COLONIZATION DYNAMICS OF ORO-GASTROINTESTINAL MICROBES CRITICAL TO INFANT HEALTH

BY

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DISSERTATION

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ABSTRACT

The gastrointestinal microbiota is intimately tied to a person’s health and well-being. This collection of bacteria, archaea, and fungi is assembled during a person’s infancy and achieves the stability of an “adult-like” microbiota in early childhood. Research has begun to uncover several host and external factors that influence the rate and course of assemblage. Historically, the immune system was considered the primary driver of microbial balance in the gastrointestinal tract. While the immune system is certainly a key component, research has now identified the importance of, among others, an infant’s route of delivery, gestational age, early-life diet, and exposure to antibiotics. This dissertation has evaluated the modulation of the gut microbiota following exposure to fermentable carbohydrates and explored the pathogenesis of two important infantile pathogens.

At birth, an infant’s gastrointestinal microbiota is largely devoid of bifidobacteria, and within the first week, this bacterial genus dominates the community. It is well-known that breastmilk contains oligosaccharides that promote the growth of bifidobacteria. However, formula-fed infants lack access to these carbohydrates and have a delayed establishment of bifidobacteria. Therefore, infant formula companies began supplementing bovine and soy-based infant formulas with prebiotics. Prebiotics, non-digestible carbohydrates that promote bacterial growth, stimulate the growth of bifidobacteria and lactobacilli in infants. Microbial analysis of infants fed prebiotics is restricted to measuring alterations in the fecal microbiota. Typically, prebiotics are fermented in the ascending colon, and the fecal microbiota may not be an appropriate representative of the colonic microbiota. In Chapter 2, the impact of galactooligosaccharides and polydextrose on the ileal and ascending colon microbiota of neonatal piglets was evaluated. More specifically, hundreds of lactobacilli were isolated and
sequenced to characterize the lactobacilli community composition. Additionally, these lactobacilli isolates were evaluated for their ability to ferment galactooligosaccharides and polydextrose. It was hypothesized that exposure to galactooligosaccharides and polydextrose would promote a lactobacilli community more capable of fermenting these prebiotics in vitro. While a significant increase of lactobacilli in the ascending colon of prebiotic-exposed piglets was observed, the lactobacilli community was not more capable of fermenting galactooligosaccharides and polydextrose than lactobacilli isolated from piglets never exposed to these prebiotics. Therefore, it was concluded that the observed increase was not due to a direct ability to ferment the provided prebiotics, but rather due to factors not directly measured.

An additional concern with prebiotic supplementation in infant formula is the promotion of pathogenic microorganisms. In preterm infants, the gastrointestinal tract lacks the maturity of term infants. A severe consequence of an immature gastrointestinal tract is an increased risk of local and disseminated infections. A common secondary complication is consumption of infant formula, lacking human milk oligosaccharides, rather than breastmilk. As observed in Chapter 2, the exposure to prebiotics did not directly stimulate lactobacilli in vivo. A potential mechanism is a cooperative breakdown of prebiotics in the gastrointestinal tract by other microbes. Several commensal microbes are strongly glycolytic and liberate mono- and disaccharides from fermentable fibers and the gastrointestinal mucin layer. These liberated carbohydrates are available for any microorganisms capable of utilization. Therefore, the presence of prebiotics in the gastrointestinal tract may lead to an increase in pathogenic microorganisms. The study presented in Chapter 3 explored the ability of several Enterobacteriaceae, a bacterial family that includes common neonatal pathogens, to ferment a variety of prebiotics and human milk oligosaccharides. The ability to utilize prebiotics for
growth was strain-dependent. Galactooligosaccharides supported the growth of many Enterobacteriaceae, whereas fructooligosaccharides were less fermentable. Fortunately, Enterobacteriaceae were incapable of fermenting the intact human milk oligosaccharides tested. Conversely, several Enterobacteriaceae were capable of fermenting the mono- and disaccharides that comprise the human milk oligosaccharides. Taken together, the inclusion of prebiotics and human milk oligosaccharides in infant formula can increase health-promoting microorganisms while conversely, promoting pathogenic microorganisms.

While prebiotics and human milk oligosaccharides can support the growth of health-promoting microbes, exposure to pathogenic microorganisms is inevitable. Once in an infant’s gastrointestinal tract, pathogenic microbes upregulate virulence traits and begin an infection. In Chapter 4, the mechanism of autoaggregation in the neonatal pathogen, *Cronobacter sakazakii* ATCC 29544 (wild-type) is described. Upon isolation of two independent nonautoaggregating clonal variants, two unique single nucleotide polymorphisms in the flagella proteins, FlhA and FliG, were identified. Furthermore, it was hypothesized that structurally intact and functional flagella were required for autoaggregation in *C. sakazakii* ATCC 29544. Several gene knockouts were constructed to target the flagella structure (ΔflhA, ΔfliG, ΔfliC, ΔflaA, and ΔfliCΔflaA) and function (ΔmotAB). A loss in autoaggregation in ΔflhA, ΔfliG, and ΔfliC gene knockouts was observed, whereas ΔflaA and ΔmotAB retained the ability to autoaggregate. Complementation of FliC restored autoaggregation to the ΔfliC and ΔfliCΔflaA strains. Therefore, it was hypothesized that a direct interaction between FliC filaments of neighboring cells allowed autoaggregation to proceed. Autoaggregation was disrupted following the addition of detached wild-type flagella in a dose-dependent manner. It was concluded that flagellation with FliC mediates direct interactions between neighboring *C. sakazakii* ATCC 29544 cells which promote
autoaggregation. Further experiments utilizing animal models will need to be conducted to determine if autoaggregation in *C. sakazakii* ATCC 29544 is necessary for its pathogenesis *in vivo*.

In Chapter 5, a model for *Candida albicans* colonization in neonatal piglets was developed. *C. albicans* is a commensal fungus for which colonization mechanisms are understudied. A surveillance analysis identified a *C. albicans*-negative piglet population at the University of Illinois-Urbana Champaign, which provided the basis for the work. Neonatal piglets were orally inoculated with either a laboratory strain, piglet, or human isolate of *C. albicans*. *C. albicans* was present on swabs of the mouth, rectum, and environment for at least 14 days post-inoculation demonstrating stable colonization of the animals. Necropsy showed that *C. albicans* most readily colonized the piglet esophagus. This model provides an opportunity for subsequent studies of *C. albicans* colonization mechanisms.
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1.1 Overview

The human’s symbiotic microbial community has evolved over thousands of generations. Upon birth, infants enter the world teeming with microbial diversity. The infant’s microbiota is assembled through a complex microbial succession that requires a homeostasis between helpful microbes, harmful microbes, and the host (1,2). Several factors including diet, environment, and host genetics influence which microbes can colonize the host. In some instances, early colonization by pathogenic (“harmful”) microbes may require antibiotic treatment which further shapes the microbiota. Eventually, the infant’s microbiota will result in a stable “adult-like” community that will remain relatively consistent during one’s life. In this review, the establishment and assembly of the infant microbiota will be discussed, along with interventions utilized to assist with the assembly process, particularly for at-risk infants. Additionally, the impacts of pathogenic colonization during assembly will be explored.

1.2 Establishment and assembly of the infant gut microbiota

A common misconception in the research literature is the claim that infants are devoid of microbial colonization until birth (1). Recent literature suggests that fetal microbial colonization may begin in utero; specifically, colonization of the placenta (3–6), umbilical cord (7), and amniotic fluid (6,8–11). Histopathological examination of vaginally and cesarean-delivered placentas (27%) had visible morphologically diverse Gram-positive and -negative bacteria (4). An in-depth microbiological analysis reported that the placenta microbiota was a diverse and
distinct community composed of common gut-associated phyla; Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria, the dominant phyla (5,6). Further classification revealed the placenta microbiota harbored a high relative abundance of Enterobacteriaceae, particularly *Escherichia coli* (5), and *Enterobacter* spp. (6). Several studies reported culturing or detecting by PCR common skin and oral microbes in the placenta (3,5,6). Specifically, *Corynebacterium* spp., *Propionibacterium* spp., *Staphylococcus* spp., and *Lactobacillus* spp. were cultured from cesarean-delivered placentas (3), low relative abundances of *Propionibacterium acnes*, *Staphylococcus epidermidis*, and *Lactobacillus iners* were reported (5), and very low relative abundances of *Lactobacillus* spp. (0.83%), *Staphylococcus* spp. (0.26%), and *Propionibacterium* spp. (1.8%) were detected in cesarean-delivered placentas (6). Similarly, a single study cultured Gram-positive bacteria, *Enterococcus faecium*, *P. acnes*, *S. epidermidis*, and *Streptococcus sanguinis*, from the umbilical cord blood of cesarean-delivered healthy neonates (7). These results are consistent with detection of skin and oral microbes in the placenta. These findings suggest that the umbilical cord may transport commensal placenta microbes from the placenta to the fetus. However, this study should be interpreted cautiously due to a very low sequencing depth (an average of 9.3 sequences (SD = 6.9) per neonate) and the lack of Gram-negative isolates (7). Furthermore, a recent study defined amniotic cavity “colonization” by relating low levels of interleukin 6 (<2.6 ng/mL), a marker of inflammation, with the presence of *Ureaplasma urealyticum*, a common genital-tract bacterium (11). Additional culture-based studies (8–10) and PCR-based studies (6,9) characterized the microbial population in amniotic fluid. Similarly to the placenta and umbilical cord, skin and oral microbes were readily cultured and detected from amniotic fluid, including *Streptococcus* spp., *Fusobacterium nucleatum*, *Staphylococcus* spp., and *Propionibacterium* spp. (6,8–10). Other microorganisms commonly reported in the
amniotic fluid were *Lactobacillus* spp. (6,8,10), *Ureaplasma* spp. (8,10,11), *Candida* spp. (8–10). Collado and colleagues (6), observed a high degree of microbial similarity between their amniotic fluid and placental samples specifically, the predominance of *Enterobacter* spp. (73% relative abundance). Although all studies reviewed report detailed methods to reduce contamination, there is the possibility that skin microbes from the mother, healthcare professionals or research staff could be isolated/detected using the techniques described. Therefore, researchers should design studies to determine links between the colonization of the placenta, umbilical cord, amniotic fluid microbiota, and the infant after birth. Specifically, the focus should be on which microbes passing from the mother to the fetus *in utero* are “colonizers” or “transients”. Also, in this era of whole genome sequencing, it is possible to determine if any of the fetal isolates are identical to isolates from any of the potential contamination sources.

Additional evidence that colonization begins *in utero* is the presence of detectable microbes in an infant’s first passage of stool, the meconium. Since the meconium is more readily available to researchers, several studies have analyzed its microbiota (2,6,12–18). The absolute quantity of microbes in the meconium ranged from $10^4$ to $10^8$ rRNA gene copies per gram of meconium (2). Additionally, a low microbial diversity has been reported for meconium samples (13,15,19). The two dominant phyla detected in meconium samples were consistently the Proteobacteria and Firmicutes (2,13–16,19). Uniquely, Collado and colleagues (6) detected very low abundances of Proteobacteria in their meconium samples and reported the Firmicutes as the primary phyla detected. Additional phyla detected were Bacteroidetes, Actinobacteria, (2,6,13,16), Tenericutes (15), and Verrucomicrobia (13,16); however, in significantly lower abundances. A consistent finding was a high abundance of Enterobacteriaceae in the meconium (2,12,16–18). Oral and skin microbes were commonly detected in the meconium and the
placenta, umbilical cord, and amniotic fluid, including Streptococcaceae (2,12,14,16,17), Staphylococcaceae (2,6,14,16–18), and Propionibacteriaceae (16), along with Lactobacillaceae (6,14,17). It is hypothesized that members of the Enterobacteriaceae, Staphylococcaceae, and Streptococcaceae families are early gut colonizers which reduce the gut environment allowing subsequent colonization by strict anaerobes (16). Taken together, these results suggest that the placental microbiota may serve as the *in utero* inoculum to prime the infant’s immune system for life outside the mother.

An infant’s gut microbiota at birth, as detected in the meconium, is naïve and composed of only a few microbial taxa. The microbiota is assembled and is influenced by several factors, such as length of gestation (20), route of delivery (21–23), infant feeding (20–25), weaning (22,23,25,26), and antibiotic use after birth (2,21,22,25). The microbiota composition fluctuates as the infant ages until it resembles an adult’s microbiota. Currently, the time to microbial maturity is unknown; however, several studies suggested that maturity occurs around 1 to 3 years (2,25,27,28) while, others observe significant alterations in the gut microbiota in young children (3 to 5 years) (26,29,30). Bacterial diversity is commonly used as a metric to determine overall similarity in microbial communities between two distinct populations. It is widely reported that the bacterial diversity in infant’s stool upon birth is low and that as an infant ages bacterial diversity increases (25,28,29,31,32). However, this upward trend in diversity is not linear (25,31,32). In general, the inclusion of solid foods and cessation of human milk corresponds with shifts in bacterial diversity (25,31,33). Furthermore, as compared to adults, bacterial diversity in infants and young children remains low (28–31). Therefore, the gut microbiota may not be fully assembled for several years.
Assembly of the infant’s gastrointestinal microbiota proceeds via a specific succession of bacterial taxa. As discussed above, the meconium is characterized by a predominance of Proteobacteria and Firmicutes (2,13–16). During the first few days of life, there is a dramatic decrease in aerobic bacteria, including Bacilli and Gammaproteobacteria (2,34), and a concomitant increase in anaerobic bacteria (20,31,34). In two cohorts studied, the relative abundance of Proteobacteria decreased rapidly and remained low during the observation period (25,30) while, in other studies Proteobacteria persisted at higher abundances (2,16,20,23,34,35). The appearance of Bifidobacteriaceae was observed during the first week after birth (16,20,25,33,36) and was slightly delayed in preterm (20) and cesarean-delivered infants (23,31,37). Additionally, a higher relative abundance of Bifidobacteriaceae was reported in breastfed infants as compared to formula-fed infants (20,22,36). Perhaps unsurprisingly, several studies reported a rapid decrease in Bifidobacteriaceae abundance at the cessation of breastfeeding (22,23,26,28,30,32,33). The emergence of Bacteroidetes is typically associated with the transition to solid food (22,25,30); however, several groups have reported its presence at and shortly after birth (2,14,16,20,23,24,27,31,35,36). Furthermore, breastfeeding (20) and vaginal delivery (22,23,32) have been associated with an early appearance of Bacteroidetes and higher abundance. The post-weaned gut microbiota is characterized by an emergence of butyrate-producing Firmicutes, specifically members of the order Clostridiales (23,26,27,29–32). This final transition represents a major compositional change that is more readily described as “adult-like”; however, other minor compositional changes occur for several years. Several studies reported instability in the gut microbiota of young children (3 to 5 years) and concluded that an adult gut microbiota composition occurs later in childhood (26,29,30). Additional
research with either a longitudinal design or with large cohorts will be useful in determining the time at which the gut microbiota matures.

1.3 Modulation of the gut microbiota with prebiotic supplementation

Human milk has evolved over a couple hundred million years and is considered the gold standard for infant nutrition (38). The macronutrient composition of human milk is lactose (≈70 g/L), lipids (≈40 g/L), human milk oligosaccharides (HMOs, 5-23 g/L), and proteins (≈8 g/L) (39,40). The HMO fraction is a pool of neutral or acidic lactose-containing oligosaccharides decorated with galactose, N-acetylglucosamine (GlcNAc), N-acetyleneuraminic acid (also sialic acid), and fucose in an assortment of glycosidic linkages. A variety of analytical methods have been used to annotate the milk glycome and have identified hundreds of structures (41,42). Fucosylated HMOs (i.e. 2'-fucosyllactose, 2'-FL) were the major fraction (≈77%), followed by sialylated HMOs (i.e. 3'-sialylactose, 3'-SL) at 16% relative abundance and the remaining 7% constituted Type I (lacto-N-tetraose) and Type II (lacto-N-neotetraose) based HMOs (42). The composition and abundance of the HMO fraction in human milk were decreased significantly during four months of lactation (43–45), but more recently, only minor changes have been reported during that same period of lactation (42). Additionally, the HMO composition of mother’s milk was predictive of the dominant members of the infant’s GI microbiota (46) and HMOs resist digestion and are excreted in feces (47) and urine (39,48). Therefore, alterations in HMO composition contribute to the assembly of the infant’s microbiota.

It is well-established that HMOs are readily fermented by members of the genera *Bifidobacterium* (49–55) and to a lesser extent *Lactobacillus* (53,55–57). Several bifidobacteria have been isolated from the feces of breastfed infants, including *B. longum* subsp. *infantis* (B.
infantis), B. breve, and B. bifidum (52), suggesting that bifidobacteria are HMO consumers. B. infantis, highly abundant in breastfed infant’s feces, is a prolific consumer of HMOs (49–51,53–55). B. breve and B. bifidum have been reported to utilize HMOs, but to a lesser extent than B. infantis, the most dynamic HMO utilizer (50–52,54,55). More specifically, in vitro fermentation studies have reported that B. infantis strains are capable of consuming fucosylated, sialylated and Type I & II HMOs (51,53–55), albeit HMOs sized less than or equal to 8 degrees of polymerization (49,50). In contrast, B. breve and B. bifidum preferentially consume Type I and Type II HMOs and their consumption of fucosylated and sialylated HMOs is strain-dependent (50–52,54). Whole-genome sequencing of B. infantis ATCC 15697 (type strain) revealed a 43 kbp gene cluster specifically for HMO utilization (58). This HMO utilization gene cluster is highly conserved among B. infantis and is missing in closely related strains, B. longum subsp. longum and B. longum subsp. suis incapable of consuming HMOs (59). Follow-up studies have begun to characterize specific loci functionally and have identified fucosidases (60), a sialidase (61), β-galactosidases (62), and β-hexosaminidases (63) in B. infantis. Similarly, a lacto-N-biosidase (64) and two α-fucosidases (65) have been described in B. bifidum. Additionally, a few lactobacilli have been found to be HMO utilizers, including Lactobacillus acidophilus, L. casei, and L. plantarum (55–57). L. acidophilus was found to cleave enzymatically fucosylated, sialylated and Type I HMOs (57); however, only Type II HMOs supported growth in vitro (55). L. plantarum had a similar capacity to cleave HMOs enzymatically (57); however, its ability to ferment HMOs for growth has not been tested. Finally, a single L. casei strain possesses an α-fucosidase capable of cleaving 2’-fucosyllactose (56) and recently, has been grown in the presence of Type I HMOs (66). It is evident that HMOs, typically delivered to the infant via breastmilk, are strongly bifidogenic and moderately lactogenic. Unfortunately, many infants are
formula-fed and therefore, lack access to these health-promoting HMOs. While there are oligosaccharides in bovine milk, the concentration and composition are vastly different from HMOs (67). Therefore, alternatives are needed to provide the benefits of HMOs to formula-fed infants.

Several researchers consider the oligosaccharides in breastmilk as prebiotics, primarily due to their bifidogenic effects (68–70). Prebiotics are defined as non-digestible carbohydrates that stimulate the growth of beneficial bacteria, thereby providing a health benefit to the host (71). Unfortunately, HMO supplementation in infant formula is extremely cost-prohibitive. Therefore, supplementation with alternative prebiotic-like carbohydrates is desired. Several commercial long-chain carbohydrates, such as galactooligosaccharides (GOS), polydextrose (PDX), and inulin-derived fructooligosaccharides (FOS) have been evaluated for their prebiotic potential. These food ingredients have GRAS (generally recognized as safe) status and have been extensively studied as functional ingredients in infant formulas (Table 1.1).

Overall prebiotic supplementation of infant formula is well-tolerated (72,73). Additionally, prebiotic supplementation did not negatively affect the weight gain of infants and was not associated with any reported adverse effects (Table 1.1). Based on the studies reviewed, a specific beneficial prebiotic dosage has not been established and in many cases, the exact dose consumed is not specified (Table 1.1). Furthermore, prebiotics are often supplemented in combination with varied ratios making comparisons difficult. GOS/FOS supplementation, either 1:1 or 9:1 ratios, significantly improved both the frequency of defecation and consistency (74–77), while some studies only observed an improvement in stool consistency (72,78–80). When GOS was in combination with PDX, stool frequency (80) and consistency (72,79,80) was improved. GOS supplementation alone can significantly impact stool frequency (81,82) and
consistency (79,82,83). Likewise, sole FOS supplementation can improve stool frequency (84,85) and consistency (85). Several studies reported no impact on stool frequency or consistency following prebiotic supplementation (86–90).

