it confers mechanical, chemical, and biological protection as well as assists with the formation of cell adhesion processes that are needed for infection (79). The specific role of cellulose in *Cronobacter* spp. pathogenesis and biofilm formation remains to be determined. Almost half (41.8%) of the isolates tested in the study were able to produce EPS. EPS prevents desiccation in the biofilm and may contribute to the tolerance of *Cronobacter* spp. in high osmotic pressure and dry conditions (20, 50).

Zogaj et al. (100) also investigated the ability of two *Cronobacter* spp. isolates to produce cellulose and curli fimbriae. They found the two *Cronobacter* isolates showed an extracellular matrix, cell clumping, pellicle formation, and biofilm formation associated with cellulose and curli fimbriae expression.

More recently, Dancer et al. (13) investigated the effect milk components have on biofilm formation of *Cronobacter* spp. They focused on three milk components: lactose (carbohydrate source), casein proteins, and whey proteins. The skim milk was diluted and added prepared solutions of lactose, casein protein, or whey protein so as to achieve the levels present in undiluted skim milk. This allowed then to target each specific component

and determine its influence on biofilm formation by *Cronobacter* spp. The nitrogen source (proteins) in skim milk has a greater influence on biofilm formation than the carbohydrate source because the bacteria grown in the diluted milk with added protein (casein or whey proteins) seemed to have increased biofilm production (13). Biofilm formation was observed under a scanning electron microscope and they found no capsule or extracellular substance present on the cells in the biofilm. However, this does not necessarily mean the capsule is not present in these isolates.

The Blood Brain Barrier

The BBB is an impermeable barrier in the brain capillaries. These capillaries are about 50-100 times more impermeable than the microvessels in the peripheral system, which is a unique characteristic of the BBB (1). It controls the flow of compounds from the bloodstream to the brain. Only small lipophillic molecules, namely oxygen and carbon dioxide, can freely diffuse across the barrier (4, 23, and 75). The presence of this impermeable barrier prevents harmful substances, for example pathogens and microbes that gain access to the bloodstream thereby invading the brain.

Cellular components

The basic cellular components of the BBB are endothelial cells, astrocytes, and pericytes. Endothelial cells line the blood vessels at the border between the blood and brain (74). They are joined together by tight junctions and form the characteristic impermeable barrier of the BBB. They perform many necessary biological functions including transport of nutrients, receptor-mediated signaling, leukocyte trafficking, and osmoregulation (regulation of the osmotic pressure in the blood) (74). Astrocyte cells are star-shaped glial cells ensheathing the blood vessels. They use their "end feet" to encircle

the endothelial cells and mediate the formation of tight junctions between these cells (4). *In-vitro* studies suggest a co-culture of endothelial cells with astrocytes is important in the maintenance of blood brain barrier tightness and function under pathophysiological and physiological conditions (29, 74). Besides mediating tight junction formation, astrocytes have many other functions in the CNS including distribution of nutrients to neurons, maintenance of extracellular ion balance, and repair of CNS tissue following traumatic injury (4, 74). Pericytes are the least studied cellular component of the blood brain barrier. They wrap around the endothelial cells in the blood vessel. It is believed they play a key role in structural support of the vessel wall and provide vasodynamic capacity to the microvasculature (4, 53). Likewise, they also may regulate endothelial cell proliferation, survival, migration, differentiation, and vascular branching (74).

Structural components

Tight junctions (or zonula occludens (ZO)) are known to form between the endothelial cells making up the BBB and directly influence its impermeability. These structures are more complex than the tight junctions found in other areas of the body (ex: testes in males and the kidneys). Tight junctions are made up of three integral membrane proteins, namely claudin, occuldin, and junction adhesion molecules (JAMs) (4). These proteins are connected to the actin cytoskeleton of the endothelial cells by peripheral membrane proteins. Claudins are responsible for regulating the diffusion of ions across the BBB (57). Occludin has been associated with regulations of the diffusion of small hydrophilic molecules and transmigration of neutrophils across the barrier (57). JAMs are members of the immunoglobulin superfamily (cell surface receptors). These are involved in cell-to-cell adhesion and leukocyte transmigration through the BBB (4, 74). A number

of cytoplasmic accessory proteins (ZO-1, ZO-2, ZO-3, cingulin, and others) are also present. These domains help organize proteins at the plasma membrane of the endothelial cells (4).

Adherens junctions are similar to tight junctions except they are usually found in the basal compartment of the cell- to- cell junction. In these junctions, the cytoplasmic catenin proteins are linked to the actin cytoskeleton via cadherin proteins (transmembrane adhesive proteins). Both tight junctions and adherens junctions are known to interact and influence tight junction assembly (58).

Use of blood brain barrier models in research

In-vivo studies of the BBB are limited because of inaccessibility of the abluminal part of the brain capillary endothelial cells. Likewise, there have been difficulties studying transport and metabolism of the *in-vivo* barrier (16). For this reason, *in-vitro* models of the BBB have been developed. Several models have been developed including monoculture, co-culture, and triple co-culture systems (7, 16, 48, 54, 55, 65, and 92). These models are cheaper than *in-vivo* models, highly repeatable, and feasible in investigating regulatory mechanisms at the molecular level (70). *In-vitro* BBB models have been used in research involving drug delivery systems, physiological mechanisms, and the permeability of the barrier in health and disease (54). A model consisting of a monolayer of HBMECs has been developed to study invasion of *E. coli* K1 through the BBB (90). The majority of these *in-vitro* models have used primary brain capillary endothelial cells from bovine, human, rodents (mostly rat) and porcine origin (15). However, a few research groups have investigated the use of secondary cell culture lines for this purpose due to cost constraints,

ease of growth, and lower risk of contamination compared with other neurovascular unit cell lines (54).

Transendothelial Electrical Resistance (TEER)

As discussed in the previous section, the BBB is a permeability barrier restricting the movement of molecules and ions into the brain. In terms of BBB models, measurement of the resistance across the layer of cells is the most straightforward method to determine the permeability of the model (15). This resistance is referred to in the literature as the transendothelial (or transepithelial if working with epithelial cells) electrical resistance commonly abbreviated as TEER or TER. The TEER system has been used extensively to measure resistance in *in-vitro* and *in-vivo* BBB models (7, 10, 15, 16, 48, 54, 55, 59, 65, and 92). The first TEER measurements were reported in 1987 by Hart et al. and Rutten et al. using the Ussing chamber setup (27, 80). For cell cultures grown on a microporous filter of culture inserts, Volt-Ohm meters are typically used to perform TEER measurements. They can be equipped with either chopstick or chamber style electrodes. Chamber style electrodes allow measurements with lower background TEER values and the chopstick style allows for fast and repeated measurements of different treatment groups in the BBB model (15). TEER measurements are expressed as the resistance measured multiplied by the area of the cell layer (i.e. the area of the membrane/filter the cells are grown on) or the Unit Area Resistance (UAR) (15).

MATERIALS AND METHODS

Cell and Bacteria Cultures

The identification code and source of the bacterial isolates used in the experiments are summarized in Table 1.

Table 1. Summary of Bacteria Isolates Used in the Study

Sample ID	Organism	Source
11775 (positive control for culture models)	*Escherichia coli* K1 (pathogenic)	American Type Culture Collection (ATCC) (Manassas, VA)
DH5α (negative control for culture models)	*Escherichia coli* K12 (non-pathogenic)	Invitrogen (Carlsbad, CA)
CT2	*Cronobacter* spp.	Meninges of infant
N72-1A	*Cronobacter* spp.	North Dakota Bovine Fecal Sample
AJW678 (positive control for biofilm quantification)	*Escherichia coli* K12 (Wild-type)	The Coli Genetic Stock Center (CGSC)- Yale University
BP1085 (negative control for biofilm quantification)	*Escherichia coli* K12 (*fimH* mutant)	Dr. Birgit Pruess North Dakota State University

The secondary cell lines used include C8-D1A astrocyte type 1 (ATCC CRL 2541) clones and EOMA (ATCC CRL 2586), which are adherent brain hemangioendotheioma endothelial cells. Both secondary cell lines were obtained from mouse. The cells were propagated in Dulbecco's Modified Eagle's Medium (DMEM) (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and antibiotics (10,000 U/ml penicillin, 10,000 μg/ml streptomycin, and 25 μg/ml

amphotericin B) (Atlanta Biologicals, Lawrenceville, GA). The media was changed every other day to maintain cell growth and viability. Some cells were cryogenically frozen back and stored in liquid nitrogen and at -130°C.

Biotyping of *Cronobacter* spp. Isolates

Biotyping had previously been done for the *Cronobacter* spp. isolates in our lab, but we wanted to validate the results for the isolates we would be using in our study. Biochemical tests were performed as follows for phenotypic characterization of the *Cronobacter* spp. isolates, as previously described by Iversen et al. (35, 36). *E. coli* K1 strain (ATCC #29522) was included as a positive control isolate. Stock bacteria cultures were stored at -80°C in Luria Bertani (LB) broth/50% glycerol (Sigma-Aldrich, St. Louis MO). Bacterial cultures were streaked for isolation onto LB agar plates (Sigma-Aldrich, St. Louis MO), grown overnight and single colonies were inoculated into the biochemical media tubes. Each bacteria strain was replicated three times to ensure consistent results. Motility was determined on motility-indole-ornithine (MIO) media. Indole production was tested by adding Kovac's reagent (Siemens, Fort Madison, IA B1010-41A) to colonies on the MIO agar. Acid production from the carbohydrates dulcitol (usb 15440) and methyl-α-D-glucopyranoside (AMG) (Sigma- Aldrich, St. Louis, MO m9376) was tested using a phenol red broth base (Acumedia, Lansing, MI 7148A) at a final concentration of 0.5%. Gas production from the carbohydrates was determined by collection in Durham tubes. Malonate utilization was assayed using sodium malonate broth (Sigma, St Louis, MO M8802).

Construction of Culture Systems and TEER

Construction of monoculture model

Millipore™ Millicell Hanging Cell Culture Inserts (for 24-well plates) containing polyethylene terephtalate (PET) membranes (1.0 μm pore-size) (Millipore Billerica, MA) were treated with 20 μg/ml fibronectin (Sigma-Aldrich, St Louis, MO). Viability of endothelial cell lines was determined using Trypan Blue dye cell counting on a hemocytometer. The cells were adjusted to a concentration of $20*10^5$ cells/ml and added to the topside of each cell culture inserts in 24 well plates containing 1.2 ml of complete DMEM (HyClone, Logan, UT) supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA) and antibiotics (10,000 U/ml penicillin, 10,000 μg/ml streptomycin, and 25 μg/ml amphotericin B) (Atlanta Biologicals, Lawrenceville, GA). DMEM media is a common cell culture media containing 40 mM L-Glutamine, 4,500 mg/L Glucose, Sodium pyruvate, and 3.7 g/L Sodium bicarbonate. This type of cell culture media was recommended by ATCC for propagation of our cell lines. Plates were incubated at 37°C, 5% CO_2 and media (DMEM, 10% FBS and antibiotics) was changed every other day. Confluency was determined by microscopic examination and trans-endothelial electrical resistance (TEER) measurements with an STX2 electrode and EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL).

Co-culture of endothelial cells and astrocytes

Viability of the astrocyte and endothelial cell lines was determined using Trypan Blue dye cell counting on a hemocytometer. The C8-D1A astrocyte cells were adjusted to a concentration of $16*10^4$ cells/ml and transferred to the underside of Millipore™ Millicell Hanging Cell Culture Inserts (for 24-well plates) containing polyethylene terephtalate

(PET) membranes (1.0 μm pore-size) (Millipore, Billerica, MA). The inserts were placed upside down in 12-well plates and incubated at 37°C with 5% CO_2 for 2 hours to allow the cells to attach to the membrane. Then, the membrane inserts were turned over and placed into 24-well plates containing about 700 μl of complete DMEM (HyClone, Logan, UT) with 10% FBS and antibiotics (Atlanta Biologicals, Lawrenceville, GA). The plates were incubated overnight at 37°C, 5% CO_2. This allows the astrocytes to grow on the bottom of the membrane. The next day, the EOMA endothelial cells were seeded onto the topside of the membrane at a concentration of $20*10^5$ cells/ml. Media was changed on both sides of the membrane inserts every 2-3 days. Confluency (100%) was determined by microscopic examination and transendothelial electrical resistance (TEER) measurements with an STX2 electrode and EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL).