Many studies reported a significant increase in the fecal bifidobacteria and lactobacilli populations (Table 1.1). The combination of GOS and FOS, either 1:1 or 9:1 ratios, correlated with a significant increase in bifidobacteria (74–77,86,89,91–94) and lactobacilli (75,76,92,95,96). A single study reported no change in fecal bifidobacteria following supplementation with GOS/FOS (97). A formula containing GOS/PDX had no impact on fecal bifidobacteria and lactobacilli (88). The fecal bifidobacteria and lactobacilli communities significantly increased in formula supplemented with GOS (81,83) alone. Sole FOS supplementation only leads to an increase in fecal bifidobacteria (84,85,87,98) and lactobacilli (87). An additional study did not detect an increase in fecal bifidobacteria or lactobacilli with sole FOS supplementation (90). Recently, a study reported that increasing dosages of GOS (2.5 – 20 g/L) or HMO (1.7 – 3.4 g/L) in infant formula over five weeks did not alter the bifidobacteria population, especially compared to their human milk reference group (99). Interestingly, several studies correlated prebiotic supplementation with a significant decrease in fecal pH (75,76,78,81–83,86,89,92,97). A decrease in pH compared with the control formula suggests that the prebiotics are fermented in the colon; however, very few studies showed direct evidence of prebiotic fermentation by bifidobacteria and lactobacilli.

Characterization of the *ex vivo* fecal microbiota following the inclusion of prebiotics has been explored. Overwhelmingly, fermentation of GOS and GOS-like prebiotics corresponds with an increase in the fecal bifidobacteria (100–106) and lactobacilli (101,103–106). Similarly, the inclusion of FOS and inulin, of varied chain lengths, led to an increase in bifidobacteria
(100,101,103–105,107) and lactobacilli (100,103–105,107). While the fecal bifidobacteria increased when PDX was added, the fecal lactobacilli remained unchanged (103,104,107), except for one study that measured an increase in lactobacilli (105). Several studies correlated the increase in bifidobacteria and lactobacilli with an increase in either acetate (100,102–107) or lactate (100,106). Therefore, it is tempting to conclude that the tested prebiotics are bifidogenic and lactogenic; however, these studies did not exclude the possibility of cross-feeding in the fecal community. Several researchers have explored direct fermentation of prebiotics by pure cultures of bifidobacteria or lactobacilli. In general, pure cultures of bifidobacteria were found to utilize GOS and FOS readily (55,108–113) and to a lesser extent, PDX and inulin (55,109,112). Prebiotic utilization was strain dependent with B. infantis strains found to be strong GOS fermenters, while B. bifidum strains were weak GOS utilizers (113,114). Lactobacilli strains, typically commercial probiotics, were good utilizers of GOS and weak to moderate utilizers of FOS (55,108,111,112). Consistently, pure lactobacilli cultures were unable to ferment PDX or inulin in vitro (55,112).

1.4 Gastrointestinal microbiota and its role in pediatric necrotizing enterocolitis

As previously discussed, the establishment of an infant’s gastrointestinal microbiota is an elaborate process that is susceptible to perturbations. Several factors, including gestational age (20), route of delivery (21–23), infant feeding (20–23,25), and exposure to antibiotics (2,21,22,25), influences the pace and direction of assembly. An improper assembly of the neonatal gastrointestinal microbiota could have severe consequences for the neonate. Specifically, a dysbiotic gastrointestinal microbiota, along with prematurity (birth before 37 weeks) and enteral feeding, puts an infant at risk of developing necrotizing enterocolitis (115–
Necrotizing enterocolitis (NEC) is a common cause of morbidity and mortality among neonates requiring extended hospitalizations. The incidence of NEC is inversely correlated with an infant’s birth weight, a consequence of prematurity (118,119). The overall incidence rate has been reported as 0.2 - 1 cases per 1,000 live births or 0.20 - 0.1% (120,121). High birth weight (HBW, >2500 g) infants incidence rate ranged between 0.01 - 0.04% (121,122), while low birth weight (LBW, 1500-2500 g) infants had an incidence rate slightly higher (0.4 – 0.7 %) than the overall average (121–123). More attention has been given to very low birth weight (VLBW, <1500 g) infants due to the significantly higher rate of incidence (4.3 - 11%) (118,119,121–126). Accordingly, mortality rates are highest among the VLBW infants (20 – 40%) (121,122,126,127) and are lowered to 5.8% in LBW infants (121). While prematurity is a key risk factor for NEC, it occurs in term infants. Interestingly, the time of onset for NEC is far quicker (7-14 days versus >18 days) for term infants than for premature infants; respectively (122,123,126). Regardless of birth weight or prematurity, a NEC diagnosis is commonly associated with co-morbidities (118), including respiratory, cardiovascular, and gastrointestinal issues (120–123,126,127). Specifically, colonization of the respiratory tract by *Ureaplasma* spp. is associated with an increased risk of developing NEC in preterm infants (128). An additional risk of mortality and morbidity occurs when infants undergo surgical procedures to treat suspected and confirmed cases of NEC (118,121,122,127).

NEC is a disease spectrum characterized by inflammatory necrosis of the gastrointestinal tissues (129). Nanthakumar and colleagues (130) hypothesized that NEC progression in premature infants results from an improper immune response during the initial stages of microbial colonization. Clinically, NEC pathology is classified by Bell’s staging criteria (three stages) based on the progression and severity of the illness (129). However, a recent review has
disseminated NEC into five subsets focusing on risk factors and differing etiologies (131). A focal point of the subsets is the role of an aberrant colonization of the gastrointestinal tract in the onset and progression of NEC (131). In neonates that do and do not develop NEC, the early gastrointestinal microbiota is composed of Proteobacteria and Firmicutes (2,13–16,116,117,132–135). Based on infants sampled before and after a NEC diagnosis, a decrease in alpha diversity (132,134–136) is associated with a change in the Proteobacteria to Firmicutes ratio (116,117,133–135). Two studies were observed cases of NEC that resulted from either an increased or decreased Proteobacteria to Firmicutes ratio, suggesting that NEC’s etiology may be a variety of causative microbial agents (134,135). When NEC is linked to an increase in Proteobacteria, it is typically at the expense of Firmicutes (116,117,133–135) and due to an increased abundance of members of the Enterobacteriaceae family (18,117,133,134). Specific Enterobacteriaceae reported in NEC infants are *Citrobacter*–like sequences (12), *Enterobacter* spp. (137), *Escherichia* spp. (136), and *Klebsiella pneumoniae*–like sequences (116,138). On the other hand, an increase in Firmicutes occurs at the expense of Proteobacteria (134,135) and is associated with an increase in Clostridia and Bacilli classes, specifically *Clostridium perfringens* (18,138) and *Enterococcus* spp. (12,134). The relative abundance of *Staphylococcus* spp. has been positively- (134,137) and negatively-associated (18) with NEC diagnosis. Interestingly, the time of onset occurred earlier in Firmicutes-associated NEC (7-21 days) than Proteobacteria-associated NEC (19-39 days) (134). Also, infants who later developed NEC were found to lack colonization by *Propionibacterium* spp. (134), which has been reported to participate in the proper assembly of the neonatal gastrointestinal microbiota (3,6). Several studies collected meconium and fecal samples before and after NEC diagnosis (18,116,133,134). In case-control studies, the fecal microbiota of infants with NEC was characterized by a predominance of
Enterobacteriaceae (139,140) or Bacilliales (139). A recent study reported the detection of *Clostridium butyricum* in feces significantly correlated with neonates with NEC as compared to case-matched control neonates (141). Finally, neonatal intestinal candidiasis, by the yeast *Candida albicans*, has been reported as an atypical cause of NEC (142). Routine monitoring of the infant fecal microbiota may assist in early detection of dramatic microbial shifts and blooms of suspected causative microbial agents; however, the fecal microbiota may not be indicative of the microbiota intimately-associated with the necrotic tissue. The microbiota of resected tissues and mucosa from NEC infants were composed primarily of Firmicutes and Proteobacteria with a lesser abundance of Bacteroidetes (143,144). Similar to fecal samples, *Clostridium* spp. and Enterobacteriaceae were found in higher abundances than control tissue (143) and mucosal samples (144). Interestingly, two cases of NEC were characterized by an overabundance of *Bacteroides* spp. which has not been observed in fecal samples (144).

The association of Proteobacteria with NEC is particularly concerning because this phylum is composed of a plethora of pathogens responsible for gastrointestinal illnesses and diseases. As discussed above, an increased abundance of members of the Enterobacteriaceae family has been linked to NEC in infants (18,117,133,134,139,140,144). Clinical presentations of NEC have been associated with culturing *Cronobacter (Enterobacter) sakazakii* (145–147), *Enterobacter cloacae* (148), *E. coli* (149–151), and *K. pneumoniae* (149,150) from blood, feces, or gastric aspirate. Furthermore, a high abundance of Enterobacteriaceae has been observed using 16S rDNA sequencing methods (117,132,138,143,144). Specifically, *Citrobacter* spp. (143), *Cronobacter* spp. (117), *Enterobacter* spp., (117,132), *Escherichial/Shigella* spp. (117,132,143,144), *Klebsiella* spp. (117,132,138,144). Additionally, animals with NEC had a significantly higher abundance of *Citrobacter* spp., *Klebsiella* spp., and *Tatumella* spp. as
compared to control animals (152). Furthermore, NEC was induced in rat pups orally-fed *Cronobacter muytjensii*, reported as *C. sakazakii* (153). Of the Enterobacteriaceae discussed, only *C. sakazakii* contamination in powdered infant formula has been epidemiologically linked to sepsis (154), meningitis (155), and NEC (146) in neonates. Therefore, additional research was conducted to understand *C. sakazakii*’s persistence in dehydrated infant formula and it’s pathogenesis in humans.

1.5 *Cronobacter sakazakii* physiology and pathogenesis

The family Enterobacteriaceae is taxonomically diverse and over the last few decades, several genera have undergone reclassification with continuing research. Specifically, *Enterobacter sakazakii*, previously known as “yellow-pigmented” *Enterobacter cloacae*, was first proposed as a new species in 1980 (156). Continued research revealed that *E. sakazakii* was phenotypically (157) and genomically (157,158) distinct from other *Enterobacter* spp. At last, *Cronobacter* gen. nov., originally consisting of five species, was proposed to describe the diverse biotypes of *Enterobacter sakazakii* properly (159). Researchers developed several biotyping (157), serotyping (160,161), and genotyping (157,162,163) methods to speciate former *Enterobacter sakazakii* isolates quickly. Utilization of multilocus sequence typing (MLST) revealed two additional *Cronobacter* species (164) and was found to speciate all seven *Cronobacter* spp. accurately (162,165). In total, 115 sequencing types (ST) represent the genetic diversity of the genus *Cronobacter*, with more than 53 sequencing types for *C. sakazakii* (162). Several studies have reported an association between *C. sakazakii* ST1 & 4 clinical isolates and severe neonatal cases (162,166–168). The *C. sakazakii* type strain (ATCC 29544), which is studied in Chapter 4, belongs to ST8 (162,166).
**C. sakazakii**, an opportunistic pathogen, is linked to fatal infections in neonates, the elderly and immunocompromised individuals (145). The first infant deaths, described in 1961, involved two infants diagnosed with general infections caused by “yellow-pigmented” *Enterobacter cloacae* (169). To date, several hundred cases of infantile *C. sakazakii* infections have been reviewed (145,147). These studies report that the majority of *C. sakazakii* infections have occurred in neonates with clinical outcomes of necrotizing enterocolitis, meningitis, bacteremia, and septicemia. Additionally, the overall fatality rate has been reported as 26.9% (147) and 50% (145) with life-long neurological and gastrointestinal complications in surviving neonates (145,147). Initially, *C. sakazakii* was thought to originate from the birthing canal; however, infections were observed in cesarean-delivered infants (170,171) and samples obtained from the mother’s cervix, vagina, and feces were negative (171). Furthermore, *C. sakazakii* has been found to persist within clinical settings for several years (172), which is the likely source for *C. sakazakii* recovered from breastfed neonates (173,174) and asymptomatic carriers (170,175). Farmer and colleagues (156), were the first to report the isolation of *C. sakazakii* from an unopened canister of dried milk. Unfortunately, it wasn’t until several years later that a global surveillance study confirmed extensive *C. sakazakii* contamination of milk powders (176). This survey (176), along with another (177), reported low levels of contamination (0.36 – 66 CFU/100g); however, both groups cautioned that the observed level of contamination might be problematic if growth were to occur during preparation or storage. Not long after, *C. sakazakii* infections in infants were epidemiologically linked to contaminated powdered infant formula (PIF) (146,154,155).

The natural reservoir for *C. sakazakii* does not appear to be a single source but rather *C. sakazakii*’s distribution in nature is ubiquitous (178–182), including raw milk (183,184). While
C. sakazakii is widespread, its association with milk powders presents a unique challenge for the populations at-risk for infection. C. sakazakii has been found to be extremely tolerant to heat, desiccation and osmotic stresses (185–191). Several studies have determined C. sakazakii’s thermal inactivation parameters (D- and z-values) in reconstituted infant formula (185–188,191,192). The D-value at 58 °C ranged from 30.5 sec to 9.85 min and was highly strain- and substrate-specific (185–188,191,192). In general, clinical isolates tended to be more heat-resistant (185,187) as well as the C. sakazakii type strain (ATCC 29544) with its D-value at 58 °C reported as 2.6 (188) and 6 min (187). As expected, the nutritional components of reconstituted milk powders significantly impacted the D-value. Osaili and colleagues (191) observed higher D-values corresponding to increasing fat contents (0, 1.29, 3.64% fat). Interestingly, three studies reported highly consistent z-values; 5.82 (185), 5.6 (187), and 5.7 °C (188). Taken together, the D- and z-values suggest that C. sakazakii, while tolerant to high temperatures, should not survive typical commercial pasteurization. Some researchers have hypothesized that contamination of milk powders with C. sakazakii occurs post-pasteurization via carriage on filth flies (193,194) or by dry mixing with heat-sensitive micronutrients (179). Therefore, understanding the desiccation and osmotic tolerance of C. sakazakii is important. A short-term survival study reported a 1 – 1.5-log decrease in several C. sakazakii isolates after storage in dehydrated PIF for 46 days (186). An additional two research studies examined the survival of C. sakazakii in mock inoculated dehydrated PIF over several years (189,190). Previously, C. sakazakii strain 607, a clinical isolate, was observed to be extremely tolerant to heat (187). Also, C. sakazakii strain 607 was found to survive 687 days (1.8 years) in dehydrated PIF (189). Following storage for five months, the C. sakazakii population decreased by 2.3-logs with an additional 1-log reduction occurred during the last 17 months (189).
additional study screened a variety of clinical and PIF C. sakazakii isolates for desiccation tolerance in dehydrated PIF for 2.5 years (190). Typically, a 3-log reduction was observed within the first six months of storage, followed by a slow decline in recoverability (190). A total of five C. sakazakii strains (clinical and PIF isolates) were recoverable at two years, with only two strains (a clinical and a PIF isolate) detectable at 2.5 years (190). Interestingly, the C. sakazakii type strain (ATCC 29544) was found to be the most sensitive to storage and was undetectable after 12 months. A single study has reported osmotic stress tolerance in C. sakazakii (186). All C. sakazakii isolates tested were detectable following extended storage in sorbitol supplemented (40 and 75% w/v) BHI (186). All told, these studies highlight the unique ability for C. sakazakii to resist heat, desiccation, and osmotic stresses which contribute C. sakazakii’s prevalence in milk powders. Additional research has shown that C. sakazakii can be easily inactivated (~4-log reduction) if PIF is reconstituted with hot water (>70 °C) (189,191) and suggests that proper handling of PIF during and after reconstitution can reduce one’s risk of C. sakazakii infections.

Since C. sakazakii does not survive pasteurization, there is interest in determining contributing factors for post-pasteurization contamination in milk powders. Commonly, biofilms formed on food contact surfaces can be a source of microbial inoculum post-pasteurization. Many foodborne pathogens are biofilm-formers, and C. sakazakii is not an exception. C. sakazakii forms biofilms on stainless steel; however, the biofilm population size is strain-, temperature-, and growth-media-dependent (188,195,196). Biofilm formation during submersion in nutrient-dense or nutrient-limiting bacterial growth media at 37 °C produced biofilms with a maximum viable cell count of 5.5 and 6.5-log CFU/cm², respectively (196). Two additional studies evaluated biofilm formation on stainless steel in the presence of infant formula
broth (188,195). *C. sakazakii* clinical, food, and environmental isolates readily adhered to stainless steel at 12 and 25 °C with on average 3.63 and 4.30-log CFU/cm² population densities, respectively (195). The biofilm population quickly reached equilibrium with the surrounding planktonic cell culture and did not change for the duration of the experiment (195). Furthermore, *C. sakazakii* strains ATCC 29544 and 823 formed sparse biofilms on stainless steel incubated in rehydrated infant formula at 37 °C (188). The final population density of *C. sakazakii* strains ATCC 29544 and 823 at 24 hours was 1.2 and 1.7-log CFU/cm²; respectively (188). While the experimental methods are slightly different between the final two studies, incubation in infant formula at 25 °C correlates with a higher population density of *C. sakazakii* in stainless steel biofilms. In addition to stainless steel, *C. sakazakii* forms biofilms on several plastic materials, including silicon (188), latex (188), polycarbonate (188), polyvinyl chloride (PVC) (195,197,198), and polyurethane (198). Importantly, these plastics are common materials in hospitals and baby products. Of particular interest are the latter two plastics as they are used for enteral feeding tubes. A surveillance study of 57 *C. sakazakii* clinical, food, and environmental isolates reported that 58.9% of the isolates adhered and formed biofilms on PVC microtiter plates (197). Additionally, a higher proportion (65%) of the food and environmental *C. sakazakii* isolates were observed biofilm formers as compared to clinical isolates (29%) (197).

*C. sakazakii* biofilms on infant-feeding bottles, including silicon, latex, and polycarbonate, were determined with *C. sakazakii* strains ATCC 29544 and 823 incubated in rehydrated infant formula (188). While *C. sakazakii* ATCC 29544 formed biofilms on all plastics tested, the total viable population was significantly lower (1.2 – 2.5-log CFU/cm²) than *C. sakazakii* strain 823 (3.6 – 3.8-log CFU/cm²) (188). Both *C. sakazakii* strain ATCC 29544 and 823 adhered best to latex submerged in infant formula at 37 °C (188). Two studies have evaluated the ability for *C.
C. sakazakii to form biofilms on enteral feeding tubes in the presence of rehydrated infant formula (195,198). C. sakazakii clinical, food, and environmental isolates readily adhered to PVC tubes at 12 and 25 °C with on average 4.98 and 5.4-log CFU/cm² population densities; respectively (195). Over a ten-day incubation in infant formula, the C. sakazakii biofilm population densities on PVC tubing increased by 1-log CFU/cm² at 25 °C (195). Meanwhile, C. sakazakii biofilm population densities on PVC tubing decreased significantly to below 4-log CFU/cm² at 12 °C (195). Furthermore, biofilms formed by several C. sakazakii isolates were observed on PVC and polyurethane tubing following incubation in rehydrated infant formula (198). The average C. sakazakii population densities on PVC and polyurethane ranged from 4.6 – 6.3-log CFU/cm and 5.3 – 6.9-log CFU/cm; respectively (198). Interestingly, silver-impregnated flexilene plastic had the highest C. sakazakii biofilm population densities (5.3 – 7.4-log CFU/cm) (198). While this tubing is not used as a feeding tube, it is advertised as having antimicrobial properties. The silver-impregnated flexilene plastic did not prevent biofilm formation by C. sakazakii and unfortunately, enhanced C. sakazakii biofilm formation (198). Furthermore, these results were supported when C. sakazakii was recovered from a discarded enteral feeding tube (199); however, the C. sakazakii isolate came from an infant who was enterally fed breastmilk (199). Taken together, these studies demonstrate the importance of proper sanitation and cleaning to prevent C. sakazakii biofilm formation following contact with rehydrated infant formula.