Preparation of bacterial cultures

The following two sections outline the procedure for bacteria treatment used for both the monoculture and co-culture systems. Each bacterial culture was inoculated into Luria Broth (LB) broth (EMD Chemicals, Gobbstown, NJ) and incubated at 37°C overnight to allow the cells to reach stationary phase growth. The bacteria cells were pelleted by centrifugation at 4000 rpm for 15 minutes. The pellet was resuspended in 5 ml of 1x PBS. To determine the CFU/ml present in the culture, the OD_{600} was measured using a BioMate 3 spectrophotometer (Thermo Scientific, Waltham, MA) and the cultures were diluted so as to obtain 1000 CFU/ml of each sample.

Infection of culture model

The complete DMEM with 10% FBS and antibiotics was removed from membrane inserts and the plate wells. The membranes were gently rinsed with 1x PBS and complete

DMEM media with 10% FBS (no antibiotics) was added to each well and insert. The plates were incubated at 37°C and 5% CO_2 for 3-4 hours. 100 µL of each bacterial culture (at 1000 CFU/ml) was added to each cell culture. Cell control treatments were incubated in the absence of bacteria and blank media control wells (inserts without cells or bacteria added) were included. Each treatment (bacteria, cell control, and media control) were done in triplicate on two plates for a total of six replicates per sample in each experiment. The plates were incubated at 37°C and 5% CO_2. TEER measurements are taken at 0, 6, 24, and 48 hours post-infection using the EVOM voltohmmeter with STX2 electrode (World Precision Instruments, Sarasota, FL). Before conducting the TEER measurements, the complete DMEM was removed from the plates and replaced with incomplete DMEM (no FBS or antibiotics added). After completion of the TEER readings, complete DMEM media was added back into the wells. Since the cell integrity seemed to decrease in the first experiment at 48 hours post-infection, the plates were only incubated for 24 hours in the second experiment taking TEER readings at 0, 6, and 24 hours.

Statistical analysis

Data were analyzed using SAS 9.2, the mean values for UAR measurements for each sample on each plate accessed. Least square means was analyzed for effect of each treatment (bacteria samples, cell control, and media control) with H_o: no difference between treatments. Student's t-test and One-way ANOVA were used to analyze the data using GraphPad Prism® 5. We also conducted a Bonferroni's Multiple Comparison Test (95% confidence interval) to examine any differences between the sample means. P-values less than 0.05 were considered significantly different.

Biofilm Quantification

Biofilm formation of the *Cronobacter* spp. isolates used in the TEER assays was quantified in sterile 96-well polystyrene microtiter plates as previously described by O'Toole et al. and Skyberg et al. (72, 86). Bacterial samples were grown overnight on Luria-Bertani (LB) agar at 37°C. A colony from each plate was transferred to 5 ml tubes of LB broth and incubated overnight at 37°C. Then, the culture was diluted 1:100 in LB broth and 200 µl of the diluted sample was transferred to a sterile 96-well plate. Sterile media was used as the blank control. An isolate known to be biofilm-forming positive (*E. coli* strain AJW678) and a non-biofilm former (BP1085) were included as controls (Table 1). Kanamycin resistance is a marker for the fimH mutant. Duplicate plates were made with 6 replicates per plate. One plate was incubated at 37°C and the other was incubated at 25°C for 48 hours without shaking. The media was removed from the wells and the plates washed with sterile PBS at a pH of 7.4. Next, the biofilms were stained with 200 µl of 0.1% Crystal violet solution for 10 minutes. After staining, the plate was washed four times with PBS and air-dried for 1 hour. The Crystal violet stain was extracted from the biofilms with 200 µl of an 80: 20 ethanol: acetone solution. The extracted stain was diluted 1:10 in new 96-well plates, which were read in a microplate reader at a wavelength of 600 nm to obtain the optical density (OD) of each well. All tests were carried out in six wells and conducted three times to ensure consistency of the results. Based upon the optical density (OD) produced by Crystal violet uptake of the bacterial biofilms, isolates were classified into four categories: non-biofilm producer, weak, moderate, or strong, using a previously described method (87). The cutoff OD (ODc) was defined as three standard deviations above the mean OD of the negative control. Strains were categorized as follows: OD<

ODc= no biofilm production; ODc< OD < (2 x ODc) = weak biofilm producer; (2 x ODc) < OD < (4 x ODc) = a moderate biofilm producer; and (4 x ODc) < OD = a strong biofilm producer.

RESULTS

Cronobacter spp. Isolates Identified as *Cronobacter sakazakii*

The results for biochemical characterization of the two *Cronobacter* spp. isolates included in this study are summarized in Table 2.

Table 2. Biochemical Characterization of *Cronobacter* spp. Isolates

Sample	Dulcitol	AMG	MIO	Malonate	Species
CT2	—	+ acid, gas	Motile, —	—	*Cronobacter*
	—	+ acid, gas	Motile, —	—	*sakazakii*
	—	+ acid, gas	Motile, —	—	
N72-1A	—	+ acid, gas	Motile, —	—	*Cronobacter*
	—	+ acid, gas	Motile, —	—	*sakazakii*
	—	+ acid, gas	Motile, —	—	

The results of this experiment indicate both isolates that were used in this study are *Cronobacter sakazakii*. These results were further confirmed with the previous phenotypic analysis performed. Throughout the remainder of this paper, we will refer to the isolates by their species names as shown in Table 2.

E. coli K1 and *C. sakazakii* Significantly Decreased TEER in Monoculture Model

A monoculture system was constructing by seed endothelial cells onto cell culture inserts containing 1.0 μm pore membranes. Bacteria were added to the monoculture models and the effect of the bacteria on the cells was determined with TEER measurements. *E. coli* K1 (11775) and *E. coli* K12 (DH5α) were included as positive and negative controls, respectively (Table 1). *E. coli* K1 is a frequent cause of bacterial meningitis, so we expected these bacteria to disrupt the endothelial cells and decrease the

TEER measurements. *E. coli* K12 is a non-pathogenic attenuated lab strain and should not have much or any effect on the BBB cells in the monoculture. *C. sakazakii* isolates (CT2 and N72-1A) were test treatment groups (Tables 1 and 2). Cell and media control were included for comparison to the bacteria treatments.

The TEER measurements for this experiment are summarized in Figure 1. The media control maintained lower TEER values than the cell control treatments and the measurements at the final reading were found to be significantly different ($P < 0.001$). This observation indicates the endothelial cells were maintained on the membranes throughout the course of the experiments. The positive control decreased the TEER over time as we expected. This was the *E. coli* K1 strain (Table 1), which is a common cause of infant meningitis (40, 90). Likewise, the two *C. sakazakii* isolates caused a decrease in TEER (Figure 1). In this case, the CT2 isolate seemed to decrease the TEER more so than the N72-1A isolate, but no significant differences were found between these isolates at the final reading (48 hours) ($P > 0.05$). At the final reading the TEER measurement both *C. sakazakii* isolates (CT2 and N72-1A) were statistically similar to the positive control (*E. coli* K1) ($P > 0.05$ for all). The TEER values for the positive control treatment were significantly different from the cell control ($P < 0.001$), which indicates the positive control did disrupt the endothelial cells. The negative control (*E. coli* K12- DH5α) (Table 1) caused TEER to decrease only at 6 hours after adding the bacteria (Figure 1). This observation could be due to the toxic effect the LPS (specifically the lipid A endotoxin) has on the endothelial cells because even though this is an attenuated, non-pathogenic strain of *E. coli* it would still possess the lipid A endotoxin in its cell wall. Lipid A endotoxin is known to be toxic to cells. In spite of this observation, the negative control did not

decrease the TEER to the same extent as the positive control, CT2 and N72-1A treatments at the final reading ($P < 0.001$). The TEER values increased at 24 hours and decreased at 48 hours in all the treatment groups (Figure 1). The increase at 24 hours could be due to the bacterial being pulled off the cells and discarded during the media change at the 6 hour reading. This increase in TEER did not occur with the *C. sakazakii* isolates because they may have adhered to the endothelial cells and formed a biofilm on them. Biofilm formation would have allowed them to resist being pulled off the cells and discarded. The *E. coli* isolates we used as control did not have this ability. The decrease in all treatment groups at the 48 hours reading could have occurred because the loss in cell viability due to excessive exposure to electrical impulses with each TEER measurement performed.

Overall, these results show the monoculture model worked as expected. The positive control (*E. coli* K1) decreased TEER measurements across the endothelial cells over time. This effect was previously described by Sukumaran et al. (90). The *C. sakazakii* isolates both had to same effect on the TEER as the positive control (*E. coli* K1) (Figure 1). We expected the CT2 isolate to have this effect because it was from a meningitis case, but to see similar results with the N72-1A isolate (from bovine fecal grab) was intriguing. This indicates the N72-1A isolate has the potential to be pathogenic and cause meningitis. The negative control (*E. coli* K12) had no effect on the TEER over time, except at the 6 hour reading. The results from the *t*-test analysis for the monoculture model are summarized in Figure 2. The cell control and negative control were not significantly different from each other ($P > 0.05$), but they both were different from the other samples (positive control, CT2, N72-1A, and media control) ($P < 0.001$) (Figure 2). The positive control, CT2, N72-1A, and media control were all not significantly different from each other ($P > 0.05$)

(Figure 2). Since this model consisted of only endothelial cells, the cell barrier may have been less impermeable, allowing the bacteria to disrupt the cells more extensively. This would cause the TEER measurements for these treatment groups to decrease to a greater extent and be similar to the media control at the final reading.

E. coli K1 and *C. sakazakii* Significantly Decreased TEER in Co-Culture Model

The co-culture model was constructed in a similar manner as the monoculture model, except astrocytes were also cultured in the underside of the cell culture insert membrane.