As previously discussed, biofilm formation and thermal, desiccation, and osmotic tolerance contribute to the virulence and persistence of C. sakazakii in the environment. A limited amount of research groups has begun to explore other factors involved in the pathogenesis of C. sakazakii. Related to biofilm formation is the production of capsule polysaccharides (CPS). The production of CPS within the C. sakazakii species is strain-specific
While capsule production did not correlate to biofilm formation on enteral feeding tubes or PVC microtiter plates (197,198), capsule production did enhanced the attachment and biofilm formation on latex, silicon, and polycarbonate (188). Additionally, capsule production was associated with thermal (188) and desiccation tolerance (190). Interestingly, the *C. sakazakii* type strain (ATCC 29544) is not capsulated (188,197). Recently, a novel K-antigen region has been identified and reported to be ubiquitous among *C. sakazakii* strains (n = 75); notably, *C. sakazakii* type strain (ATCC 29544) was included in the analysis (200). Additionally, this study correlated the presence of a K2 antigen with highly virulent *C. sakazakii* strains, those causing meningitis and NEC (200). Like other Gram-negative microorganisms, *C. sakazakii* expresses and presents lipopolysaccharides on its extracellular surface (160,201). Initially, two serotypes were identified based on sequence variation of the O-antigen locus (201). The *C. sakazakii* type strain (ATCC 29544) expresses O1 antigen (160,201). Further research has revealed a total of seven serotypes in *C. sakazakii* with O1 & O2 representing the majority of isolates (160). An O-antigen deficient *C. sakazakii* strain (ATCC BAA-894) had increased biofilm formation compared to wild-type (202). *C. sakazakii* strains have peritrichous flagella and are motile (159). The *C. sakazakii* flagella are associated with biofilm formation, intestinal adhesion, and inducement of an immune response (203,204). Aflagellate gene knockouts of *C. sakazakii* strain ES5 were deficient in biofilm formation and adhesion to the human cell line Caco-2 (203). Additionally, detached flagella from *C. sakazakii* strains (ATCC BAA-894 and ATCC 29004) induced a TLR5-dependent immune response in monocyte-derived macrophages (204). Since, the primary route of entry into an infant is through the oro-gastrointestinal tract, *C. sakazakii* must utilize mechanisms to promote adhesion and invasion of the gastrointestinal tract. A surveillance study analyzed the ability for 50 clinical,
food, and environmental to adhere to the human epithelial cell line, Caco-2 (205). A total of 29
*C. sakazakii* strains adhered to Caco-2 cells with no reported differences due to their source of
isolation (205). Furthermore, the maximum percent adhesion of *C. sakazakii* strains to Caco-2
was 1.9% (206) and 2.6% (207) of the inoculum. The later study demonstrated that *C. sakazakii*
percent adhesion was similar to the positive controls, *Salmonella enterica* ser. Enteritidis (1.4%)
and *Enterobacter cloacae* (3.7%) and much higher than the *E. coli* K12 negative control (0.04%)
(207). The maximum percent invasion of Caco-2 cells by *C. sakazakii* was 0.23% (207) and 1%
(208) of the inoculum. Invasion of Caco-2 cells by *C. sakazakii* was significantly higher than
noninvasive controls (207,208). Several studies have demonstrated that invasion of Caco-2 cells
is mediated by outer membrane proteins, including OmpA (209,210), OmpX (209), and Inv
(210). These *in vitro* studies provide insights into the mechanisms *C. sakazakii* utilizes during
pathogenesis in humans.

1.6 *Candida albicans* physiology and oro-gastrointestinal colonization

The *Candida* genus is a morphologically and genetically diverse group of fungal
microorganisms. Traditionally, the *Candida* genus served as a reservoir taxon for “imperfect
fungi” without a defined sexual cycle (211). Consequently, the phylogeny of *Candida* species
and closely related fungi has been challenging to resolve. Recently, DNA-based typing methods
confirmed that *Candida* species are polyphyletic and distributed across the two main clades,
CTG and *Saccharomyces*, of the Saccharomycotina subphylum (212). Several common
pathogenic *Candida* species, including *C. albicans*, *C. parapsilosis*, and *C. tropicalis*, anchor the
CTG clade characterized by an alternative codon assignment (the CTG codon codes for serine,
not leucine). Other *Candida* species, such as *C. glabrata* and *C. krusei*, are genetically distinct
from the CTG clade *Candida* species and are more closely related to *Saccharomyces cerevisiae*. Clinically *C. albicans* is responsible for > 65% of candidiasis cases (213) and has garnered the most attention. *C. albicans* is a diploid yeast with eight pairs of chromosomes (214) and predominately undergoes asexual clonal replication. Although rare, a parasexual cycle is stress-induced (215) and contributes to the genetic diversity of *C. albicans*. Early work found significant karyotypic variation in clinical (214,216–218) and laboratory (214,216) *C. albicans* isolates; however, karyotyping can be affected by *C. albicans*’ penchant for phenotypic switching (219). Subsequent fingerprinting methods were developed to resolve the phylogenetic diversity of *C. albicans*. A comparison of methods, including randomly amplified polymorphic DNA (RAPD), multilocus enzyme electrophoresis (MLEE), Southern blot hybridization with the Ca3 DNA probe confirmed the clonal nature of *C. albicans* (220). Additionally, this study demonstrated that the Ca3 probe was able to discriminate microevolutionary changes (220). These authors further identified five distinct geographical clusters of *C. albicans* using the Ca3 probe, representing North America (groups 1, 2, and 3) (220), South Africa (221), and Europe (222). The *C. albicans* lineage has been expanded to 17 different clades using an optimized set of MLST loci (223,224). Recently, whole genome sequencing of *C. albicans* has allowed for more thorough analysis of its evolutionary past. The diploid genome of *C. albicans* type strain SC5314, utilized in Chapter 5, is publically available (225). *C. albicans* SC5314 belongs to clade 1 which represents ~40% of all *C. albicans* isolates analyzed (224,226). Additionally, clade 1 *C. albicans* isolates are commonly disease-causing (227) and correlated with higher mortality in neonates under one year of age (228).

*Candida* spp. are commensal fungi that innocuously reside on and within the human body (229); however, under certain circumstances many *Candida* spp. can become pathogenic to their
hosts (230). *Candida* spp., of which *C. albicans* is the most clinically relevant (213), cause self-limiting fungal infections of the skin and mucous membranes or life-threatening disseminated and systemic infections (230). Oro-gastrointestinal colonization by *Candida* spp. is common in neonates with a total incidence rate of 4.6% (231) to 62% (232). Most studies observed incidence rates between 12.8% and 29% (233–238). While *Candida* was readily isolated from neonates, the relative abundance of oro-gastrointestinal *Candida* is low (<10^6 cells per gram) (229). *C. albicans* was the most prevalent yeast isolated from the mouth/oropharynx (231,233,235–240) and rectum/stool (231,233–240) of neonates followed by *C. parapsilosis* (233,234,236–238). In contrast, a single study reported slightly higher isolation of *C. parapsilosis* than *C. candida*, though both were isolated from 85% and 77% of neonates, respectively (232). Several studies observed *C. albicans* colonization by two weeks of age (231–237,239,240); however, detailed experiments regarding the timing of *C. albicans* colonization in neonates are limited. Overall *Candida* spp. colonization was correlated with, birth weight (235,238), gestational age (235,238), and vaginal delivery (236,238). A decreased birth weight and gestational age are significantly negatively correlated with *Candida* spp. colonization (235,238). The correlations reported in these studies should be interpreted with caution due to their small samples sizes. Additionally, birth weight and gestational age are inherently correlated and therefore, it is not surprising that both were found to be correlated with *Candida* spp. colonization. Furthermore, vaginal delivery was significantly positively correlated with *Candida* spp. colonization (236,238), suggesting transmission of maternal vaginal *Candida* spp. to the neonate during birth. In a cohort of 347 mother/neonate pairs, all 16 *C. albicans*–positive neonates were born to *C. albicans*–positive mothers with identical pulsotyopes (231). Another study found 84.6% of their mother/neonate (11/13) pairs had identical CARE-2 DNA banding
patterns of *C. albicans* (240). The authors concluded that vertical transmission occurred in these 11 neonates within one week postnatal. Neither study robustly demonstrated that direct vaginal transmission occurred. Limited research suggests that vertical transmission may occur before birth. *Candida* spp. (9), including *C. albicans* (8,10) have been cultured from amniotic fluid samples. More compelling research suggests that horizontal transmission (nosocomial) of *Candida* spp. readily occurs among neonates admitted to a neonatal intensive care unit (233,234,241). Specifically, the hands of healthcare professionals, 29% positive for *Candida* spp., were strongly implicated in the transmission of *C. albicans* and *C. parapsilosis* (234). As scientists gain more knowledge on *C. albicans*, and other *Candida* spp., role as a commensal microbe and its colonization in humans, healthcare professionals will be better suited to handle *C. albicans* infections and outbreaks.

As an opportunistic pathogen and near ubiquitous association with the human orogastrointestinal tract, studying *C. albicans* colonization in humans is limited to observational studies. Therefore, researchers began investigating *C. albicans* colonization in rodents following oral administration of the fungus. Unfortunately, *C. albicans* is not a member of the commensal murine mycobiota and is unable to displace the resident bacterial and fungal community. Several groups found that reducing the resident microbiota by pretreatment with antibiotics (242–247), steroids (242,244), X-ray irradiation (243,244) increased the susceptibility of the adult rodents to colonization by *C. albicans*. In pretreated rodents, the gastrointestinal *C. albicans* density ranged from $10^6$ – $10^7$ cells per gram feces/contents/tissue and remained stable for 3 (246), 12 (243) and 21 days (242,247). In one study, *C. albicans*-positive mice were treated daily with antibiotics (243) which continually depressed the resident microbiota. Other groups colonized neonatal rodents with *C. albicans* while the resident microbiota was naïve and
susceptible (248–251). The gastrointestinal *C. albicans* density at $10^2$ - $10^5$ cells per gram feces/contents/organ at 20 days (248), 2 (250), 4 (251), or 10 weeks (249) was significantly lower than the pretreated adult mice. To better model human colonization with *C. albicans*, adult mice with resident microbiota were orally inoculated with *C. albicans*. Without pretreatment, the gastrointestinal *C. albicans* density at $10^2$ – $10^4$ cells per gram feces/contents/tissue (252–255) was similar in *C. albicans* inoculated neonatal mice (248–251). While these studies demonstrate *C. albicans* colonization in adult mice without pretreatment, the *C. albicans* inoculum was administered for 3 days (252) to 2 weeks (253–255). Additionally, these studies reported significant increases in gastrointestinal *C. albicans* density to $10^5$ – $10^8$ cells per gram feces/contents/tissue following treatment with antibiotics (245,252,254,255) and immunosuppressants (252). While these rodent models were designed to study *C. albicans* colonization in humans, the artificiality of these models is problematic, particularly, due to the natural resistance of the rodent gastrointestinal tract to *C. albicans* colonization. Neonatal piglets are often used as human biomedical models, including microbial colonization. While dissemination of *C. albicans* was investigated in gnotobiotic neonatal piglets (256), no current piglet models have been developed to examine *C. albicans* colonization.
Table 1.1. Human clinical trials evaluating prebiotic supplementation in infant formula. Studies were included if prebiotics were clearly identified, were the sole supplement, and were delivered to healthy infants. More specifically, excluded studies supplemented prebiotics in combination with a probiotic or other micro- or macronutrients or included infants with medical conditions.

<table>
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<th>Dosage</th>
<th>Microbial Response</th>
<th>Stool Properties</th>
<th>Infant Response</th>
<th>References</th>
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<td>Lactobacilli</td>
<td>Frequency</td>
<td>Consistency</td>
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<td>Down*</td>
</tr>
<tr>
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<td>Dosage²</td>
<td>Microbial Response³</td>
<td>Stool Properties³</td>
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<td>GOS/PDX (1/1)</td>
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<td>(72)</td>
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Table 1.1. (cont)

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</table>

1 All studies reported either no adverse effects with prebiotics or no differences between groups; 2 Prebiotics ratio and dosage is listed if reported by authors; 3 Outcomes were limited to the headings to ease comparisons; 4 Improvement in outcome as compared to control formula; 5 No dif = no statistical differences from control formula; 6 No change = no statistical differences from baseline; 7 GOS served as the formula control as there was no formula group without prebiotics; Abbreviations used: FOS = fructooligosaccharides, GOS = galactooligosaccharides, PDX = polydextrose; * P < 0.05; † P < 0.1; Blank space = outcome was not measured.
1.8 References


55. Thongaram T. Probiotic metabolism of human milk oligosaccharides (HMOs) and prebiotics. University of Illinois Urbana Champaign; 2014.


CHAPTER TWO
CHARACTERIZATION OF THE INTESTINAL LACTOBACILLI COMMUNITY FOLLOWING GALACTOOLIGOSACCHARIDES AND POLYDEXTROSE SUPPLEMENTATION IN THE NEONATAL PIGLET

2.1 Abstract

Recently, prebiotic supplementation of infant formula has become common practice; however, the impact on the intestinal microbiota has not been completely elucidated. In this study, neonatal piglets were randomized to: formula (FORM, n=8), formula supplemented with 2 g/L each galactooligosaccharides (GOS) and polydextrose (PDX, F+GP, n=9) or a sow-reared (SOW, n=12) reference group for 19 days. The ileal (IL) and ascending colon (AC) microbiota were characterized using culture-dependent and -independent methods. 16S amplicon sequencing identified no differences at the genera level in the IL. Interestingly, six genera in the AC were significantly different between FORM and F+GP (P<0.05): Lactobacillus, Ruminococcus, Parabacteroides, Oscillospira, Hydrogenoanaerobacterium, and Catabacter. In particular, the relative abundance of AC Lactobacillus was higher (P=0.04) in F+GP as compared to FORM. Culture-dependent analysis of the IL and AC lactobacilli communities of FORM and F+GP revealed a Lactobacillus spp. composition similar to 16S amplicon sequencing. Additional analysis demonstrated individual Lactobacillus isolates were unable to ferment PDX. Conversely, a majority of lactobacilli isolates could ferment GOS, regardless of piglet diet. In addition, the ability of lactobacilli isolates to ferment the longer chain GOS...
fragments (DP 3 or greater), which are expected to be present in the distal intestine, was not different between FORM and F+GP. In conclusion, prebiotic supplementation of formula impacted the AC microbiota; however, direct utilization of GOS or PDX does not lead to an increase in *Lactobacillus* spp.
2.2 Introduction

Infants are readily colonized following birth from exposure to the environment, most notably, microbiota from their mother’s vagina, skin, feces and potentially human milk[1-4]. After initial colonization, the infant microbiota is assembled through a complex succession of microbiota that is rapidly changing [5,6]. Additionally, the intestinal microbiota is influenced by infant nutrition [7]. Human milk is the gold standard for infant nutrition; however, formula feeding early in life is common and often required. Human milk oligosaccharides (HMOs) and their related glycoproteins and glycolipids are a significant component of human breast milk [8]. HMOs are a complex mixture of structurally diverse oligosaccharides that influence the gut microbial community, reviewed by Coppa and colleagues [9]. Bovine milk-based infant formulas are nearly devoid of complex oligosaccharides [10,11], which may negatively influence microbial colonization. Therefore, there has been interest to supplement infant formulas with alternative prebiotic oligosaccharides to provide similar functional benefits as HMOs [12-14].

Prebiotics are non-digestible carbohydrates that stimulate the growth of beneficial bacteria, thereby providing a health benefit to the host [14-17]. Several prebiotics are generally recognized as safe (GRAS) and are used as food ingredients, such as galactooligosaccharides (GOS), polydextrose (PDX) and fructooligosaccharides (FOS) [17-19]. Prebiotic supplementation of infant formula is well tolerated and stool softness and frequency are similar to breast-fed reference groups [20-22]. Additionally, GOS, commonly supplemented with FOS, increases counts of bifidobacteria [14,20,22,23] and to a lesser extent lactobacilli [23] in infant feces. A recent study reported a dose-dependent increase in lactobacilli in response to PDX supplementation in neonatal piglets [24]. Both bifidobacteria and lactobacilli have been used as...
commercial probiotics [25] and it is hypothesized that their health promoting functions in the gut may be enhanced by prebiotic supplementation.

Prebiotic supplemented formula is best suited for testing in infants; however, microbial community analysis is limited to feces. Therefore, a suitable analogue for infant studies is the neonatal piglet as it allows for more invasive sampling [26-28]. Findings in human infants show that bovine milk-based formula supplemented with prebiotics does not negatively affect piglet development [24,29-32]. While several studies have investigated the impact of prebiotics on the piglet gut microbiota by older traditional and molecular techniques [24,29,30,32,33], there remains a need for a more descriptive analysis.

In this study, vaginally-delivered neonatal piglets were used to model the development of the infant microbiota in response to GOS/PDX supplemented formula. We utilized culture-dependent and -independent methods to perform a broad analysis of the ileal and colonic microbiota. Additionally, we report several species of lactobacilli that are responsive to GOS supplementation.

2.3 Materials and Methods

2.3.1 Ethics statement

The experimental procedure and the use of animals were approved by the University of Illinois Institutional Animal Care and Use Committee (IACUC) in accordance with all laws and regulations pertaining to research with animals. The approved protocol was #09268.
2.3.2 Animals and diets

Neonatal piglets (n=29) were obtained from the University of Illinois Swine Research Center at 48 h-of-age to allow for ingestion of colostrum. Piglets were transported to animal facilities on campus and were individually-housed in custom cages in rooms maintained at 25 °C with 12 h light-dark cycles. Additional heat was provided to an ambient temperature of 30 °C within the piglet cages. The study was conducted in one replicate and used 3 litters (littermates were randomized into each treatment group). All piglets were randomized to receive a non-medicated bovine milk-based formula (Advance Baby Pig Liqui-Wean, Milk Specialties Company, Dundee, IL) alone (FORM, n=8) or formula supplemented with 2 g/L each GOS (FrieslandCampina, Meppel, Netherlands) and PDX (Danisco, Tarrytown, NY, USA) (F+GP, n=9). Sow-reared (SOW, n=12) piglets were included as a reference group. Formula was offered immediately after transport to the animal facility and delivered twenty-times daily by pump for a total volume of 360 mL/kg/d for 19 d.

2.3.3 Sample collection

On d 21, the piglets were lightly sedated with Telazol (7 mg/kg bodyweight; Fort Dodge Animal Health, Fort Dodge, IA, USA) and were euthanized by an intracardiac injection of sodium pentobarbital (72 mg/kg bodyweight; Fatal Plus, Vortech Pharmaceuticals, Ltd., Dearborn, MI, USA). Blood and tissue samples were collected for analysis as previously described [32]. Briefly, the small intestine was quickly excised from the pyloric sphincter to the ileocecal valve and its total length was measured. The intestine was cut into three segments at 10% and 85% of the total small intestine length to separate duodenum, jejunum and ileum. Ileal and AC contents were collected for bacterial enumeration, measurement of pH and percent dry
matter or were flash frozen and stored at -80 °C for 16S amplicon sequencing. The pH was measured using meter (Mettler Toledo, Columbus, OH, USA) equipped with a combination pH electrode (Hanna Instruments, Woonsocket, RI, USA). Percent dry matter of IL and AC contents was calculated following drying at 100 °C for 24 h. Following the removal of contents, intestinal sections were flushed with ice-cold phosphate buffered saline (PBS) and fixed in 10 % (v/v) formalin.

2.3.4 Intestinal histomorphology

Formalin-fixed IL and AC tissue samples were embedded in paraffin wax, sliced to approximately 5 μm with a Leica RM2255 microtome (Leica Geosystems, Norcross, GA, USA), mounted on glass microscope slides and stained with hematoxylin and eosin. Images were captured using the Nanozoomer Digital Pathology suite (Hamamatsu, Bridgewater, NJ, USA) and measurements were determined with AxioVision (Carl Zeiss Microscopy, Thornwood, NY, USA). Images were analyzed for 8-10 well formed villi or cuffs. IL villus height and crypt depth; AC cuff width and depth were measured.

2.3.5 Fermentation products

IL and AC contents were analyzed for volatile fatty acid (VFA) products by gas chromatography, as previously described [34]. VFAs measured were acetate, propionate and butyrate (short-chain fatty acids [SCFA]). Additionally, several branch-chain fatty acids (BCFA) isobutyrate, isovalerate and valerate were measured.
2.3.6 Culture-dependent characterization of the microbiome

2.3.6.1 Bacterial enumeration

Ileal and AC contents were homogenized in PBS by vortex and aseptically plated in triplicate using an Eddy Jet spiral plater (Neu-Tec, Farmingdale, NY, USA) on *Lactobacillus* selective (LBS) agar (BD, Franklin Lakes, NJ, USA). The agar plates were incubated for 48 h at 37 °C anaerobically (5 % CO₂, 5 % H₂ and 90 % N₂). Bacteria were counted using the spiral plater manufacturer’s methods and expressed as colony forming units (CFU) per gram of homogenized sample. Ten colonies per piglet per sample were randomly selected, cultivated in MRS, resuspended in 12.5 % glycerol and stored at -80 °C.

2.3.6.2 DNA extraction

Putative lactobacilli isolates were then grown in 2 mL MRS to stationary phase. Following growth, bacterial genomic DNA was isolated using a modified bead-beating technique [35]. Briefly, 2 mL of cells were pelleted at 10,000 x g and washed twice with molecular biology grade water (Mo Bio Laboratories, Carlsbad, CA, USA). The pellets were resuspended in 750 µL of 0.5 M EDTA buffer pH 8. The resuspended cells were subjected to bead-beating in a FastPrep-24 Instrument (MP Biomedicals, Solon, OH, USA) at 6 m/s for 1 min. The samples were then centrifuged at 10,000 x g at 4 °C for 3 min. A 600 µL aliquot of the supernatant was removed and frozen at -20 °C for subsequent analysis.

2.3.6.3 Identification

A partial HSP60 sequence was amplified using lactobacilli specific HSP60 primers, LB308F (5’-TGAAGAAYGTNRYNGCYGG-3’) and LB806RM (5’-
AANGTNCCVCGVATCTTGTGT-3’), previously described by Blaiotta and colleagues [36]. The PCR amplification of a 499 bp amplicon was performed with a 50 µL total volume. The reaction system consisted of 3 µL of target DNA, 5 µL of each primer (10 mM), 25 µL of EconoTaq Plus Green 2X Master Mix (Lucigen Corporation, Middleton, WI, USA). The PCR consisted of an initial step of 2 min at 95 °C, followed by 35 cycles (30 s at 95 °C, 30 s at 50 °C, and 1.5 min at 72 °C) and completed with an extension step at 72 °C for 15 min. PCR products were purified using the Zymoclean DNA Clean & Concentrator Kit (Zymo Research Corp., Irvine, CA, USA) according to the manufacturer's instructions. PCR products were quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE, USA). Sanger sequencing was done with either primer (LB308F or LB806RM) using an ABI 3730XL capillary sequencers (Life Technologies). The sequences were trimmed for quality and uploaded to the BLAST database (NCBI) to determine identity. Species identity was determined at 97 % sequence similarity.

To confirm identity, a few isolates from each identified species was further sequenced by amplification of a partial 16S rRNA sequence by using the 16S rRNA primers, 8F (5’-AGAGTTTGATCCTGGCTCAG-3’) [37] and 1392R (5’-GACGGGCGGTGWGTRCA-3’) [38]. The same PCR conditions, sequencing and identification was performed as outlined above.

2.3.6.4 In vitro carbohydrate utilization

Lactobacilli isolated from IL and AC were tested for the ability to utilize glucose (Fisher Scientific), Purimune galactooligosaccharides (GOSP, Ingredion, Westchester, IL, USA), and PDX as the sole carbohydrate source. In preparation for the assay, isolates were revived from -80 °C storage by culturing in 2 mL of MRS anaerobically for 24 or 48 h. Isolates were passaged (1%, v/v) one time into 2 mL semi-synthetic MRS medium (sMRS) containing per liter: 20 g
peptone, 5 g yeast extract, 5 g sodium acetate, 2 g disodium phosphate, 2 g ammonium citrate, 1 mL tween 80, 0.1 g magnesium sulfate, 0.05 g manganese sulfate. Glucose (10 g/L) was added when necessary. Isolates were further incubated overnight (16-24 h). Following growth, a 1 mL aliquot of sMRS grown cells were washed twice using PBS, pH 7.0 by centrifugation at 13,000 x g for 5 min and resuspended in 1 mL sterile carbohydrate-free sMRS.

Stock sMRS solutions containing either a 2 % (w/v) of the carbohydrates tested or blank (no added carbohydrate) were prepared and 130 µL aliquots were dispensed into a 96-well plate. The washed isolates were diluted 10-fold in 1 mL of carbohydrate-free sMRS and 20 µL was used to inoculate each well. Experimental plates were pre-incubated anaerobically for 30 min, followed by the addition 50 µL of mineral oil (bioMérieux, Craponne, France) to each well. Immediately, the initial optical density (OD) at 595 nm was measured using a Multiscan Ascent 96-well plate reader (MTX Lab Systems, Vienna, VA, USA). The plates were incubated anaerobically at 37 °C for 48 h. After 24 and 48 h incubation, the plates were vortexed to resuspend the cells and OD was recorded. Each well was blank-corrected at each time point (0, 24 and 48 h) and the maximum OD (ODmax) was calculated at 24 and 48 h. Utilization cutoffs were defined by the ODmax obtained and are as follows; low utilization: OD<0.4, moderate utilization: 0.4>OD<0.7 and high utilization: OD>0.7. The relative utilization of GOS and PDX was calculated to adjust for isolates incapable of growing in vitro.

2.3.6.5 Thin layer chromatography

Consumption of GOSP was determined by measuring the GOS fractions remaining in the post-fermentation supernatants of lactobacilli isolated from the IL and AC as previously described [39]. Briefly, 2 µL of a 1 % (v/v) carbohydrate standard or bacterial post-fermentation
supernatant was spotted onto TLC silica gel plates (Sigma-Aldrich, St. Louis, MO, USA). Glucose, galactose (Sigma-Aldrich), lactose (Fisher Scientific) and GOSP were included as carbohydrate standards. The TLC developing solvent was composed of a 2:1:1 mixture of n-propanol (Fisher Scientific), acetic acid (Fisher Scientific) and water. TLC plates were further prepared by spraying an ethanol based solution containing 0.5% (w/v) α-naphthol (Alfa Aesar) and 5% (v/v) H$_2$SO$_4$ (Sigma-Aldrich). TLC plates were visualized by heating at 100°C for 5 min.

2.3.7 Culture-independent characterization of the microbiome

2.3.7.1 DNA extraction

Bacterial genomic DNA was isolated from 200-300 mg of IL and AC contents using a modified bead beating method [40,41]. The modifications included: (1) IL and AC content samples were lysed using a 3 min homogenization step with beads using a vortexer and (2) extracted DNA was resuspended in TE buffer overnight at 4 °C instead of mechanical pipetting. These modifications were used to minimize DNA shearing for downstream applications. The DNA was purified using QIAmp DNA Stool Kits (Qiagen, Valencia, CA, USA). Extracted DNA was quantified by NanoDrop.

2.3.7.2 Quantitative PCR

Butyrate productivity was quantified from extracted bacterial genomic DNA from AC contents. Butyrate productivity was determined by amplification of the Butyryl-Coenzyme A transferase using previously published methods [42]. Standard curves of $10^2$ to $10^8$ CFU/mL were produced using a 573 bp gene product synthesized and ligated into a plasmid (GeneArt,
Carlsbad, CA, USA). The minimum detectable CFU/mL was $10^3$. The AC bacterial genomic DNA was diluted 10-fold prior to quantification. Quantitative PCR was performed using the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Each 10 µL qPCR reaction included 5 µL of 2x Power SYBR Green PCR Master mix (Applied Biosystems), bovine serum albumin at a final concentration of 1 µg/µL (New England Bio Labs, Ipswich, MA, USA), 0.5 µM of each primer and 2 µL of the DNA sample. The PCR conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 53 °C for 1 min and 72 °C for 30 s. Following amplification, a dissociation step was included to analyze the melting profile of the amplified products and ensure that no extraneous products were generated.

2.3.7.3 16S amplicon sequencing

Genomic DNA isolated from IL and AC contents were analyzed by 16S amplicon sequencing at the Research and Testing Laboratory (RTL, Lubbock, TX) based upon RTL protocols [43]. Briefly, the V1-V3 hypervariable region of the 16S rRNA gene was amplified using the Gray28F (5’-GAGTTTGATCNTGGCTCAG-3’) and Gray519R (5’-GNTTACNGCGGCKGCTG-3’) primer set. Sequencing was performed using the Roche 454 FLX instrument (Roche, Indianapolis, IN, USA). For a detailed description of the sequencing and bioinformatic analyses, refer to Ishak and colleagues [44]. Raw read data was submitted to the Sequence Read Archive (NCBI) and can be accessed with the accession number SRP049961.
2.3.8 Statistical analyses and illustrations

Analysis of piglet weight, weight gain, intestinal lengths and weights, intestinal histomorphology, fermentation products and bacterial enumeration was performed using the general linear model (GLM) procedure within SAS (Version 9.2, SAS Institute, Cary, NC, USA). When significant comparisons (P<0.05) were observed, post hoc analysis using the least significant difference (LSD) was used. Lactobacilli communities are presented as stacked bar charts produced with R [45] using the gplots [46] package. Dendrograms were constructed using the Bray-Curtis Dissimilarity index using the vegan [47] R package. Prior to analysis of the 16S amplicon sequencing data, rarefaction was used to standardize richness to 3118 sequences per sample, thus facilitating comparisons among groups. In addition, IL samples were analyzed separately from AC samples. Microbial diversity and richness was quantified as the number of operational taxonomic units (OTUs) or with Chao1’s richness estimator (each were defined based on 3% divergence). The impact of diet on bacterial diversity and total estimated richness was evaluated using one-way ANOVA. The overall variability within the microbiota was illustrated using Principle Coordinates Analysis (PCoA), and multivariate differences among diet groups were evaluated using distance-based redundancy analysis (dbRDA) [48]. For PCoA and dbRDA, phylogenetic distances among samples were first calculated using the UniFrac distance measure, unweighted UniFrac distance considers only changes in composition (i.e., identities) [49]. UniFrac was calculated using QIIME [50], and all other analyses were conducted in R [45], using the vegan [47] and labdsv [51] packages. Individual bacterial taxa were screened for differences among diets using ANOVA. Prior to analysis, relative abundances were transformed using an arcsin transform (p’ = arcsin (√p)). To control for errors due to multiple testing and to limit the amount of false positives, ANOVA model p-values were adjusted to maintain a false
discovery rate (FDR) of 10%. Indicator species analysis [52] was used to identify individual species within the genus *Lactobacillus* that were indicative of each of the diets. Indicator species analysis synthesizes information about occurrence and abundance of individual taxa, and this information is summarized as an indicator score. The analysis also provides a randomization test of the degree to which taxa are indicative of a particular state. Here, all species are shown, regardless of the results of the randomization test.

2.4 Results

2.4.1 Animal observations

The final body weight and overall weight gain of the piglets were not different between the groups (P>0.05, data not shown). Additionally, the total intestinal weight and length did not differ between groups and averaged 196 ± 41.3 g/kg and 148.8 ± 40.3 cm/kg; respectively. Intestinal histomorphology demonstrated that the ileal (IL) villus length, crypt depth and ascending colon (AC) cuff width were not significantly different among the groups (P>0.05; Table 2.1). However, the SOW group had significantly deeper AC cuff depth as compared to FORM and F+GP groups (P<0.0001). While there were no differences in the percentage of dry matter in the AC contents (P>0.05, Table 2.1), the percentage of dry matter in the IL was lower in the F+GP group than the SOW group (P=0.04), but not the FORM group.

2.4.2 Isolation and characterization of intestinal lactobacilli

Based on published research, we hypothesized that supplementation of GOS/PDX should selectively stimulate the growth of lactobacilli. Therefore immediately following euthanasia, IL and AC contents were plated on *Lactobacillus* selective (LBS) agar to obtain lactobacilli isolates.
Total presumptive *Lactobacillus* counts (CFU/g) were not significantly different between the groups or location (P>0.05, data not shown). While the absolute abundance of lactobacilli was not different between groups, it may be the composition of the lactobacilli community that is important. Therefore, approximately 10 isolates per piglet were cultivated and speciated using a combination of the HSP60 and 16S rDNA gene sequences. All of the isolates identified were of the order *Lactobacillales* and were represented by eight *Lactobacillus* species and two non-*Lactobacillus* genera. Unfortunately, the co-isolation of *Pediococcus* and *Weisella* with *Lactobacillus* in the formula-fed piglets was quite significant (range 10-37% of total isolates). The *Pediococcus* and *Weisella* isolates were removed from further analyses.

Culture-dependent analysis showed a variable lactobacilli community between groups (Figure 2.1). The SOW group was dominated by two lactobacilli: *L. johnsonii* (IL: 64%, AC: 50%) and *L. reuteri* (IL: 33%, AC: 44%). While *L. johnsonii* was also highly abundant in the FORM and F+GP groups, these two groups had a more diverse lactobacilli community as compared to the SOW group. *L. vaginalis* was solely detected in the FORM and F+GP groups, representing 7-13% (IL and AC) of the isolates.

To determine if the supplementation of GOS/PDX selectively enriches for a GOS/PDX fermenting community, carbohydrate utilization was conducted with the FORM and F+GP lactobacilli isolates (Figure 2.2 and Table A.1). The majority of lactobacilli isolates from both the FORM and F+GP piglets were found to utilize GOS well (ΔOD>0.7); whereas, the utilization of PDX was minimal. While a larger proportion of lactobacilli isolates were defined as high utilizers of GOS in the IL (FORM: 87.5% vs. F+GP: 97.3%), this was not the case in the ascending colon (FORM: 86% vs. F+GP: 70.7%). Thin layer chromatography was conducted to determine if the ability to consume different GOS fractions was different due to GOS
supplementation (data not shown). In general, the lactobacilli isolates with a low (ΔOD<0.4) or moderate (0.4>ΔOD<0.7) ability to utilize GOS corresponded with a reduction in the mono- and disaccharide fractions (DP1-2). The lactobacilli isolates classified as high utilizers (ΔOD>0.7) demonstrated partial consumption of trisaccharide GOS (DP3) as well as complete consumption of the DP1-2 fractions. We did not observe any reduction in the longer GOS chains (DP4-6) by any of the isolates. Interestingly, *L. vaginalis* was observed to differentially utilize GOS *in vitro*. The few *L. vaginalis* isolates that grew to an OD>0.4 showed at least partial consumption of GOS (DP1-3); whereas, the majority of isolates were not capable of fermenting GOS as a sole carbon source. Overall, the consumption of GOS was not different between the FORM and F+GP piglets regardless of location.

2.4.3 Characterization of intestinal bacterial community

The IL and AC microbiota was analyzed by 16S amplicon sequencing which generated a total of 292,547 sequences with an average of 5,519 sequences per sample. Following sequence normalization, overall microbial diversity was determined by calculating the total number of operational taxonomic units (OTUs) detected and total richness was estimated by the Chao1 index (Table 2.2). The SOW group had higher IL microbial diversity and total richness as compared to the formula-fed piglets (OTUs: P=0.01, Chao1: P=0.025; respectively). The microbial diversity and total richness were not significantly different in the AC.

The overall variation in the piglet microbiota among the three groups was evaluated using a distance-based redundancy analysis (dbRDA). Distances were calculated using the unweighted Unifrac distance measure and were illustrated using Principle Coordinates Analysis (PCoA). The PCoA of the piglet microbiome shows a clear divergence in location of samples (IL vs. AC)
and between the SOW piglets and the formula-fed piglets (Figure 2.3a). This divergence was evident following characterization at the taxonomic level. A heat map was constructed with the relative abundances of the dominant taxa in both locations (Figure A.1). Groups were clustered using the unweighted Unifrac distances. In the IL, the SOW group was characterized by a high abundance of *Clostridium* spp. whereas the formula-fed piglets were defined more by the presence of *Weissella* spp. In the AC, the differences in relative abundance of *Faecalibacterium* and *Parabacteroides* showed separation of the SOW group from the formula-fed groups (Figure A.2).

In general, our data (Figure 2.3 and A.1-A.2) demonstrates that sow-reared piglets may not be an appropriate reference group. The primary focus of this study was to analyze the bacterial composition due to a prebiotic supplementation. The differences between the formula-fed piglets and the sow-reared piglets were not solely dietary; therefore, the 16S amplicon sequencing data was reanalyzed without the SOW group in order to observe differences due solely to the prebiotic supplementation.

Consistent with the previous 16S amplicon sequencing data, the microbial diversity and total richness of the FORM and F+GP groups were not significantly different in the IL or AC (P>0.05, data not shown). Similar clustering between the FORM and F+GP groups by location was observed when determining the overall variation of the microbiome with a PCoA of the unweighted Unifrac distances (Figure 2.3b). In the IL, no detected genera had a FDR less than 10%; therefore further statistical analysis was not performed. However, six genera were detected in the AC that had an FDR less than 10% and were determined to be significantly different (P<0.05) between the FORM and F+GP groups based on differences in relative abundance (Table A.2 and Figure A.2). *Ruminococcus, Oscillopira, Hydrogenoanaerobacterium* and
Catabacter were significantly more abundant in the FORM group, while Parabacteroides and Lactobacillus were significantly more abundant in the F+GP group. Interestingly, the relative abundance of Parabacteroides more than two times greater in the F+GP piglets (FORM: $8.67 \pm 2.22$ vs. F+GP: $18.33 \pm 3.98$; Table A.2).

2.4.4 Culture-independent examination of lactobacilli community

16S amplicon sequencing analysis identified that the Lactobacillus genus differed between the FORM and F+GP groups in the AC (SOW group is included for reference). Eight major species of Lactobacillus, having a relative abundance above 1% of the total lactobacilli community, were detected in IL and AC (Figure 2.4). Consistent with the culture-dependent results, L. johnsonii is a dominant member of the lactobacilli community in all piglets. On the other hand, L. mucosae was detected in all piglets by 16S amplicon sequencing; however, was not among the lactobacilli isolated from either the IL or AC.

Further analysis was conducted to determine if any lactobacilli were predictive of a particular treatment (Figure 2.5a-b). Indicator species analysis showed that L. plantarum, L. amylovorus and L. pentosus to be indicative of the group FORM but not the F+GP group in the IL (Figure 2.5a). Conversely, L. vaginalis, L. johnsonii and L. reuteri were more indicative of the F+GP group in the IL. In the AC, L. johnsonii, L. mucosae, L. agilis and L. reuteri were highly indicative of the F+GP group as compared to the FORM group; whereas, L. vaginalis and L. rogosae, were slightly indicative of the FORM group (Figure 2.5b).
2.4.5 Fermentation products

Volatile fatty acids (VFA) were determined from the IL and AC contents (Figure 2.6). In the IL, propionate was higher (P=0.028) in the FORM group as compared to the F+GP and SOW groups; (Figure 2.6a) and isovalerate concentration was lower (P=0.004) in the F+GP group when compared to the SOW group, but not the FORM group (Figure 2.6b). The IL pH ranged from 7.7-7.8 and was not different among the treatment groups (P=0.892). In the AC, there were no significant differences in the measured SCFAs (P>0.05, Figure 2.6c). Furthermore, the SOW group had higher observed levels of isobutyrate (P=0.004), isovalerate (P=0.001) and total BCFA (P=0.008) than the formula-fed groups (Figure 2.6d). The AC pH tended (P=0.055) to be lower in both formula-fed groups (FORM: 6.8, F+GP: 6.6) than in the SOW group (7.3).

The total butyrate productivity was evaluated in the AC contents using quantitative PCR. The absolute abundance of the butyryl-Coenzyme A transferase gene was not significantly different between the three groups (P=0.4281, data not shown).

2.5 Discussion

Prebiotic supplementation of infant formula is well tolerated, improves the consistency and frequency of stools and provides a bifidogenic effect [14,20-22,53-57]. Additionally, our group has shown that prebiotic supplementation does not increase bacterial translocation in neonatal piglets [32]. Taken together, these studies demonstrate the nutritional benefits and safety for prebiotic supplementation of infant formula. However, a detailed examination of the impact of prebiotics on the composition of the gut microbiome and the products of microbial fermentation is lacking. In addition, identification of prebiotic-responsive lactobacilli could assist in determining which organisms are enhanced by prebiotic supplementation. In this study,
the impact of GOS and PDX (2 g/L each) provided in a bovine milk-based infant formula was examined in neonatal piglets, a relevant infant model [26-28]. Since prebiotic fermentation is restricted to the distal GI tract [58], the IL and AC were targeted for analysis.

Prebiotic supplementation did not significantly affect the body weight gain or intestinal weight or length compared to SOW or FORM. Formula feeding minimally impacted the intestinal histomorphology, compared to SOW with no impact of prebiotic supplementation. The AC crypt depth was significantly deeper in the SOW piglets as compared to the formula-fed piglets, which is consistent with our previous study that investigated bacterial translocation of GOS/PDX-supplemented formula-fed piglets [32]. Another study that supplemented formula with inulin and GOS did not detect differences in colonic histomorphology due to prebiotic supplementation; however a sow-reared group was not included [59]. Additional piglet studies that measured intestinal histomorphology following prebiotic supplementation only reported ileal measurements [24,31]. Therefore, we can conclude that the addition of GOS and PDX to formula is well tolerated and minimally impacts piglet intestinal development.

Changes in the IL and AC microbiota due to prebiotic supplementation were evaluated by high-throughput 16S amplicon sequencing. Significant differences in the intestinal microbiota composition were observed between the SOW and formula-fed piglets. In this study, there were differences in nutrition (sow’s milk versus bovine milk-based formula), housing (group housing with siblings and sow versus individual-housing of formula-fed piglets) and exposure to novel microbiota. Previous work has shown that formula-fed and breast-fed infants have different microbial composition [7,60], that stress can affect the microbial composition [61] and that homogenization of the gut bacterial community may occur in co-housed animals [62].
Therefore, future microbial studies examining formula-fed versus mother-fed animals should take care to minimize the variation between groups.