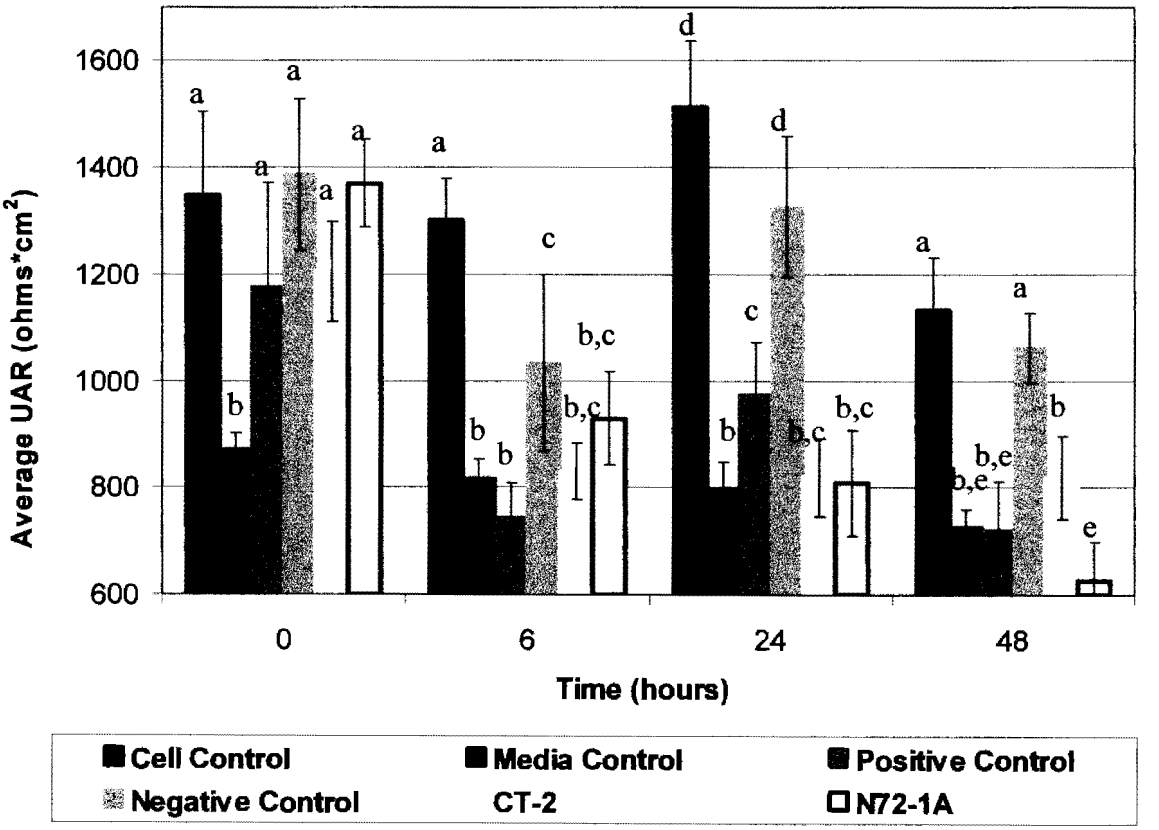

Figure 1. TEER measurements for monoculture system. UAR= Unit Area Resistance. Letter superscripts (a, b, c, d, and e) signify the statistical relationship between the data points.

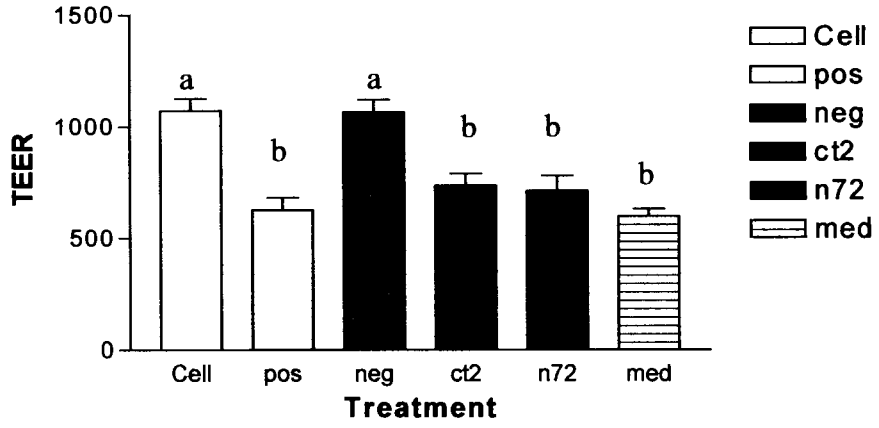

Figure 2. Student *t*-test data for monoculture model. Letter subscripts (a and b) signify the statistical relationship between the data points
Cell = cell control, pos = positive control, neg = negative control, ct2= CT2, n72 = N72-1A, and med = media control

The 1.0 μm pores in the membranes allowed interaction between the endothelial cells and astrocytes to still occur as it does in the *in-vivo* BBB, but still keeps the cell types physically separated as they are found in the BBB. A total of two experiments were conducted using this model. In the first experiment, TEER measurements were taken at the same timepoints as the monoculture (0, 6, 24, and 49 hours). For the second experiment, TEER measurements were taken at 0, 6, and 24 hours. Statistical analysis was performed on the final reading TEER values for the final reading in the second experiment (24 hours).

TEER measurements for the first co-culture model constructed are summarized in Figure 3. The positive control sample (*E. coli* K1) significantly decreased the TEER values across the co-culture over time as seen with the monoculture model. The greatest decrease was observed at 6 hours after addition of the bacteria. The *C. sakazakii* treatments revealed similar effect on the TEER readings as the positive control (*E. coli* K1) treatment. The negative control (*E. coli* K12) did not significantly affect the TEER measurements until after 24 hours.

As seen with the monoculture model, an increase in the TEER values for the positive control (*E. coli* K1) and the negative control (*E. coli* K12) was observed at 24 hours and decrease in the TEER measurements at 48 hours was observed in all treatment groups. The increase at 24 hours could be due to the bacterial being pulled off the cells and discarded during the media change at the 6 hour reading. This increase in TEER did not occur with the *C. sakazakii* isolates because they may have adhered to the endothelial cells and formed a biofilm on them. Biofilm formation would have allowed them to resist being pulled off the cells and discarded. The *E. coli* isolates we used as control did not have this ability. The decrease in all treatment groups at the 48 hours reading could have occurred because the loss in cell viability due to excessive exposure to electrical impulses with each TEER measurement performed.