Due to the distinct differences between SOW and the formula-fed piglets, the 16S amplicon sequencing data was reanalyzed without SOW in order to isolate differences solely due to prebiotic supplementation. The bacterial diversity and richness did not differ between FORM and F+GP; however, the relative abundance of several bacterial genera were significantly different in the AC. Interestingly, \textit{Parabacteroides} and \textit{Lactobacillus} were identified as being significantly more abundant in the AC of F+GP piglets. \textit{Parabacteroides}, closely related to \textit{Bacteroides} \cite{63}, may possess enzymes capable of utilizing either GOS or PDX; however, there is currently no published data to confirm this hypothesis. Martinez and colleagues \cite{64} did report a significant increase of \textit{Parabacteroides distasonis} in human feces following consumption of resistant starch type 4 but not resistant starch type 2. Future studies should evaluate the \textit{Parabacteroides} community in relation to GOS/PDX supplementation in greater detail.

Historically, lactobacilli are considered health promoting microorganisms and have recently gained notoriety as probiotics \cite{65}. Additionally, \textit{Lactobacillus} sp. have been shown to be stimulated by prebiotic inclusion in formula milk \cite{23,24}. Therefore, we hypothesized that the supplementation of GOS/PDX may alter the \textit{in vivo} lactobacilli community. Following speciation of presumptive lactobacilli isolates, we observed that \textit{L. johnsonii} was the predominant member in the IL and AC of all piglets. While the formula-fed piglets had a more diverse lactobacilli community, it was not apparent if any \textit{Lactobacillus} sp. isolated were prebiotic-responsive. Therefore, the lactobacilli isolates were tested for their ability to utilize GOS and/or PDX as a sole carbon source (direct fermentation). We observed extensive
utilization of GOS but minimal utilization of PDX by *Lactobacillus* sp. isolated from the IL and AC of all formula-fed piglets. Therefore, we further hypothesized that GOS supplementation may select for lactobacilli isolates that are capable of consuming longer chain GOS (DP3-6) rather than the mono- and disaccharide fractions (DP1-2) that are mostly removed by digestion prior to the IL and AC. To test this hypothesis, the post-fermentate was analyzed by TLC for consumption of GOS fractions (DP1-6). In general, most lactobacilli isolates were capable of consuming the mono- and disaccharide fractions (DP1-2); however, a smaller fraction of lactobacilli were capable of consuming trisaccharide GOS (DP3). Importantly, there was no difference regarding the number of FORM and F+GP lactobacilli isolates that could ferment the longer GOS fractions. Direct fermentation of GOS or PDX doesn’t appear to be responsible for the increase in the lactobacilli population in the AC of the F+GP piglets.

Consistent with culture-dependent analysis, *L. johnsonii* was highly abundant in both FORM and F+GP piglets and was indicative of the F+GP treatment in the IL and AC. Various strains of *L. johnsonii* have been investigated in depth for their health promoting properties. In a rodent model for diabetes mellitus type 1 (T1DM), *L. johnsonii* was differentially abundant in the T1DM-resistant rats versus their T1DM-prone cohorts [66]. Furthermore, transmission of the strain *L. johnsonii* N6.2 can delay the onset of T1DM in prone rats [67]. The potential mechanisms of action of *L. johnsonii* N6.2 in this rodent model have been investigated and include enhancement of intestinal barrier function [67], reduction of intestinal oxidative stress [67] and modulation of the immune system [68-70]. Administration of *L. johnsonii* (La-1) to mice with branchial induced asthma dampened the autoimmune response by reducing the proportion of CD4+ T lymphocytes expressing IL-4 and increasing CD4+ T lymphocytes
expressing IFN\(\gamma\) [71]. Thus, promoting the growth of \emph{L. johnsonii} by feeding PDX and GOS has the potential to modulate immune development; however, this remains to be directly tested.

Indicator species analysis of the 16S amplicon sequencing data identified two additional \emph{Lactobacillus} species that were characteristic of the F+GP piglets. \emph{L. vaginalis} was strongly indicative of the F+GP piglets in the IL, whereas \emph{L. mucosae} was strongly indicative of the F+GP piglets in the AC. Surprisingly, the majority of \emph{L. vaginalis} representative isolates (79 \%) were not GOS utilizers \textit{in vitro} suggesting that direct GOS utilization did not lead to an increase in \emph{L. vaginalis} \textit{in vivo}.

Commonly, changes in microbial communities are coupled with the measurement of select byproducts of fermentation. Prebiotics, resistant to host digestion in the proximal GI of piglets and humans, are selectively fermented by the microbial community in the distal GI where the primary metabolites produced are SCFA from carbohydrate fermentation, [72] and BCFA from protein fermentation [73,74]. In the IL, no increase in total SCFA due to the prebiotics was detected; F+GP had the lowest SCFA concentration. Mono- and disaccharides are the primary substrate responsible for SCFA production in the IL, whereas prebiotics are largely fermented in the large bowel [58]. Total SCFA concentrations were not significantly different among the three groups in the AC; however, it is estimated that >95 \% of SCFA are rapidly absorbed by colonocytes [58]. Without the ability to measure the rate of host absorption of SCFA, and the total amount of SCFA in AC contents, we cannot determine the degree to which GOS and PDX were fermented \textit{in vivo}. In young pigs fed a transgalactooligosaccharide-supplemented casein-cornstarch diet [75], ileal SCFA concentrations were similar to the amounts observed in the current study. Additionally, Fava and colleagues [76] reported that PDX supplementation did not affect SCFA in the distal small intestine or proximal colon of piglets. Thus, our results are
consistent with these studies. The production of BCFA is primarily due to the fermentation of amino acids rather than prebiotics [73,74]. The SOW group had significantly higher concentrations of isobutyrate, isovalerate and total BCFA. This is consistent with additional piglet studies performed in our group [77], and may be due to proteins in sow milk that are resistant to small intestinal digestion, such as immunoglobulins.

This study provided a descriptive analysis of changes in the intestinal communities of neonatal piglets fed formula milk supplemented with GOS and PDX. Most interestingly were the significant increases in Parabacteroides and Lactobacillus sp. in the F+GP piglets. We did not observe an increase in the amount of lactobacilli that can utilize GOS nor the DP of GOS that was consumed. Therefore, we conclude that direct utilization of GOS was not responsible for the observed increase in Lactobacillus sp. Additional studies should be conducted to determine if GOS/PDX utilization by Parabacteroides sp. in vitro and to identify if this genus has physiological benefits to a neonatal piglet.

2.6 Acknowledgements

MJM, SMD and ZEJ designed the research; JLH, DOK and SD conducted research; JLH, DOK and SBC performed statistical analysis; JLH and MJM wrote the manuscript; SMD and MJM made revisions and additions to the manuscript. MJM had primary responsibility for final manuscript content. This study was supported by Mead Johnson Nutrition. The authors would like to acknowledge Latrice Tynes and Joseph Murray for their contributions to the analysis of IL and AC histomorphology and Josh Warren for his assistance with the thin layer chromatography. We would also like to acknowledge Dr. Marcia Monaco for assistance in caring for the piglets.
2.7 Tables and Figures

**Table 2.1.** Histomorphology of ileum and ascending colon of 21-d-old piglets fed formula (FORM), formula supplemented with GOS and PDX (F+GP) or sow-reared (SOW).

<table>
<thead>
<tr>
<th>Diets</th>
<th>FORM</th>
<th>F+GP</th>
<th>SOW</th>
<th>GLM ANOVA</th>
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<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
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<tr>
<td>Ileum</td>
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<tr>
<td>Villus Length (µm)</td>
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<td>Crypt Depth (µm)</td>
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<td>177</td>
<td>30</td>
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<td>3.6</td>
<td>9.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.9</td>
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<tr>
<td>Ascending Colon</td>
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<td></td>
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<td></td>
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<tr>
<td>Cuff Width (µm)</td>
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<td>58&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6</td>
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<tr>
<td>Cuff Depth (µm)</td>
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<td>Dry Matter (%)</td>
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<sup>a,b</sup> values with the same letters are not different at α=0.05 (Tukeys LSD).
Table 2.2. Microbial analysis from ileal and ascending colon contents of 21d old piglets fed formula (FORM), formula supplemented with GOS and PDX (F+GP) or sow-reared (SOW) as determined by 16S amplicon sequencing.

<table>
<thead>
<tr>
<th>Diets</th>
<th>FORM</th>
<th>F+GP</th>
<th>SOW</th>
<th>GLM ANOVA</th>
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<td>SD</td>
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</tr>
</tbody>
</table>

¹ OTU and Chao1 normalized to 3118 sequences per sample.
² ND = not determined.

⁻⁻ values within the row with the same letters are not different.
Figure 2.1. Lactobacilli population is dominated by few members. Lactobacilli communities isolated from the ileum (IL) and ascending colon (AC) of 21 d old piglets fed either a bovine milk-based formula (FORM), formula supplemented with GOS and PDX (F+GP) or sow-reared (SOW). Stacked bar chart represents the percent relative abundance of each lactobacilli detected in the community. Beta diversity was calculated using the Bray-Curtis Dissimilarity Index and is visualized by a dendrogram.
Figure 2.2. Several isolated lactobacilli identified as GOS utilizers. Carbohydrate utilization of lactobacilli communities isolated from the ileum (IL) and ascending colon (AC) of 21 d old piglets fed either a bovine milk-based formula (FORM), formula supplemented with GOS and PDX (F+GP). (A) Percent relative utilization (OD>0.7) of glucose, Purimune galactooligosaccharide (GOSP) or PDX. To account for isolates not capable of in vitro fermentation, utilization was normalized to maximum number of isolates capable of utilizing glucose as a sole carbon source. (B) Maximum optical density (595 nm) of Lactobacilli sp. when GOSP is supplemented as a sole carbon source. Colored bar indicates the mean maximum optical density. Not all Lactobacillus sp. were detected in each treatment or location.
Figure 2.3. Bacterial communities vary greatly by location, but less by diet. Ileal (IL) and ascending colon (AC) microbiome of 21 d old piglets fed either a bovine milk-based formula (FORM), formula supplemented with GOS and PDX (F+GP) or sow-reared (SOW) illustrated by Principle Coordinate Analysis (PCoA). PCoA was constructed from the unweighted Unifrac distance measure which only considers the change in composition not abundance. PCoA of the 16S amplicon sequencing data set: (A) all three treatments; (B) formula-fed piglets only.
Figure 2.4. Culture-independent analysis reveals a more diverse lactobacilli community. Lactobacilli communities from the ileum (IL) and ascending colon (AC) of 21 d old piglets fed either a bovine milk-based formula (FORM), formula supplemented with GOS and PDX (F+GP) or sow-reared (SOW) as determined by 16S amplicon sequencing. Stacked bar chart represents the percent relative abundance of each lactobacilli detected in the community. Beta diversity was calculated using the Bray-Curtis Dissimilarity Index and is visualized by a dendrogram. Lactobacilli represented by the label Lactobacillus sp. were unable to be assigned a species. Minor Lactobacillus sp. is an aggregate of lactobacilli present in > 1 % percent relative abundance (Table A.3).
Figure 2.5. Identification of the predominant *Lactobacillus* spp. in each group. Indicator species analysis (ISA) of the lactobacilli community of 21 d old piglets fed either a bovine milk-based formula (FORM), formula supplemented with GOS and PDX (F+GP) or sow-reared (SOW, included for reference). Color change demonstrates the more a species is indicative of a diet. The higher the indicator score (i.e. darker color), the more that species is indicative of that group. ISA scores for the genera *Lactobacillus* detected in the (A) IL; (B) AC.
Figure 2.6. Prebiotic supplementation leads to minimal changes in static volatile fatty acid concentration. Volatile fatty acids detected in ileal (IL) and ascending colon (AC) contents of 21 d old piglets fed either a bovine milk-based formula (FORM), formula supplemented with GOS and PDX (F+GP) or sow-reared (SOW). VFAs detected in IL: (A) Short-chain fatty acids; (B) Branched-chain fatty acids. VFAs detected in the AC: (C) Short-chain fatty acids; (D) Branched-chain fatty acids. Values with different superscripts are significantly different (p<0.05).
2.8 References


CHAPTER THREE

IN VITRO IMPACT OF HUMAN MILK OLIGOSACCHARIDES ON ENTEROBACTERIACEAE GROWTH

3.1 Abstract

Human milk oligosaccharides (HMOs) function as prebiotics in the infant gut by selecting for specific species of bifidobacteria and bacteroides, but little is known about their potential utilization by Enterobacteriaceae, the relative numbers of which have been linked to the onset of necrotizing enterocolitis in preterm infants. In this study, the \textit{in vitro} growth of purified HMOs and other related carbohydrates was evaluated using individual strains of Enterobacteriaceae and an Enterobacteriaceae consortia enriched from piglet feces. None of the Enterobacteriaceae strains grew on 2'-fucosyllactose, 6'-sialyllactose or lacto-N-neotetraose (LNnT); however, several strains were capable of utilizing galactooligosaccharides, maltodextrin, and the mono- and disaccharide components of HMOs for growth. The enriched fecal consortia also did not grow on 2'-fucosyllactose or 6'-sialyllactose, but there was limited growth on LNnT. It was concluded that 2'-fucosyllactose and 6'-sialyllactose supplementation of preterm infant formula should not lead to an increase in Enterobacteriaceae; however, supplementation with LNnT may require further study.

\footnote{This chapter was published in its entirety in the Journal of Agricultural and Food Chemistry. Hoeflinger JL, Davis SR, Chow J, Miller MJ. (2015) In vitro impact of human milk oligosaccharides on Enterobacteriaceae growth. J Agric Food Chem 63(12):3295-3302. This article is reprinted with permission from the publisher.}
3.2 Introduction

Human milk is replete with biological compounds that not only provide neonates with an optimal nutrition source\textsuperscript{1} but are associated with proper intestinal, and immunological development\textsuperscript{2}. Of particular interest are the human milk oligosaccharides (HMOs), a group of complex carbohydrates produced by the mother and abundantly expressed in breast milk at levels ranging from 5 to 23 g/L\textsuperscript{3,4}. Significant work has led to the annotation of the structural diversity of HMOs with over 200 unique compounds identified\textsuperscript{5}. Regardless of their structural complexity, HMOs are not digestible by human neonates and have been detected in feces\textsuperscript{6}.

Over the past two decades, an extraordinary amount of work has been done to elucidate the relationship between HMOs and infant health. The research shows that HMOs function as part of a neonate’s innate immune system and protect the neonate while the adaptive immune system is being developed\textsuperscript{7,8}. Furthermore, HMOs act as soluble receptor analogues to prevent invasion by common pathogenic microorganisms\textsuperscript{4,9}. Specifically, neutral and acidic HMO fractions altered the adhesion of enteropathogenic \textit{Escherichia coli} to Caco-2 cells\textsuperscript{10,11}, whereas fucosylated HMOs reduced colonization of \textit{Campylobacter jejuni} in mice\textsuperscript{12}. Finally, HMOs have been shown to contribute to the establishment of a healthy gut microbiota, in particular, by selectively promoting the colonization of specific \textit{Bifidobacterium}\textsuperscript{13-15} and \textit{Bacteroides} species\textsuperscript{16}.

Improper establishment of the neonatal gastrointestinal (GI) microbiota has been implicated as one of several contributing factors to necrotizing enterocolitis (NEC),\textsuperscript{17} a highly detrimental and often fatal necrosis of intestinal tissue that is primarily observed in preterm infants. Fecal microbiome studies in preterm neonates have documented a bloom in relative abundance of \textit{Proteobacteria}, specifically members of the class \textit{Gammaproteobacteria}, at the
expense of *Firmicutes*.\textsuperscript{18-21} It is hypothesized that this microbial imbalance increases intestinal inflammation and promotes development of NEC.\textsuperscript{22}

Although there is a growing amount of literature regarding the utilization of HMOs by bifidobacteria and *Bacteroides*,\textsuperscript{15,23,23} little is known about the ability of Enterobacteriaceae, members of the *Gammaproteobacteria* class of bacteria, to metabolize or grow on individual HMOs. Marcobal and colleagues\textsuperscript{16} observed that two strains of *E. coli* (OP50 and EC100) showed limited growth when provided with a complex mixture of HMOs that was purified from pooled human milk samples. Hence, more data are needed to verify that other members of this family are unable to use individual purified HMOs.

In this study, several Enterobacteriaceae strains were selected and evaluated for their ability to grow in the presence of purified HMOs, prebiotics, and other related carbohydrates. In addition, carbohydrate utilization was assessed using an Enterobacteriaceae consortia enriched from piglet feces. Finally, the impact of HMOs on the ability for Enterobacteriaceae strains to utilize glucose, lactose, and maltodextrin for growth was investigated.

### 3.3 Materials and Methods

#### 3.3.1 Bacterial strains

Enterobacteriaceae strains (Table 3.1) were chosen on the basis of published data showing their relationship with the incidence of necrotizing enterocolitis in mice\textsuperscript{24} and infants.\textsuperscript{18-20} Bacterial strains were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Confirmation of bacterial identity was completed prior to the bacterial growth assays. Briefly, bacterial cultures were inoculated from frozen stock (12.5% glycerol, v/v) into brain-heart infusion (BHI; Difco, Franklin Lakes, NJ, USA) broth and incubated
overnight aerobically with shaking at 200 rpm at 37 °C. Genomic DNA was isolated from stationary bacterial cultures. Briefly, a 1.8 mL aliquot was centrifuged at 10,000g in a 2 mL centrifuge tube containing approximately 100 μg of 0.25 μm silicon beads (RPI Corp., Mount Prospect, IL, USA) for 1 min and the bacterial pellet was resuspended in nanopure water. The tubes were boiled for 5 min (additional kill step for pathogens) and were then centrifuged at 7000g for 5 min. The bacterial pellet was resuspended in nanopure water and vortexed at maximum speed for 10 min. The lysed bacterial cells were centrifuged at 12,000g for 3 min, and the supernatant was frozen at -20 °C. An approximately 1400 bp amplicon of DNA was amplified from the crude DNA extract by 16S rRNA specific primers, 8F (5’ – AGAGTTTGATCCTGGCTCAG – 3’) and 1391R (5’ – GACGGGCGGTGWGTRCA – 3’). The PCR reaction mixture contained 3 μL of target DNA, 5 μL of each primer (10 mM), and 25 μL of EconoTaq Plus 2X Master Mix (Lucigen Corp., Middleton, WI, USA). The PCR cycling parameters consisted of an initial step of 5 min at 95 °C, followed by 35 cycles (30 s at 95 °C, 60 s at 54 °C, and 2 min at 72 °C) and an extension step at 72 °C for 7 min. PCR products were purified using the Zymoclean DNA Clean & Concentrator Kit (Zymo Research Corp., Irvine, CA, USA) according to the manufacturer's instructions. Sanger sequencing was done with either primer (8F or 1391R) using an ABI 3730XL capillary sequencer (Life Technologies). The sequences were trimmed for quality and uploaded to the BLAST database (NCBI) to determine identity (>97% similarity). All strains were confirmed as the species indicated in Table 3.1.

3.3.2 Bacterial growth assays

Bacterial growth due to the addition of a carbohydrate was determined using a Bioscreen C (Growth Curves USA, Piscataway, NJ, USA). Bacterial cultures were inoculated from frozen
stock (12.5% glycerol, v/v) into BHI broth and grown overnight aerobically with shaking at 200 rpm and 37 °C. A 1% (v/v) inoculum was passed into 5 mL of ZMB-1 broth, a chemically defined medium allowing for carbohydrate utilization experiments, supplemented with 1% glucose and incubated overnight aerobically with shaking at 200 rpm at 37 °C. To prepare the bacterial inoculum for the Bioscreen C growth assay, bacterial cultures were centrifuged at 4000g for 10 min and washed twice with phosphate-buffered saline (PBS; MP Biomedicals, Santa Ana, CA, USA), pH 7.4. The washed bacterial pellets were resuspended in 5 mL of carbohydrate free ZMB-1 broth. The final bacterial inoculum was prepared by transferring a 1% (v/v) volume of ZMB-1 suspended cells into a fresh aliquot of ZMB-1 broth. The honeycomb plate was prepared with each well containing a total of 300 μL: 225 μL of washed cells in ZMB-1 broth and 25 μL of 10% (w/v) sugar solution (Table 3.2), and 50 μL of mineral oil (to prevent evaporation). Plates were preincubated anaerobically (90% N₂, 5% CO₂, 5% H₂; Coy Laboratory Products, Grass Lake, MI, USA) for 1 h prior to the addition of mineral oil. Plates were incubated anaerobically for 24, 48, 72 or 96 h (varied by experiment). Optical density at 600 nm was measured at 30 min intervals, which were preceded by 30 s of shaking at maximum speed. Each carbohydrate was tested with a minimum of three independent replications.