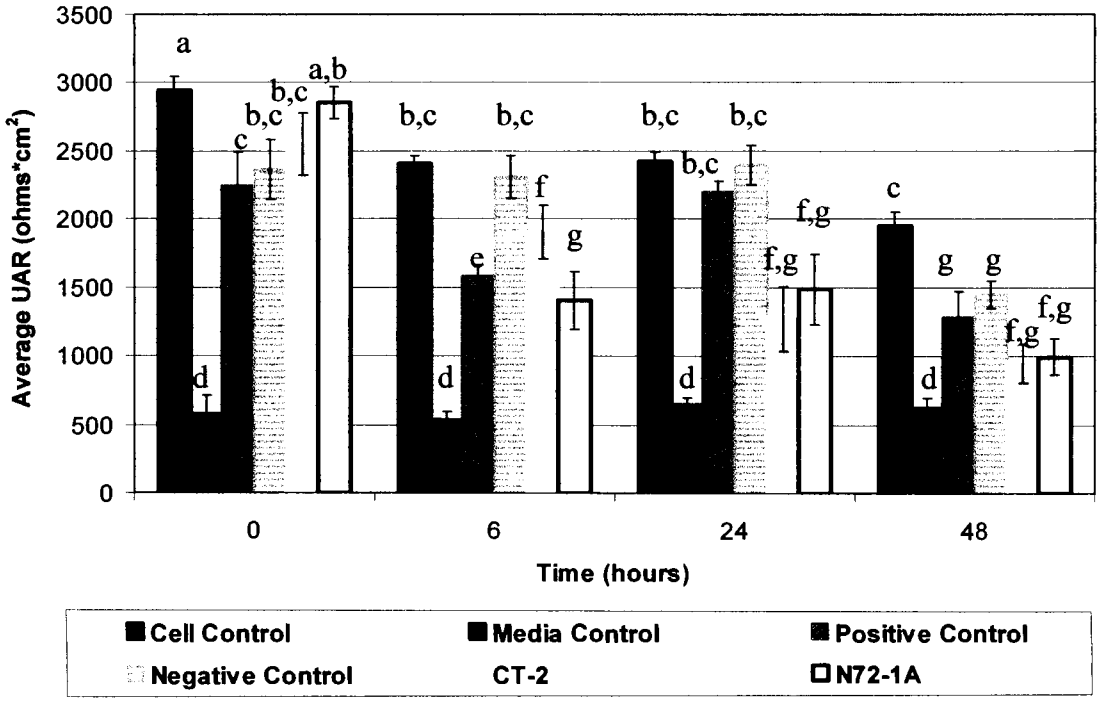

Figure 3. TEER measurements for co-culture model- Experiment 1. UAR = Unit Area Resistance. Letter superscripts (a, b, c, d, e, f, and g) signify the statistical relationship between the data points.

For the second experiment using the co-culture system, TEER measurements were taken up to 24 hours since the cell integrity seemed to decrease in all sample wells at 48 hours in experiment 1 (Figure 3). The TEER measurements for this experiment are illustrated in Figure 4.

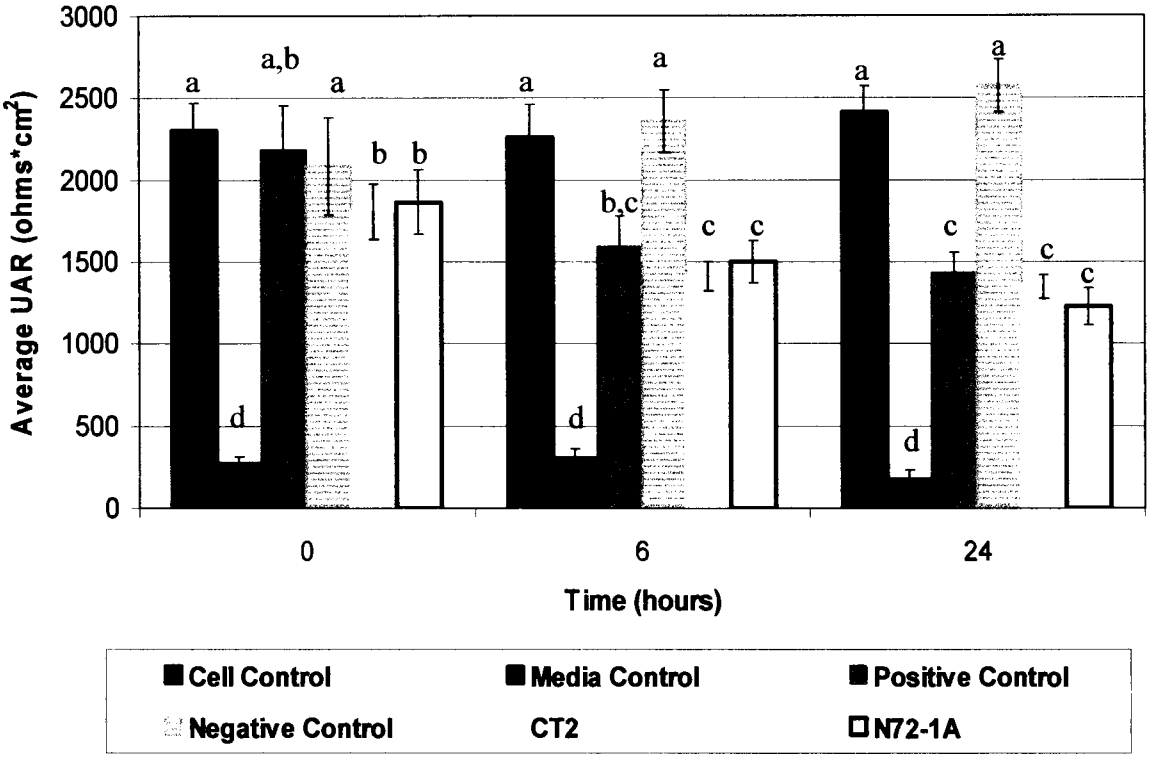

Figure 4. TEER measurements for co-culture model- Experiment 2. UAR= Unit Area Resistance. Letter superscripts (a, b, c, and d) signify the statistical relationship between the data points.

Similar trends can be seen in the TEER measurements in the second experiment as with the first experiment. The increase in TEER measurements at the 24 hour reading is absent in these results because the bacteria treatments were saved and added back to the appropriate wells at the 6 hour media change (Figure 4).

Statistical analysis was performed on the TEER values at the final reading (24 hours) for this experiment and the results are summarized in Figure 5 and discussed below.