3.3.3 Enrichment of Enterobacteriaceae consortia

The following protocol was utilized to enrich and isolate Enterobacteriaceae from feces of weaned piglets (n = 10) aged 42 days. Fresh fecal samples (~1 g) were collected from piglets raised at the University of Illinois Swine Research Center. Samples were collected from their source (piglets) and stored on ice until use.
Enrichment of an Enterobacteriaceae consortium was performed as follows. Approximately 200 mg of feces was added to 2 mL (10-fold dilution) of prewarmed sterile buffered peptone water (BPW), pH 7.0. The fecal slurry was vortexed vigorously for complete homogenization. The BPW slurry was incubated overnight aerobically with shaking at 200 rpm and 37 °C. Following incubation, the BPW fecal slurry was vortexed and set aside for 4 min to allow settling of the solid material. A 1 mL aliquot was combined with 9 mL (10-fold dilution) of Enterobacteriaceae Enrichment (EE) Broth (Oxoid, Basingstoke, UK) and incubated for 24 h aerobically with shaking at 200 rpm and 37 °C. A 5 mL aliquot of EE cultures was used for a Bioscreen C growth assay (procedure same as above). Upon completion of the Bioscreen C growth assay, 150 μL of cell suspension was centrifuged at 12,000 g for 3 min. A 100 μL aliquot was collected for HPLC analysis and stored at -20 °C. The remaining fermentate was pipetted off, and the cell pellets were frozen at -20 °C for archiving.

3.3.4 High-performance liquid chromatography (HPLC)

Selected fermentates from the in vitro growth assays were analyzed by HPLC using an Agilent Technologies 1260 Infinity HPLC (Agilent, Santa Clara, CA, USA) equipped with a refractive index detector using a Rezex ROA-Organic Acid H+ (8%) column (Phenomenex Inc., Torrance, CA, USA). The column was eluted with 0.005 N H₂SO₄ at a flow rate of 0.6 mL/min at 50 °C. Baseline samples were diluted 10-fold (20 μL of 10% sugar stock solution with 180 μL of ZMB-1), whereas fermentate samples were diluted 4-fold (50 μL of fermentate with 150 μL of water) prior to analysis.
3.3.5 Co-substrate growth assay

Bacterial growth resulting from the combination of a fermentable carbohydrate and an HMO was determined using a Bioscreen C as previously described. Washed cells were grown in ZMB-1 media supplemented with 1% (w/v) fermentable carbohydrate (Glu, Lac, or Mal) and 1% HMO (2’-FL, 6’-SL, or LNnT). Plates were incubated anaerobically for 48 or 72 h (varied by experiment). Each combination of carbohydrates was tested with three independent replications.

The impact on bacterial growth due to the presence or absence of HMOs was evaluated using a growth curve regression script written in MATLAB (version R2013a, The MathWorks, Natick, MA, USA) with the curve fitting toolbox. This script implements the modified logistic regression model which provides a quantitative assessment of bacterial growth. Additional heuristics were included in the modified logistic regression model to more accurately approximate the bacterial growth curves: (a) the death phase of the bacterial growth curve was not included in the regression, and (b) a threshold to detect cases where no growth occurred was set to an OD = 0.4 (600nm). All regressions were judged by “goodness of fit” metrics, which were provided by the MATLAB curve fitting toolbox. Acceptable regression curves were selected on the basis of an \( R^2 > 0.98 \).

The growth curve was distilled into four calculable parameters: lag time (amount of time for bacteria to enter exponential growth phase), maximum specific growth rate (\( \mu_{\text{max}} \)), doubling time (db time, also known as generation time), and delta OD (\( \Delta \text{OD, 600 nm} \)). The lag time is defined as the x-intercept of the tangent line at the inflection point of the logistic regression curve (LRC) and is reported in hours. The maximum specific growth rate is defined as the slope of the tangent line at the inflection point of the LRC and is reported in hours\(^{-1}\). The doubling
time is mathematically related to the maximum specific growth rate and can be calculated using eq 1 (reported in hours):

\[ \text{dbtime} = \frac{\ln(2)}{\mu_{\text{max}}} \]

The \( \Delta \text{OD} \) is calculated as the difference between the asymptote of the logistic curve (maximum OD) and the minimum OD, and is reported as unit OD 600 nm.

### 3.3.6 Statistical analysis

Statistical differences in bacterial growth parameters due to the presence/absence of HMO were determined using SAS (version 9.2, SAS Institute, Cary, NC, USA). Following satisfaction of the requirements of normality and homogeneity of variance, the bacterial parameters were evaluated using the generalized linear model (GLM). In cases when statistical significance was seen (\( p < 0.05 \)), a post hoc mean separation was done using Dunnett’s test. This test determines the statistical significance of the difference of each treatment (addition of HMO) to the control (fermentable sugar). Data visualized by heat map and dendrogram were conducted in R\(^2^9\) using the gplots package.\(^3^0\) The presented dendrogram (Figure 3.1) shows the relatedness of organisms by their pattern of growth.

### 3.4 Results

#### 3.4.1 In vitro growth of selected carbohydrates

Nine pathogenic and two nonpathogenic Enterobacteriaceae strains (Table 3.1) were evaluated for their ability to grow in vitro using a selection of carbohydrates (Table 3.2). Preliminary experiments revealed that all tested Enterobacteriaceae were able to grow anaerobically in ZMB-1 supplemented with 1% glucose, whereas no growth was observed in the
absence of a carbohydrate (data not shown). The resulting carbohydrate utilization data are displayed as a heat map (Figure 3.1). Interestingly, three distinct groups were identified on the basis of the observed patterns of growth and demonstrated significant overlap with phylogeny. Group 1 is represented by the closely related *E. coli* strains and *Shigella dysenteriae*. Group 2 is represented by the two *Klebsiella* strains. Whereas the two *Cronobacter sakazakii* strains are grouped together, the other three strains, *Citrobacter freundii*, *Cronobacter muytjensii* and *Enterobacter cloacae*, in group 3 are not grouped according to phylogeny.

Bacterial strains that are represented by group 1 are EC1000, EC11775, EC29425, and SD13313. Their inability to utilize Suc and Lac (except EC11775) and weakened ability to grow in the presence of almost all carbohydrates tested are likely driving their clustering. Within the *in vitro* testing environment, these organisms were observed to grow on Glu, Fru, and GlcNAc; however, they did not grow in the presence of the tested prebiotics or HMOs.

The bacterial strains included in group 2 are KP13883 and KO13182. These two *Klebsiella* strains were strong utilizers of all carbohydrates tested except the HMOs. The unique ability for KP13883 and KO13182 to utilize FOS and Inu for growth likely contributed to their clustering. Interestingly, only SA and PDX were differentially fermented between KP13883 and KO13182. Because KO13182 was observed to have a minimal increase in optical density in ZMB-1 supplemented with 1% LNnT (OD$_{av}$ = 0.386), the bacterial growth curve was scrutinized. It was determined that an OD = 0.4 was an appropriate threshold because curves below this OD did not have a “characteristic” logistic curve with a distinct lag, exponential, or stationary phase. Additionally, the increase in OD occurred incrementally throughout the duration of the experiment (up to 96 h) until the maximum OD reading observed. We hypothesize that this small increase in growth was due to the presence of a small fraction of
mono- or disaccharides in the LNnT we used. To test this hypothesis, the fermentate samples (n = 3) from KO13182 grown in ZMB-1 supplemented with 1% LNnT were analyzed by HPLC. The average percent decrease of LNnT detected in the fermentate as compared to the baseline (1% LNnT in ZMB-1) was 11.22 ± 0.29% (SEM) and was not considered significant.

Bacterial strains that are represented by group 3 are CSBAA894, CF8090, CS29544, CM51329 and EC13047. Interestingly, all five bacterial strains were unable to grow on \(\text{L-fuc, fructooligosaccharides, and HMOs. Additionally, CF8090, CSBAA894, and CS29544 were observed to grow weakly on SA, whereas EC13047 and CM51329 were unable to utilize SA for growth. Conversely, these strains readily utilized Lac and Gal. Both CSBAA894 and CS29544 were observed to be GOS utilizers due to their ability to ferment the UGOS-P.}

3.4.2 Enterobacteriaceae consortia growth

Enterobacteriaceae consortia were enriched from the feces of 42 day-old piglets (n = 10) who were weaned prior to fecal collection. These fecal consortia (PFEC) readily utilized Glu, Gal, Lac, GlcNAc, SA, and Mal for growth (data not shown). Interestingly, seven PFEC were capable of growing on UGOS-P with an extended lag time (Figure 3.2A), with three of the seven PFEC (4, 8 and 10) demonstrating multiphasic growth on UGOS-P. None of the PFEC grew when 2’-FL or 6’-SL (OD < 0.3) was supplemented as a sole carbon source. Conversely, three PFEC (1, 3, and 9) grew using 1% LNnT as a sole carbon source, albeit with a significant lag time (> 60 h, Figure 3.2B). Subsequent HPLC analysis of the fermentate of all PFEC and two controls (EC29425 or no bacteria added) indicated that all of the PFEC, including the seven (piglets 2, 4, 5, 6, 7, 8, and 10) that did not show detectable growth (all OD < 0.25, Figure 3.2B), utilized at least some LNnT. More specifically, approximately 39 ± 8.8% of the LNnT
disappeared from the fermentate of the seven PFEC that did not grow (OD < 0.25) on LNnT, and 62.8-97.7% of LNnT disappeared from the three PFEC that grew on LNnT (OD > 0.4). By contrast, the bacteria-free control (10.05 g/L) and EC29425 (9.4 g/L) showed no change in LNnT.

3.4.3 *In vitro* co-substrate growth

Because HMOs are not the sole carbohydrates present in the diet of infants, we investigated the impact of providing various fermentable carbohydrates in combination with the HMOs on the growth of selected Enterobacteriaceae strains. Bacterial strains were grown in ZMB-1 supplemented with 1% of a fermentable carbohydrate (Glu, Lac, or Mal) and compared to 1% fermentable carbohydrate + 1% HMO (2’-FL, 6’-SL, or LNnT). The impact on bacterial growth was evaluated by determination of the bacterial lag time, doubling time, and ΔOD and is presented in Figure B.1-B.3, respectively. To ease comparisons, the statistically significant percent of change between the experimental condition (addition of HMO) and control condition (fermentable carbohydrate) was illustrated using a heat map (Figure 3.3).

Combining individual HMOs with another fermentable carbohydrate affected lag time in only a few instances. The lag time for EC1000 was significantly decreased in the presence of Mal supplemented with 6’-SL and LNnT but not 2’-FL. Conversely, only Lac supplemented with 2’-FL caused a significant decrease in CSBAA894’s lag time. Interestingly, both KP13883 and KO13182 had shorter (p < 0.05) lag times with all three HMOs in the presence of Lac, although it was more pronounced with 6’-SL (p < 0.005). Uniquely, EC11775 had a significantly increased lag time (6 h) when grown in the presence of Mal and 6’-SL.
The bacterial db time was differentially affected depending on the strain/carbohydrate combination. Several strains exhibited an increase in db time in the presence of an HMO. Specifically, the db time was significantly longer when an HMO was combined with Mal (EC13047: 2’-FL and LNnT, EC11775 and KP13883: 6’-SL and LNnT). Additionally, several strains (CS29544, EC13047, and EC11775) were significantly affected by the presence of LNnT in combination with Lac but to a lesser degree. Interestingly, only two strains (EC11775 and EC29425) had a significant decrease in db time. A significant decrease in db time for EC11775 was observed only when Glu was in combination with all three HMOs, whereas EC29425’s db time decreased 69.7% when Mal was combined with 6’-SL. The largest percent change in db time was observed for EC11775 when Mal was combined with 6’-SL and LNnT (increases of 284% and 186%, respectively).

High-throughput experiments use the ΔOD to describe the magnitude of bacterial growth achieved over the course of a carbohydrate utilization experiment. It was observed that the ΔOD was differentially affected on the basis of the strain/carbohydrate combinations; however, the percent change was minimal (range -25 to 10%). In general, the ΔOD was decreased in the presence of an HMO; however, no clear pattern was found. Interestingly, the ΔOD of CM51329 was significantly increased when Lac was in combination with 2’-FL. Conversely, when Glu and Lac were in combination with LNnT the ΔOD of CM51329 was significantly decreased.

3.5 Discussion

Human milk oligosaccharides are believed to contribute to the health and development of the infant GI tract by multiple mechanisms. During the establishment of the intestinal microbiota,^{2,11} HMOs function as prebiotics by selecting for certain Bifidobacterium and
Bacteroides species. Although it has been assumed that HMOs do not support the growth of Enterobacteriaceae, which are thought to promote the inflammatory response that occurs during NEC, the ability of various members of this family to utilize individual HMOs has not been thoroughly examined. In this study, we assessed the ability of several Enterobacteriaceae strains and enriched piglet fecal Enterobacteriaceae consortia to utilize 2′-FL, 6′-SL, and LNnT as a sole carbohydrate for growth. Additionally, we examined the impact of adding glucose, lactose, and maltodextrin on HMO utilization by the various Enterobacteriaceae strains.

Several Enterobacteriaceae tested were able to readily utilize mono- or disaccharides (Glu, Gal, Lac, Fru, and Suc) for growth; however, growth on prebiotic carbohydrates (FOS, Inu, UGOS-P, Mal, and PDX) was lessened or absent (Figure 3.1). Utilization of prebiotics for growth may be limited due to an organisms’ inability to transport larger carbohydrates (> 3 monomers) intracellularly. Therefore, the presence of extracellular glycosidases may assist transportation by cleaving the prebiotics into mono- or disaccharides. The extracellular glycosidases from Bifidobacterium bifidum have been studied extensively. The extracellular lacto-N-biosidase allows for the intracellular transport of lacto-N-biose by removing the terminal galactose from type 1 HMOs.\textsuperscript{13} We hypothesized that KP13883 and KO13182 likely possess several extracellular glycosidases due to their ability to use a wide range of prebiotics (FOS, Inu, UGOS-P, Mal, and PDX) tested regardless of chain length. On the basis of the inability of any of the selected Enterobacteriaceae to detectably grow when 2′-FL, 6′-SL, or LNnT was the sole carbon source, we also hypothesized that these organisms do not possess the appropriate HMO-specific transporter or extracellular enzymes necessary for catabolism. Our data do demonstrate the potential of cross-feeding, whereby commensal GI organisms could liberate monosaccharides from HMOs, thereby providing a substrate for pathogenic Enterobacteriaceae. Ng and
colleagues demonstrated that *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Clostridium difficile* advantageously catabolize liberated muco-glycans in a *Bacteroides thetaiotaomicron* monoassociated mouse model. *Salmonella* Typhimurium and *C. difficile* were observed to have a growth advantage and induction of genes associated with the sialic acid (nan) operon when *B. thetaiotaomicron* was present.

Analysis of the infant fecal microbiome in infants diagnosed with NEC detected a bloom in proteobacteria (>50% relative abundance) in the GI of infants diagnosed with NEC. Interestingly, an analysis of an infants’ GI metabolome pre-NEC and following diagnosis revealed a significant increase in carbohydrate utilization genes mapped to members of the Enterobacteriaceae family. Although none of the selected Enterobacteriaceae were able to grow on the HMOs tested, it would be premature to assume that no Enterobacteriaceae strains are capable of utilizing HMOs for growth. Therefore, Enterobacteriaceae consortia were enriched with piglet feces, a rich source of Enterobacteriaceae, and evaluated for their ability to ferment selected carbohydrates including 2’-FL, 6’-SL, and LNnT. All PFEC tested were able to ferment HMO-associated mono- and disaccharides, whereas there was no detectable growth when 2’-FL or 6’-SL was supplied as a sole carbon source (data not shown). Conversely, all PFEC were observed to reduce LNnT detectably; however, not all PFEC had detectable growth (Figure 3.2B). Those PFEC that had detectable growth showed the largest reduction in LNnT (>63%); meanwhile, the other seven PFEC with no detectable growth converted only <40% of LNnT. Therefore, supplementation of preterm infant formula with LNnT may require further study. Additionally, seven PFEC were observed to utilize UGOS-P for growth (Figure 3.2B). Because galactooligosaccharides supplementation of infant formula did not cause a reduction in the incidence of NEC, we conclude that supplementation poses no additional risk.
Considering that supplementation of HMOs in infant formula would likely be done in the presence of additional carbohydrates, we evaluated the impact of Enterobacteriaceae growth on Glu, Lac, and Mal in combination with HMOs. Although the inclusion of HMOs would likely not affect Glu utilization \textit{in vivo}, Glu was included as a control. On the other hand, colonic lactose fermentation has been observed in premature infants.\textsuperscript{34,35} Maltodextrin was also included because it is often added to infant formula. Because all included Enterobacteriaceae showed no detectable growth when 2'-FL, 6'-SL, or LNnT was provided as the sole carbon source, any change in growth was not attributed to direct fermentation of the tested HMOs. The resulting bacterial growth was analyzed using a logistic regression analysis to obtain the lag time, db time and ΔOD. On the basis of these three parameters, two patterns can be used to determine the impact of growth in the presence of HMOs. A decrease in lag time and db time and a decrease in ΔOD would indicate an environment that favors bacterial growth, whereas an increase in lag time and db time and a decrease in ΔOD would indicate an environment that disfavors growth. Interestingly, no strain/carbohydrate combination tested had a significant fold change in all three parameters that favored growth. Conversely, EC11775 was the only organism to have a significant fold change in all three parameters that did not favor growth. The combination of Mal and 6'-SL led to a significant increase in lag time (44\%) and db time (284\%) and a significant decrease in ΔOD (-23\%). Hunt and colleagues\textsuperscript{36} reported that pooled HMOs in combination with glucose and lactose resulted in an enhanced growth of \textit{Staphylococcus epidermidis} and \textit{Staphylococcus aureus} breastmilk isolates. The two \textit{Staphylococcus} species did not directly catabolize the pooled HMO fraction, but rather metabolized amino acids.\textsuperscript{36} It appears that 6'-SL, an acidic HMO, may disturb EC11775’s ability to grow on complex carbohydrates; however, the impact of 6'-SL on gene expression was not tested.
In conclusion, this study demonstrates that individual Enterobacteriaceae strains do not detectably grow in the presence of 2’-FL, 6’-SL, and LNnT, whereas three PFEC did have detectable growth when LNnT was added as a sole carbon source. Conversely, no PFEC had detectable growth on 2’-FL and 6’-SL. In addition, no Enterobacteriaceae strain had significantly enhanced growth in all three calculated bacterial growth parameters (lag time, db time and ΔOD) when HMOs were combined with a fermentable carbohydrate. On the other hand, Mal in the presence of 6’-SL did not favor the growth of EC11775. Therefore, we conclude that supplementation of preterm infant formula with 2’-FL and 6’-SL should not lead to an increase in Enterobacteriaceae; however, supplementation with LNnT may require further exploration.

3.6 Acknowledgements

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3.7 Funding

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3.8 Tables and Figures

Table 3.1. Selected Enterobacteriaceae strains associated with necrotizing enterocolitis

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¹ Origin as listed on ATCC Web site (http://www.atcc.org/).
² Control strains classified as non-pathogenic Biosafety Level 1.
³ NA, information is not available
⁴ Organism designated as the species ATCC type strain.
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<td>Galactooligosaccharides-purimune</td>
<td>GOS-P</td>
<td>prebiotic</td>
<td>GTC Nutrition</td>
</tr>
<tr>
<td>Ultrapurified galactooligosaccharides-purimune</td>
<td>UGOS-P</td>
<td>prebiotic</td>
<td>GTC Nutrition 37</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>Mal</td>
<td>prebiotic</td>
<td>Ross Products</td>
</tr>
<tr>
<td>Polydextrose</td>
<td>PDX</td>
<td>prebiotic</td>
<td>Danisco</td>
</tr>
</tbody>
</table>

1 Component sugars are mono- or disaccharides present in HMO sugars
2 HMO, human milk oligosaccharides
Figure 3.1. Maximal growth, OD 600 nm, achieved on 1% carbohydrate (glucose, galactose, lactose, N-acetylglucosamine (GlcNAc), N-acetylneuraminic acid (sialic acid), L-fucose, fructose, sucrose, short-chain fructooligosacharides (FOS), inulin, galactooligosaccharides-vivinal (GOS-V), galactooligosaccharides-purimune (GOS-P), ultrapurified galactooligosaccharides-purimune (UGOS-P), maltodextrin, polydextrose (PDX), 2'-fucosyllactose (2'-FL), 6'-sialyllactose (6'-SL) and lacto-N-neotetraose (LNnT)) in ZMB-1 media. No growth was observed in the absence of carbohydrates. Blank is corrected by subtraction of wells containing no cells and no carbohydrate. Dendrogram shows relatedness by pattern of growth. Average of three or more independent experiments.
**Figure 3.2.** Growth curves of enriched piglet fecal Enterobacteriaceae consortia (n = 10), OD 600 nm, achieved at 96 h on 1% carbohydrate in ZMB-1 media. (A) ultrapurified galactooligosaccharides-purimune (UGOS-P); (B) lacto-N-neotetraose (LNnT). Blank is corrected by subtraction of wells containing no cells and no carbohydrate. One independent Enterobacteriaceae consortia enriched per piglet.
Figure 3.3. Statistically significant percent change in growth parameters determined from co-substrate bacterial growth. Bacterial cultures were grown in ZMB-1 with 1% fermentable carbohydrate (glucose, lactose and maltodextrin) and 1% HMO (2’-fucosyllactose (2’-FL), 6’-sialyllactose (6’-SL), lacto-N-neotetraose (LNNt)). Heat map was generated on the basis of the percent change of either an increase or a decrease in the growth parameters of co-substrate wells compared to wells containing only the fermentable carbohydrate (ANOVA with Dunnett’s mean separation). No growth was observed in the absence of carbohydrates. Blank is corrected by subtraction of wells containing no cells and no carbohydrate. Average of three independent experiments. Only organisms that were found to have a statistically significant percent change are shown. An organism’s inability to grow in either lactose or maltodextrin is represented by gray boxes.
3.9 References


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4.1 Abstract

*Cronobacter sakazakii* is an opportunistic nosocomial and foodborne pathogen that causes severe infections with high morbidity and mortality rates in neonates, the elderly and immunocompromised individuals. Little is known about the pathogenesis mechanism of this significant pathogen and if there are any consequences of *C. sakazakii* colonization in healthy individuals. In this study, we characterized the mechanisms of autoaggregation in *C. sakazakii* ATCC 29544 (CS29544). Autoaggregation in CS29544 occurred rapidly, within 30 mins, and proceeded to a maximum of 70%. Frameshift mutations in two flagella proteins (FlhA and FliG) were identified in two nonautoaggregating CS29544 clonal variant isolates. Strategic gene knockouts were generated to determine if structurally intact and functional flagella were required for autoaggregation in CS29544. All structural knockouts (*ΔflhA, ΔfliG, and ΔfliC*) abolished autoaggregation, while the functional knockout (*ΔmotAB*) did not prevent autoaggregation. Complementation with FliC (*ΔfliC/cfliC*) restored autoaggregation. Autoaggregation was also disrupted by the addition of exogenous wild-type CS29544 filaments in a dose-dependent manner. Finally, filament supercoils tethering neighboring wild-type CS29544 cells together were observed by transmission electron microscopy. *In silico* analyses suggests that direct

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1This chapter has been prepared for publication under the title “*Cronobacter sakazakii* ATCC 29544 autoaggregation requires FliC flagellation, not motility” with co-authors Hoeflinger JL and Miller MJ.
interactions of neighboring CS29544 FliC filaments proceed by hydrophobic bonding between the externally exposed hypervariable regions of the CS29544 FliC flagellin protein. Further research is needed to confirm if flagella-mediated autoaggregation plays a prominent role in *C. sakazakii* pathogenesis.
4.2 Introduction

*Cronobacter* spp., reclassified from *Enterobacter* (1), are motile, biofilm-forming, facultative anaerobic Gram-negative bacilli. *Cronobacter sakazakii*, the most prominent species, is an opportunistic pathogen associated with fatal infections in neonates and immunocompromised children and adults (2). Most notably, *C. sakazakii* infections in neonates have been linked epidemiologically to the consumption of powdered infant formula (PIF) (3–5). Furthermore, *C. sakazakii* withstands desiccation in PIF and thrives in reconstituted PIF, especially when PIF is temperature-abused (6–8). In response, medical and health professionals were cautioned regarding the use of PIF; however, *C. sakazakii* infections in neonatal units are not solely due to consumption of contaminated PIF (9). *C. sakazakii* was reported in infants exclusively breastfed (10–12). Another concern is the frequency with which nasogastric tubes are used to deliver enteral nutrition in premature neonates (13). A surveillance study reported that several species of Enterobacteriaceae, including a single *C. sakazakii* isolate, were recovered from used nasogastric enteral feeding tubes (10). These researchers cautioned that microbial biofilms on nasogastric enteral feeding tubes might serve as a continuous inoculum during bolus feedings while the tube is in place. A simple solution may be to switch from indwelling nasogastric tubes to insertion of a nasogastric tube at each feeding; however, the comfort of the neonate and associated economic costs must be considered (14). A multifactorial approach to protecting neonates from microbial infections associated with feedings is needed, including identification of the mechanisms *C. sakazakii* utilizes during biofilm formation and gastrointestinal colonization.

Many bacteria, especially pathogens, have developed elaborate mechanisms to permit attachment to and formation of dense sessile mono- or polymicrobial aggregates on biotic and
abiotic surfaces (15–17). Following this initial attachment, bacterial aggregates may cooperatively form biofilms, whereby increasing their chance of survival. Herein, the formation of monospecies aggregates is referred to as autoaggregation. Autoaggregation is common in the Enterobacteriaceae family, including *Escherichia coli* (18–24), *Salmonella* spp. (25), *Klebsiella pneumoniae* (26), *Edwardsiella tarda* (27), *Citrobacter freundii* (28), *Yersina pestis* (29), and *Proteus mirabilis* (30,31) and often occurs via self-recognizing cell-surface appendages. Autoaggregation is mediated by adhesins (22,23,31–34) and other cell-surface molecules, such as surface-associated proteins (19,27), pili (18), fimbriae (20,21,25,35), flagella (36,37) and lipopolysaccharides (24,38). Several studies observed by microscopy supercoiling between neighboring microorganisms promoted by pili in *Escherichia coli* (18) and flagella in *Pseudomonas marina* (36) and *Pyrococcus furiosus* (37). The gastrointestinal colonization of two *Escherichia coli* pathotypes occurs via different fully characterized mechanisms of autoaggregation. Bundle-forming fimbriae (AAF/I and AAF/II) in enteroaggregative *E. coli* promote autoaggregation and biofilm formation along the intestinal surface (20,35), while enteropathogenic *E. coli* adheres to the intestinal surface via interactions between Intimin and Tir (39) and establishes three-dimensional microcolonies using bundle-forming pilus (18). While autoaggregation was observed in some *C. sakazakii* strains (38,40,41); the extracellular factor mediating autoaggregation in *C. sakazakii* and its biological function remains unknown.

The bacterial flagellum’s role in motility and bacterial chemotaxis is well characterized (42), but motility is not its sole biological function. Bacterial flagella contribute to the virulence of bacterial pathogens, including adhesion, microcolony formation, invasion, and biofilm formation, reviewed by Haiko and Westerlund-Wikström (43). Unlike other Enterobacteriaceae, the contribution of *C. sakazakii*’s flagellum to its virulence has received little attention. The
flagella of C. sakazakii ES5 are required for adhesion to Caco-2 monolayers and biofilm formation to microtiter plates (44). This study describes the role the bacterial flagella plays in the autoaggregation of C. sakazakii ATCC 29544 (CS29544). A collection of gene knockout and complementation strains revealed that structurally intact FliC containing filaments were required for autoaggregation. Additionally, we provide evidence to suggest that direct interactions between neighboring filaments promote autoaggregation of liquid CS29544 cultures.

4.3 Materials and Methods

4.3.1 Bacterial strains and growth conditions

CS29544 was cultured in brain heart infusion (BHI) broth (BD, Franklin Lakes, NJ, USA), pH 7.38 at 37°C overnight aerobically with agitation (250 rpm) unless specified. CS29544 was enumerated and spread plated on BHI agar plates following serial dilution in 1X phosphate buffered saline (PBS, Dulbecco’s Formula), pH 7.4. Escherichia coli was cultured in lysogeny broth (LB, Miller’s formula, Thermo Fisher Scientific, Waltham, MA, USA) at 37°C overnight aerobically with agitation (250 rpm) unless specified. When necessary, ampicillin (Thermo Fisher Scientific) or chloramphenicol (Thermo Fisher Scientific) were added to BHI or LB at a final concentration of 100 μg/mL and 35 μg/mL, respectively. To test for motility, CS29544 were grown on 0.4% agar composed of 3 g/L beef extract, 10 g/L Bacto peptone, and 5 g/L sodium chloride (BPN) supplemented with 1% of 2,3,5-triphenyltetrazolium chloride (redox indicator) or observed microscopically by wet mount.
4.3.2 Autoaggregation assays

Stationary phase CS29544 cultures, grown in 10 mL BHI at 37°C, were held stagnantly at room temperature for 6 h to allow for autoaggregation. The change in optical density (OD) at 600 nm was gently measured at 30 min intervals for 2 h followed by 1 h intervals. Autoaggregation was reported as the maximum percent autoaggregation; typically the endpoint was used, and calculated by equation 1.

\[
\text{Percent of autoaggregation} = \frac{1 - OD_{\text{time point}}}{OD_{\text{initial}}} \times 100
\]  

Several additional autoaggregation assays were conducted with modifications after growth of CS29544 in 10 mL BHI, including stagnant incubation at different temperatures (4°C and 37°C), the addition of 50 mM EDTA or PBS, and before and after blending on “whip” speed for 30 s (BL113SG, Black and Decker, Towson, MD, USA). Furthermore, autoaggregation assays were completed with CS29544 following growth in 10 mL BHI at different pH values (pH = 4, 5, 6, 7.38, and 8) or incubated anaerobically (90% N2, 5% CO2, 5% H2; Coy Laboratory Products, Grass Lake, MI, USA). Finally, autoaggregation assays were run with CS29544 following growth (10 mL) in different media, including Miller and Lennox LB formulations (LB10 and LB5, respectively), tryptic soy broth (TSB), and BPN broth.

Stationary phase CS29544 and nonautoaggregating clonal variant cultures (described below) grown in BHI were mounted and held stagnantly at room temperature for 1 h to allow for autoaggregation. Still images were taken every 10 s for a total of 1 h by a stationary DSLR camera (Rebel T2i, Canon, Melville, NY, USA) with an intervalometer. Images (360 frames) were stitched together to create a video file with 24 frames/s. An additional time lapse video was constructed as previously described with the CS29544 and flagella competition assays (described
below) with still images were taken every 20 s for a total of 6 h. Images (1080 frames) were stitched together to create a video file with 72 frames/s.

4.3.3 Isolation of nonautoaggregating CS29544 variants

Stationary phase CS29544 cultures were allowed to autoaggregate for 2 h. Then, two separate 100 μL (1%, v/v) aliquots, one from the top fraction of the CS29544 culture and one from the bottom fraction (autoaggregating control), were passed into two fresh tubes of 10 mL BHI broth and incubated as described above. Successive passage following autoaggregation continued until autoaggregation was arrested. Two independent nonautoaggregating variants were isolated and characterized.

4.3.4 DNA extraction and whole-genome sequencing

Genomic DNA was isolated from CS29544 and clonal variants using the UltraClean® Microbial DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) according to manufacturer’s instructions. High-quality genomic DNA libraries were prepared and sequenced using the Illumina platform (Illumina, San Diego, CA, USA) by the DNA Services group affiliated with the Roy J. Carver Biotechnology Center at the University of Illinois-Urbana Champaign. Paired-end libraries of CS29544 and clonal variants were prepared with the TruSeq Genomic DNA Sample Prep Kit (length: 200 - 600 bp) and sequenced on a HiSeq2500 with the TruSeq SBS Sequencing Kit v1 producing an average read length of 160 nt. An additional mate-pair library of CS29544 was prepared with the Nextera Mate Pair Library Sample Prep Kit (length: 3 – 8 kbp) and sequenced on a MiSeq V3 with the MiSeq 600-cycle Sequencing Kit v3 producing an average read length of 300 nt. Paired-end reads were imported into CLC Genomics
Workbench v7.5, quality and adapter trimmed using default settings. Mate-pair reads were quality and adaptor trimmed using Cutadapt (45) and an in-house Perl script provided by the Roy J. Carver Biotechnology Center. Processed mate-pair reads were imported into CLC Genomics Workbench, and de novo assembled using default parameters, only contigs larger than 1000 bp were kept. The paired-end reads were mapped to the CS29544 de novo assembly and single nucleotide polymorphisms (SNPs) were identified (> 90% frequency) using the Basic Variant Detection tool with default parameters. Putative SNPs were identified and confirmed by targeted Sanger sequencing using an ABI 3730XL capillary sequencer (Life Technologies, Carlsbad, CA, USA).

4.3.5 Construction of CS29544 gene knockout strains and complementation vector

Targeted gene disruptions (flhA, fliG, motAB, fliC, and flaA) were constructed in the wild-type CS29544 using the lambda Red recombinase system (46,47). All bacterial strains, plasmids, and primers used in this study are listed in Tables 4.1 & 4.2. The FRT-Cm\textsuperscript{r}-FRT cassette in the recombinant mutants was cured by transformation and subsequent removal of the temperature-sensitive flippase (FLP) recombinase helper plasmid. The double gene knockout (fliC and flaA) was constructed as described above in the CS29544 ΔflaA strain. Gene disruptions were confirmed by junction fragment PCR using the appropriate primer sets.

A fliC complementation vector was constructed by GenScript (Piscataway Township, NJ, USA). Briefly, a 1011 bp sequence, containing the fliC coding sequence and native promoter, was obtained from the publically available CS29544 genome (NCBI Reference Sequence: NZ_CP011047.1). The entire DNA fragment was synthesized and cloned into the pET-11a vector with the restriction enzymes, Bg\textsuperscript{II} and BamHI. Upon receipt, the cfliC vector was
electroporated into *E. coli* Top10 and subsequently electroporated into the CS29544 Δ*fliC* and Δ*flaA*fliC strains using LB broth. Putative compliments were grown in BHI or on motility agar plates supplemented with 50 μg/mL ampicillin. Restoration of wild-type function was assessed by autoaggregation assays, motility assays, microscopy and flagella harvest as detailed above and below.

4.3.6 Flagella staining and microscopy

The presence of extracellular flagella of CS29544, gene knockout, and complementation strains were determined by a combination of imaging techniques. Log or stationary phase CS29544, gene knockout, and complementation cultures were stained using a crystal violet-based flagella stain (Hardy Diagnostics, Santa Maria, CA, USA) according to manufacturer’s instructions. Stains were visualized using a light microscope at 1000x total magnification (BA210, Motic, Richmond, British Columbia, CA). Images were captured with a 2-megapixel camera (Motic).

Several overnight colonies of CS29544, gene knockout, and complementation strains were gently lifted from BHI agar plates and suspended in phosphate buffered Karnovsky’s fixative containing 2% glutaraldehyde and 2.5% paraformaldehyde. Transmission electron microscopy (TEM) was completed by the Beckman Institute’s Microscopy Suite at the University of Illinois-Urbana Champaign. Briefly, the samples were stained with 2% uranyl acetate for 1 min and visualized using a CM200 LaB6 transmission electron microscope (FEI Co., Hillsboro, OR, USA). TEM was conducted at 120 kV and images were captured with a 2 k x 2 k digital camera (Tietz, Gauting, Germany). Several locations on the grids were examined, and the pictures are representative of the whole sample.
4.3.7 Flagella harvest and filament protein identification, sequencing, and in silico analysis

The extracellular protein fraction of CS29544, gene knockout, and complementation strains was harvested by differential centrifugation (48). Bacteria were cultured in two baffled flasks containing 500 mL of BHI each and incubated overnight at 37°C with agitation (250 rpm). Stationary phase cultures (1 L total) were centrifuged at 3220 xg for 10 min at 4°C. Bacterial pellets were resuspended in 250 mL total of 0.1 M Tris-HCl, pH 7.8 and blended at room temperature for 30 s on “whip” speed. Blended suspensions were centrifuged at 12000 xg for 10 min at 4°C. The supernatant was further ultracentrifugated at 55000 xg for 1 h at 4°C. Protein pellets were resuspended in a total of 1 mL 0.1 M Tris-HCl, pH 7.8 containing 50% glycerol (v/v) and stored at -20°C. Total protein was quantified with the Bradford Assay (BioRad Laboratories, Hercules, CA, USA) and visualized with SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Typical flagella protein recovery was 0.5 - 0.7 mg/mL from 1 L of cell mass (~10^12 cells).

The putative FliC (28.9 kDa) band from CS29544 flagella preparation was excised from an SDS-PAGE gel and treated in-gel with trypsin (G-Bioscience, St. Louis, MO, USA) by the DNA Services group affiliated with the Roy J. Carver Biotechnology Center at the University of Illinois-Urbana Champaign. Protein was digested at a ratio of 1:20 (trypsin:protein) in 25 mM ammonium bicarbonate at 55°C for 30 min. Following lyophilization, peptides were analyzed by liquid chromatography-mass spectrometry (LC-MS). A total of 1 – 2 μg of digested peptides were loaded into a Dionex Ultimate 3000 RSLCnano connected directly to a Thermo LTQ-Velos-ETD Pro Mass Spectrometer (Thermo Fisher Scientific). Peptide were run on an Acclaim 300 C18 nano-column (Thermo Fisher Scientific) using a gradient of 100% A (water + 0.1%
formic acid) to 60% B (acetonitrile + 0.1% formic acid) at a flow rate of 300 nL/min. Raw data were collected by Xcalibur (Thermo Fisher Scientific) and processed with an in-house Mascot Distiller and Mascot Server (Matrix Science, Boston, MA, USA) and identified with the NCBI database.

The secondary structure of the CS29544 FliC protein was predicted from the amino acid coding sequence (NCBI Reference Sequence: NZ_CP011047.1) using the Iterative Threading Assembly Refinement (I-TASSER) method (49) with default parameters. The I-TASSER method is publicly available at http://zhanglab.ccmb.med.umich.edu/I-TASSER/, accessed 10/15/2015. The theoretical secondary structures of FliC were visualized and modified using UCSF Chimera v.1.10.2 (50), publicly available at https://www.cgl.ucsf.edu/chimera/. The hydrophobicity index of the primary FliC amino acid sequence was determined using the ProtScale tool from the ExPASy Bioinformatics Resource Portal (51), publicly available at http://www.expasy.org/, accessed 06/01/2016. The hydrophobicity index was calculated using the Kyte & Doolittle amino acid scale (52) with a window size of 15 amino acids.

4.3.8 CS29544 and flagella competition assays

A 3 mL aliquot of stationary phase CS29544 culture was mixed with detached flagella pieces at a concentration of 0.1, 1, 5, 10, or 20 μg/mL of total flagella protein and allowed to autoaggregate for 6 h. Controls included, addition of 20 μg/mL of bovine serum albumin (BSA, New England BioLabs, Ipswich, MA, USA) or equal volume of protein storage buffer (no protein).
4.3.9 CS29544 biofilm formation to polyvinyl chloride tubing

Polyvinyl chloride tubing (PCV, 0.318 cm outer diameter, 0.159 cm inner diameter, U.S. Plastic Corporation, Lima, OH, USA) was cut with a sterile blade into 5 cm long pieces (external surface area ~5.15 cm$^2$). The PVC tube pieces were disinfected with submersion in 70% ethanol for 10 min and aseptically dried. Two PVC tube pieces were aseptically transferred to a 15 mL centrifuge tube containing 10 mL of BHI, supplemented with 50 μg/mL ampicillin for $\Delta$fliC/cfliC. Each experimental vessel was inoculated with 1% (v/v) stationary phase CS29544, $\Delta$motAB, $\Delta$fliC, and $\Delta$fliC/cfliC cells and incubated vertically at 37°C aerobically with agitation (250 rpm) for 24 h. After incubation, each PVC tube piece was transferred with sterile forceps and washed three times in 5 mL PBS, pH 7.4. After washing, each PVC tube piece was placed into 30 mL PBS, pH 7.4 containing 3 g of autoclaved 0.1 mm diameter glass beads (Research Products International, Mount Prospect, IL, USA). Biofilms were subsequently disrupted by vortex at maximum speed for 1 min. Bacterial biofilm populations were enumerated by serial dilution ($10^{-1} – 10^{-3}$) in 0.1 % peptone water, spread plated (0.1 mL in triplicate) on BHI agar plates, and incubated at 37°C overnight aerobically.