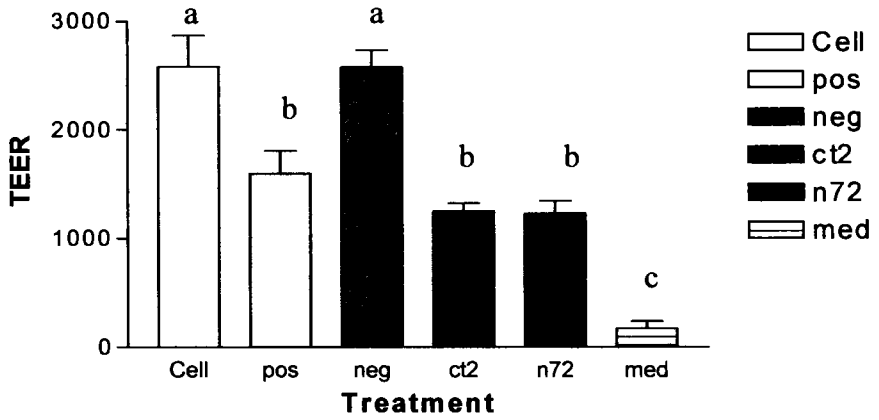

Figure 5. Student *t*-test data for co-culture model. Letter subscripts (a, b, and c) signify the statistical relationship between the data points
Cell = cell control, pos = positive control, neg = negative control, ct2= CT2, n72 = N72-1A, and med = media control

The results shown here are similar to the monoculture model, aside from the initial measurements at 0 hours (Figure 2). The cell control and negative control are statistically similar ($P > 0.05$). Unlike with the monoculture model, the positive control, CT2, and N72-1A are significantly different from the media control ($P < 0.001$). This observation could have occurred because the endothelial cell barrier formed in this co-culture model is supported by the astrocyte cells, so the pathogenic bacteria (positive control, CT-2 and N72-1A) could not disrupt it to as great of an extent as with the monoculture model where astrocytes were absent. The positive control was different from the cell and negative control treatments as well, which was also seen in the monoculture ($P < 0.001$ and $P < 0.001$, respectively). This means the positive control was able to disrupt the endothelial cells in the co-culture in the same manner as those in the monoculture system. The *C. sakazakii* isolates were also different from the cell and negative control treatments ($P < 0.001$ and $P < 0.001$, respectively).

According to these results, the *C. sakazakii* isolates were able to have the same effect on the endothelial cells in the co-culture model as those in the monoculture system. Since the co-culture model is a more realistic representation of the BBB, the *C. sakazakii* isolates would have the potential to be pathogenic and cross the BBB during infection.

CT-2 and N72-1A Found To Be Non-Biofilm Formers with Crystal Violet Assay

The Crystal violet staining assay used had been previously described by O' Toole et al. (72) and Skyberg et al. (86). Overnight bacteria cultures were diluted 1:100 and transferred to two 96-well plates. The positive control was a known biofilm former (AJW678) and the negative control was a *fimH* mutant (BP1085) (Table 1). Six replicates of each sample were included in each plate and sterile media represented the blank control. One plate was incubated at 25°C and the other at 37°C for 48 hours. The media was removed from the wells and the plates were washed with sterile PBS. The biofilms were stained with Crystal violet for 10 minutes and then the plates were washed four times with PBS. After air drying for 1 hour the Crystal violet stain was extracted from the biofilms with a 80:20 ethanol:acetone solution. The extracted stain was diluted 1:10 in new 96-well plates and these were read on a spectrophotometer at a wavelength of 600 nm to determine the OD value for each well. The samples tested were classified according to the method described by Stephanovic et al. (87). The cutoff OD (ODc) value was determined for the negative control, which was defined as three standard deviations above the OD value of this sample. Then, the other samples were categorized as follows: OD < ODc= no biofilm production; ODc < OD < 2x ODc= weak biofilm producer, (2x ODc) < OD < (4x ODc) = moderate biofilm producer, and (4x ODc) < OD = a strong biofilm producer. The results obtained for the quantification of biofilm formation is listed in Table 3. In the first column

of the table are the bacteria samples tested. The second column contains the OD values for each of the bacteria tested. The ODc, 2x ODc, and 4x ODc values were calculated for the negative control (bottom row of Table 3) to compare to the OD of the other isolates as described by Stephanovic et al. (87). These comparisons are represented in column 3, 4, and 5 of Table 3. According to these results, the *C. sakazakii* isolates we used in this study were non-biofilm producers. Although this was the only biofilm quantification assay used in this study, there are many other ones described in the literature. Thus, another biofilm quantification assay should be performed on these samples before it can be concluded they are indeed non-biofilm producers.

Table 3. Biofilm Quantification Results of *Cronobacter sakazakii* Isolates.

Sample ID	OD		ODc		2x ODc		4x ODc		Biofilm production	
	25 °C	37°C	25 °C	37°C	25 °C	37°C	25 °C	37°C	25 °C	37°C
CT2	0.158	0.265	**0.158** < **0.183**	**0.265** < **0.856**	0.158 < 0.366	0.265 < 1.713	0.158 < 0.732	0.265 < 3.435	None	None
N72-1A	0.104	0.243	**0.104** < **0.183**	**0.243** < **0.856**	0.104 < 0.366	0.243 < 1.713	0.104 < 0.732	0.243 < 3.435	None	None
Positive control	1.015	0.549	1.015 > 0.183	**0.549** < **0.856**	1.015 > 0.366	0.549 < 1.713	**1.015** > **0.732**	0.549 < 3.435	Strong	None
Negative control	0.102	0.285	0.183	0.856	0.366	1.713	0.732	3.435	None	None

OD= optical density of samples, ODc= cutoff OD (calculated for negative control on bottom row of table and compared to OD values of samples in middle rows of table)

DISCUSSION

Cronobacter spp. is an emerging foodborne pathogen and the cause of rare, but serious CNS disease in neonates. The specific pathogenesis of *Cronobacter* spp. is largely unknown. In this study, an *in-vitro* co-culture model was developed to assist with characterizing the pathogenicity of certain *Cronobacter* spp. strains. No other study in the literature has reported developing such a model of the BBB to study this microorganism. An endothelial monolayer model was developed for studying the invasion of HBMECs by *E. coli* K1 strains (90) and *Cronobacter* spp. (93). This paper proposes a co-culture model consisting of endothelial cells and astrocytes because this would be a more realistic representation of the actual BBB *in-vivo*. Other studies examining the function of the BBB have unanimously stated astrocytes do significantly increase the integrity of the endothelial cells making up the BBB (4, 7, 16, 29, and 74). Monoculture, co-culture, and triple co-culture models have been previously developed for used to study BBB physiology, drug delivery systems, and BBB permeability under physiological or pathological conditions (7, 16, 48, 54, 55, 65, and 92).

E. coli K1 and *C. sakazakii* Significantly Decreased TEER in Monoculture Model

A preliminary experiment using a monoculture system containing only endothelial cells was performed for comparison to the co-culture system. The purpose of this was to justify the advantage of using a co-culture system rather than a monoculture system. In addition of constructing the co-culture model, biofilm quantification was also performed on the isolates (Table 3) for comparison with the co-culture results as previously explained.

We tested the monoculture model previously described in the literature for *Cronobacter* spp. We used *E. coli* K1 strain as the positive control because this was a

pathogenic strain known to cause meningitis. The negative control was a nonpathogenic *E. coli* K12 strain. The monoculture model worked as expected because the positive control did disrupt the endothelial cells as indicated by the decrease in TEER values, and the negative control has no effect on the TEER measurements of the treated cells (Figure 1). The *C. sakazakii* isolates had the same effect on the TEER measurements of the endothelial cells in the monoculture model as the positive control. We expected to see this with the CT2 isolate since this was obtained from the meninges of an infant with meningitis. It was intriguing to discover the N72-1A isolate also decreased the TEER values and disrupted the cells in the same manner as the CT2 and positive control isolates. This observation does indicate that the N72-1A isolate would have the potential to be pathogenic and cause meningitis. Although this type of assay has been described in the literature, it has limitations. Most importantly, it does not include astrocyte cells, which have been described as critical components of the BBB. Therefore, we developed our co-culture system and tested it with the same isolates used with the monoculture model.

E. coli K1 and *C. sakazakii* Significantly Decreased TEER in Co-Culture Model

When we conducted testing using the co-culture system, we obtained greater TEER values overall (Figures 3 and 4) with this model than the monoculture model (Figure 1). This observation was most likely due to the presence of the astrocytes, which are known to stimulate formation of tight junctions between the endothelial cells and increase barrier integrity (29, 74). In spite of the increased barrier integrity, the positive control, CT2, and N72-1A isolates were still able to disrupt the endothelial cells in this model, but overall TEER values were higher than found in the monoculture model (i.e. in monoculture model these treatments were statistically similar to the media control ($P > 0.05$) at the final

reading, in the co-culture they were not ($P < 0.001$)). This indicated that the co-culture model constructed is a more realistic representation of the actual BBB structure than the monoculture model previously described in the literature (48, 57, and 85). This system would be a more useful tool for *in-vitro* research of BBB pathogens. We plan on using this assay to characterize the pathogenicity of other *Cronobacter* spp. isolates in our lab.

CT-2 and N72-1A Found To Be Non-Biofilm Formers with Crystal Violet Assay

A simple Crystal violet staining assay was used to quantify the biofilm production of the C. *sakazakii* isolates used in this study (Table 1). Although this method of biofilm quantification did not detect biofilm formation with the CT2 and N72-1A isolates it does not rule out the possibility that they may form a biofilm under different conditions and/or the Crystal violet assay simply did not detect it. Therefore, further testing in the future needs to be done on CT2 and N72-1A using other biofilm quantification assays to confirm these results. The Bact-Titer Glo Microbial Cell Viability assay available from Promega (Madison, WI) is one possible alternative biofilm assay. Sule et al. (91) has shown the BacTiter-Glo assay can be used to determine the attached biomass (number of cells) by detecting the ATP level. This method would allow us to assay the population of cells in the biofilm itself.

Conclusion and Future Research

Taken together, these results indicate the co-culture model worked similar to the monoculture model. The negative control had no significant effect on the TEER measurements, so it did not disrupt the *in-vitro* BBB. The positive control, CT2 and N72-1A isolates did significantly decrease the TEER over time and, consequently, did disrupt the *in-vitro* BBB. It was expected the positive control and CT2 would have this effect on

the model because they were both known to cause meningitis. Since N72-1A also was found to significantly decrease the TEER (although not to the same extent as CT2) this would imply that N72-1A is also a pathogenic *C. sakazakii*. With this co-culture assay, we can determine the pathogenicity of *Cronobacter* spp. isolates and their potential to disrupt the BBB during infection. The development of this co-culture assay was a monumental step in *Cronobacter* spp. research. This system is a more realistic model of the BBB and would allow researchers to screen *Cronobacter* spp. isolates for potential BBB pathogenicity. However, much more work needs to be done with this research.

Taking TEER measurements at different time points would be a potential modification for this assay. We based our measurements here on the growth curve of the *C. sakazakii* isolates we used from our lab. Examining the effect of the bacteria on TEER at different time points than described in this study would help further characterize the effect these bacteria have on the endothelial cells in the model. As previously mentioned, pericytes are another component of the BBB and they are thought to also influence the BBB integrity by mediating tight junction formation.

A triple co-culture model consisting of endothelial cells, astrocytes, and pericytes has been described in the literature (65). However, it has not been used to test the pathogenicity of meningitis causing bacteria as described in this paper. This model has the potential to be an even better representation of the *in-vivo* BBB than the co-culture system described here. In future studies, a triple co-culture model could be developed to test the pathogenicity of *Cronobacter* spp. bacteria and this would allow us to determine which isolates do have to potential to cross the BBB *in-vivo*.

In terms of the biofilm quantification portion of this research, more assays will need to be done to confirm that CT2 and N72-1A are non-biofilm producers as shown in this study. For this reason, no conclusion can be drawn as far as determination of the effect biofilm formation has on pathogenesis, if any. As previously mentioned, the Bact-Titer Glo Microbial Viability assay would be a good assay to run with these *C. sakazakii* isolates. In addition, the biofilm formation of these *C. sakazakii* isolates could be assayed in actual PIF. Dancer et al. (13) found proteins in milk can influence the biofilm production by *Cronobacter* spp. It would be interesting to see the effect PIF has on *Cronobacter* spp. biofilm production.

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