4.3.10 Statistical Analysis

Autoaggregation assays and biofilm formation assay result from of three independent replicates. The differences between the average maximum percent autoaggregation due to temperature, redox balance, pH, blending, and various media and the differences between biofilm formation due to the presence of FliC were determined with SAS® (Version 9.4, SAS Institute, Cary, NC) using the generalized linear model (GLM). When statistical significance was observed ($p < 0.05$), a post hoc mean separation was run using the Tukey’s Honest
Significance Difference test. All data satisfied the assumptions of normality and homogeneity of variance.

4.4 Results

4.4.1 Characterization of autoaggregation in CS29544

Stationary phase BHI cultures of CS29544 autoaggregated at 25°C within 30 – 60 min (Figure 4.1A). Following 6 h stagnant incubation, the average maximum percent autoaggregation was 70.3 ± 2.2%. Various growth media and physiological conditions were tested to better understand CS29544 autoaggregation (Figure 4.1B-F). Autoaggregation of BHI grown cells at 37°C was significantly higher when stagnantly incubated at 37°C than 4 or 25°C (p = 0.0022, Figure 4.1B) or in the presence of EDTA (p = 0.0092; Figure 4.1C) compared to the PBS-added control. Although the maximum percent autoaggregation was lower for cells grown in a reduced environment, the difference was not significant (p = 0.3003, Figure 4.1D). Autoaggregation following overnight growth in BHI was significantly higher than overnight growth in LB10, LB5, TSB and BPN (p = 0.0203, Figure 4.1E). The difference in autoaggregation was not readily explained by the presence of salt, extracts, protein sources, or inclusion of phosphates but rather the initial pH of the media. Therefore, CS29544 was grown in BHI with varying initial pH values (range pH 4 – 8). CS29544 did not grow at pH 4, but its growth was not affected by BHI at pH 5 – 8 (Figure 4.1F). As predicted, the average maximum percent autoaggregation decreased with decreasing pH (p < 0.0001, Figure 4.1F). The average maximum percent autoaggregation in BHI at an initial pH 6 was 51.9 ± 1.6% which was slightly lower than the average maximum percent autoaggregation observed in the various media tested. During this initial characterization, an abolishment of autoaggregation was only achieved when
grown in BHI at pH 5; however, this observation did not clearly point to a specific mechanism. Since the average maximum percent autoaggregation never reached 100%, we hypothesized that there might be a nonautoaggregating subpopulation of CS29544 mediated by an identifiable genetic variation.

4.4.2 Nonautoaggregating CS29544 are a stable genetically-distinct subpopulation

Following five successive passages selecting against autoaggregating CS29544, we were able to isolate two independent nonautoaggregating clonal variants (CV) of CS29544 (Figure 4.2A & B, Video C.1). De novo assembly of the mate-pair library preparation from wild-type CS29544 was used as the reference genome for comparative genomic analysis with the nonautoaggregating CV’s (2.10 & 3.6) assemblies. A single unique SNP was detected in each of the nonautoaggregating CV. Strain 2.10 contained a putative deletion of two consecutive base pairs (GC), while strain 3.6 contained a putative deletion of a single base pair (C). Further corroboration by Sanger sequencing revealed the two nonautoaggregating CV contained frameshift mutations and NCBI BLAST analysis revealed these mutations were located in the open reading frames of two flagella proteins, FlhA (2.10) and FliG (3.6), referred to as flhA.CV and fliG.CV. Accordingly, we assessed the flagellation of the wild-type CS29544 and the CV with motility assays and microscopy (Figure 4.2C). Both the flhA.CV and fliG.CV were nonmotile and aflagellate by staining and TEM (Figure 4.2C). As a result, we constructed a variety of gene knockouts to determine if structurally intact and functional flagella were required for autoaggregation in CS29544.
4.4.3 Flagella structure, not function, is required for autoaggregation in CS29544

Published C. sakazakii genomes, available from NCBI, have annotated some 40 genes related to their flagella’s structure, function, and regulation. Therefore, gene knockout strains (Table 4.1) were constructed to disrupt the structure and function of the CS29544 flagella, refer to Figure 4.3A-C for a simplified diagram of the bacterial flagella outlining our knockout strategy. Two basal body proteins, FlhA and FliG (same genes as the nonautoaggregating clonal variants) were targeted. Previously, FlhA truncation mutants in Campylobacter jejuni resulted in aflagellate cells lacking flagellar components past the inner membrane (53). FliG, along with FliN and FliM, forms the C ring of the basal body (54). Proton-driven conformational changes in the MotA and MotB stator (motor) proteins (55) directly interacts with the C-terminus of the FliG protein of the C ring to provide rotation to the flagella (56,57). While FliG is integral for the function of the flagella, its necessity for assembly is disputed (56,57). It was hypothesized that disruption of motAB would render the cells nonmotile while retaining the structural components (Figure 4.4B). Additionally, disruption of flhA and fliG would block the early assembly of the flagella and the cells would be aflagellate (Figure 4.4C). Based on the published CS29544 genome, CS29544 has redundant filament proteins; therefore, both FliC and FlaA single and double gene knockouts were constructed. Finally, the function of the flagella was disrupted by targeting the two motor proteins, MotA and MotB.

The ΔflhA, ΔfliG, ΔfliC, and ΔflaAΔfliC strains did not autoaggregate and were aflagellate by staining and TEM and nonmotile (Figure 4.4A-C). Conversely, the ΔmotAB and ΔflaA strains remained autoaggregative and had visible flagella by staining and TEM (Figure 4.4A-C). These two strains differed in their motility: ΔflaA was motile while ΔmotAB was not motile. Therefore, we concluded that motility was not required for autoaggregation in CS29544. Based on the
phenotypes of the structural gene knockouts, we hypothesized that filaments composed of FliC, not FlaA, were required for autoaggregation. Upon mechanical removal of the filaments, only the autoaggregating CS29544, ΔmotAB, and ΔflaA strains possessed a heavily dense 28.9 kDa protein band (Figure 4.5). The band was confirmed as FliC by a total of five peptides having individual ion scores of 44 and a Mascot ion score of 1191. Furthermore, the ΔfliC strain did not contain a 50.1 kDa band, the predicted size for FlaA. The autoaggregation phenotype, motility, and flagellation were restored in the ΔfliC/cfliC and ΔflaAΔfliC/cfliC complementation strains (Figure 4.6A-C). Finally, we confirmed a loss of autoaggregation in CS29544 after mechanical removal of flagella from 79.4 ± 4.4% to 2.2 ± 2.2% with no reduction in cellular viability (data not shown).

4.4.4 Flagella-mediated autoaggregation occurs by flagella-flagella interactions

Visual analysis of the CS29544 TEM image revealed supercoiled flagella linking several bacteria together in a cluster (Figure 4.7A). Therefore, it was hypothesized that autoaggregation in CS29544 proceeds as flagella from one cell becomes entangled with the flagella from neighboring cells. To test this hypothesis, mechanically detached flagella pieces were added to stationary phase CS29544 cultures. Autoaggregation of CS29544 was not affected by the addition of 0.1 or 1.0 μg/mL of detached flagella, 20 μg/mL BSA, or the no-protein control (PSB, Figure 4.7B). Autoaggregation was prevented when 20 μg/mL of detached flagella was added. The addition of detached flagella altered the manner by which the CS29544 autoaggregated. The controls autoaggregated as before by forming small flocs of cells that settled to the bottom of the tube. In the presence of 5 and 10 μg/mL of detached flagella flocs did not form, rather a large mass of cells settled gradually to the bottom of the tube (Video C.2).
The predicted secondary structure of the CS29544 FliC protein was analogous to homologous flagellins with a highly conserved flagellin N- and C-termini linked by a hypervariable region (Figure 4.8A). Analysis of the hydrophobicity scores of the linear amino acid sequence revealed a peak in hydrophobicity followed by a valley in hydrophobicity (Figure 4.8B) located in the hypervariable region of the CS29544 FliC. The bacterial filament is composed of several thousand flagellin proteins with the conserved regions stacked laterally. The hypervariable region is externally exposed and able to interact with its environment. We hypothesize that the hypervariable hydrophobic peaks and valleys of neighboring cells interact laterally to promote autoaggregation in CS29544. Further work will be required to test this hypothesis.

4.4.5 Biofilm formation to polyvinyl chloride is not mediated by flagella

The total cellular population of wild-type CS29544 biofilms on PVC was $1.5 \times 10^4 \pm 3.0 \times 10^3$ CFU/cm$^2$. The total cellular populations of ΔmotAB, ΔfliC, and ΔfliC/cfliC on PVC biofilms were $4.8 \times 10^3 \pm 2.2 \times 10^3$, $2.4 \times 10^4 \pm 7.0 \times 10^3$, and $1.0 \times 10^4 \pm 6.5 \times 10^2$ CFU/cm$^2$, respectively. CS29544 biofilm formation on PVC was not mediated by FliC under the conditions tested (p = 0.0928, Figure 4.9).

4.5 Discussion

While autoaggregation was demonstrated in C. sakazakii ATCCBAA-894 (38,41), the molecular basis for autoaggregation was not described. To understand the genetic determinants of autoaggregation in CS29544, a set of structural and functional flagellar mutants were constructed. These flagellar mutants revealed the requirement of FliC containing filaments in the
autoaggregation of CS29544. Additionally, these results suggest an additional biological function for the CS29544 flagellum rather than its contributions to motility.

Protein-protein interactions, such as flagella-mediated autoaggregation, may require specific environmental conditions. Previously, protein-protein aggregation in bacteria was influenced by altering the growth media (18), sodium chloride concentrations (23), pH (22,31), or the presence of divalent cations (32,36). In this study, autoaggregation assays were conducted to identify nutritional dependencies and provide insights into potential mechanisms. Different growth media containing various nutrient extracts, proteins sources, salts (NaCl and phosphates), and carbohydrates (dextrose) were tested. Additionally, autoaggregation was observed under a variety of temperatures, redox potentials, and pH values. Wild-type CS29544 was flagellated and highly motile under all growth conditions tested, except growth in BHI at pH 5. In hindsight, it is not surprising that autoaggregation in CS29544 had only minimal nutritional or conditional dependencies, even though flagellar expression is a tightly regulated system that quickly responds to changes in the bacterium’s surrounding environment (58). In favorable environmental conditions, such as nutrient-dense media, motility may be arrested following the downregulation of flagellar genes. However, nonmotile bacteria do not immediately shed their structurally intact flagella and these flagella may participate in other biological functions. While abolishment of autoaggregation in CS29544 was observed in structural mutants ($\Delta$flhA, $\Delta$fliG, $\Delta$fliC, and $\Delta$flaA$\Delta$fliC), autoaggregation was not affected in the functional mutant ($\Delta$motAB) which retained the structural components. These results suggest that autoaggregation in CS29544 may serve as an additional biological function for the CS29544 flagellum in environmental conditions that favor the downregulation of motility but not the loss of structure.
The CS29544 genome encodes < 40 genes that are required for the assembly, function and regulation of its flagellum. In this study, autoaggregation was only mediated by the loss of structural proteins, specifically, the lack of the FliC containing filament. Four structural mutants, two direct (ΔfliC and ΔflaAΔfliC) and two indirect (ΔflhA and ΔfliG), resulted in aflagellate nonautoaggregating CS29544 cells. Since the extracellular filament, comprised of several thousand FliC monomers, extends several microns from the cell, it is physically able to promote cell-cell interactions. Upon close examination of wild-type CS29544 cells by TEM, neighboring cells appeared tethered by their filaments. Similar bundles were observed in *Escherichia coli* (18), *Pseudomonas marina* (36) and *Pyrococcus furiosus* (37). Furthermore, flagella-mediated autoaggregation was disrupted in a dose-dependent manner by the addition of exogenous wild-type FliC filaments. Protein-protein interactions may be mediated by several factors, including ionic and hydrophobic bonds. As discussed above, only growth in BHI at pH 5 abolished flagella-mediated autoaggregation in CS29544 and no other nutritional or conditional dependencies were observed. Previously, TibA-mediated autoaggregation in an enterotoxigenic *E. coli* was affected by changes in pH (22). The authors speculated that TibA-mediated autoaggregation might be promoted by pH-mediated ionic bonds between charged amino acid side chains. It is tempting to conclude that flagella-mediated autoaggregation involves ionic bonding due to abolishment at pH 5; however, our observations do not support this conclusion. CS29544 cells grown in BHI at pH 5 were growth-impaired, had no visible flagella by staining, and were nonmotile by wet mount.

The CS29544 genome encodes two different flagellin proteins, *fliC* and *flaA*; however, only FliC monomers were incorporated into the harvested CS29544 filaments under the conditions tested. Consistent with this study, FliC is the sole *C. sakazakii* flagellin protein
reported in the literature (59,60). FliC flagellin proteins and its homologs have highly conserved N- and C-termini connected by a hypervariable region. The conserved domains of several flagellin proteins self-assemble and form the internal channel of the filament during elongation. The conserved domains exposure to the bacterium’s environment is limited and should not contribute to flagella-mediated autoaggregation. Conversely, the hypervariable region is invariably externally exposed and likely interacts with components of the bacterium’s surroundings. As a result, our in silico methods centered on the secondary structure and hydrophobicity of the hypervariable region. The entire CS29544 FliC flagellin protein is composed of 278 amino acids, of which 50 amino acids comprise the hypervariable region. Of note, the hypervariable region in the C. sakazakii FliC flagellin protein is far shorter than FliC flagellin proteins of related Enterobacteriaceae (60). As seen in the predicted secondary structure, the hypervariable region is relaxed and spatially aligned with the conserved regions. Unfortunately, the predicted FliC secondary structure did not reveal any obvious structural contributions to flagella-mediated autoaggregation. Rather alterations in hydrophobicity along the amino acid sequence illustrated the potential of hydrophobic interactions. It is hypothesized that along the length of every filament are thousands of FliC monomers with alternating hydrophobic peaks and valleys (colored in red and yellow, respectively in Figure 4.8). The following hypothesis can be best illustrated by the act of zipping a zipper. When filaments are in close proximity, it is hypothesized that these hydrophobic peaks and valleys interact to allow rapid and reversible supercoiling. Once a sufficient number of CS29544 cells are tethered together by their FliC filaments, autoaggregation by this mechanism proceeds. Further work is needed to test these hypotheses and to more precisely define which amino acids interact during flagella-mediated autoaggregation in CS29544.
C. sakazakii strains form biofilms on a variety of abiotic surfaces, including stainless steel (61–63), silicon (61), latex (61), polyvinyl chloride (40,62,64), and polyurethane (64). The latter two plastics are used for enteral feeding tubes and formation of C. sakazakii biofilms on these plastics is concerning. Hurrell and colleagues (10) isolated C. sakazakii, along with other pathogenic Enterobacteriaceae from used enteral feeding tubes. Biofilm formation on enteral feeding tubes is problematic for several reasons. First, enteral feeding tubes typically reside within an infant at body temperature (37°C) for several days (65). Secondly, infant feeds are nutrient-dense and provide sufficient growth substrate for bacteria. Lastly, with every feeding bacteria might dislodge from the biofilm and continuously inoculate the neonate (10,65). To determine the impact of flagella-mediated autoaggregation on C. sakazakii biofilm formation, the biofilm formation in the wild-type CS29544 was compared to the ΔmotAB, ΔfliC, and ΔfliC/cfliC strains. To model C. sakazakii biofilm formation on neonatal enteral feeding tubes, flagella-mediated biofilm formation was tested in a nutrient-dense environment (BHI broth) at 37°C using PVC tubing. In this study, the total cellular biofilm population on PVC tubing ranged from 3.7-log CFU/cm² in the ΔmotAB strain to 4.4-log CFU/cm² in the ΔfliC strain. There was no significant difference in biofilm formation between the wild-type CS29544 and the ΔmotAB, ΔfliC, and ΔfliC/cfliC strains under the conditions tested. The observed C. sakazakii population density was consistent with the average biofilm population of 4.0-log CFU/cm² on PVC tubing of five strains of C. sakazakii grown in TSB at 12°C reported by Kim and colleagues (62). Additionally, that study reported an approximately 1.5 log increase in the average biofilm population (5.7-log CFU/cm²) on PVC tubing when C. sakazakii strains were grown in TSB at 25°C. Since 27°C is the optimum temperature for C. sakazakii exopolysaccharide production, this result is not surprising. Admittedly, C. sakazakii biofilm formation due to differences in
flagella-mediated autoaggregation phenotype was not robustly tested. To date, a single study has demonstrated the importance of *C. sakazakii* strain ES5’s flagellum in biofilm formation and adhesion to microtiter plates (44). The data presented here demonstrates that additional research into *C. sakazakii* flagella-mediated autoaggregation, biofilm formation, and gastrointestinal colonization is critically needed.

A significant shortcoming of this study is its limited scope. A single strain of *C. sakazakii* was characterized, and generalization to all *C. sakazakii* strains should be avoided. Currently, our collective understanding of *C. sakazakii* pathogenesis is insufficient. Several decades of work were completed to characterize the diverse pathotypes in *E. coli*, and it is tempting to speculate that *C. sakazakii* may have definable pathotypes of which flagella-mediated autoaggregation is important. Future studies should be designed to characterize flagella-mediated autoaggregation contributions to *C. sakazakii* pathogenesis *in vivo* with suitable animal models. Concurrently, autoaggregation, not necessarily flagella-mediated, should be characterized in several clinical, environmental, and laboratory *C. sakazakii* strains. This current study contributes much-needed knowledge to the *C. sakazakii* literature.

4.6 Acknowledgments

We acknowledge the assistance of staff of the DNA and Protein Services Lab at the Roy J. Carver Biotechnology Center and the Beckman Institute Microscopy Suite, particularly Catherine Wallace. We thank Dr. James Slauch for the Lambda Red Recombinase System and Daniel Hoeflinger for compiling the time lapse photos into videos.
### 4.7 Tables and Figures

**Table 4.1.** Bacterial strains and plasmids used in this study.

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<th>Source or reference</th>
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Table 4.2. Primer sequences used to create and confirm gene knockouts.

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¹ 40 bp gene homology was added to the 5’ end of the pKD3 homologous sequences; Forward: TGTAGGCTGAGCTGCTTGC and Reverse: CATATGAAATATCCTCCTTTCG
Figure 4.1. Autoaggregation of stationary phase CS29544 cultures. (A) Time course of autoaggregation in BHI broth over 6 h. Maximum percent autoaggregation (B) in BHI broth during stagnant incubation at different temperatures, (C) in BHI broth in the presence of 50 mM EDTA or PBS, (D) in BHI broth following growth aerobically or anaerobically, (D) in a variety of growth media; LB10 = LB Miller’s formula (10 g/L NaCL), LB5 = LB Lennox’s formula (5 g/L NaCL), TSB = tryptic soy broth and BPN = motility broth, pH of growth media is reported for reference, (E) in BHI at different initial pH values. Maximum optical density (600 nm) is reported for reference. All experiments are mean ± standard error of three independent replicates. Values with no letters in common are significantly different (p < 0.05).
Figure 4.2. Autoaggregation, motility and flagellation of non-autoaggregating CS29544. (A) Time course of autoaggregation in BHI broth over 6 h. CS29544 (included for reference) and clonal variants: flhA.CV and fliG.CV. Experiment is mean ± standard error of three independent replicates. (B) Autoaggregation tube assays in BHI broth after stagnant incubation for 6 h; WT = CS29544. (C) Motility agar plates centrally inoculated and imaged after 24 h, flagellation as detected by crystal violet staining at 1000X total magnification and TEM, bar = 4 μm.
Figure 4.4. Autoaggregation, motility and flagellation of CS29544 and gene knockout strains. (A) Time course of autoaggregation in BHI broth over 6 h. Gene knockouts: ΔflhA, ΔfliG, ΔmotAB, ΔfliC, ΔflaA, ΔfliCΔflaA. Experiment is mean ± standard error of three independent replicates. (B) Autoaggregation tube assays in BHI broth after stagnant incubation for 6 h. (C) Motility agar plates centrally inoculated and imaged after 24 h, flagellation as detected by crystal violet staining at 1000x total magnification and TEM, bar = 4 μm.
Figure 4.5. Harvested filaments from CS29544, gene knockout, and complementation strains. Gene knockouts: ΔflhA, ΔfliG, ΔmotAB, ΔfliC, ΔflaA, ΔfliCΔflaA and FliC complements: ΔfliC/cfliC and ΔfliCΔflaA/cfliC. Each lane was loaded with approximately 2 μg of total protein. FliC expected size 28.9 kDa.
Figure 4.6. Autoaggregation, motility and flagellation of FliC complementation strains. (A) Time course of autoaggregation in BHI broth over 6 h. FliC complements: ΔfliC/cfliC and ΔfliCAflaA/cfliC. Experiment is mean ± standard error of three independent replicates. (B) Autoaggregation tube assays in BHI broth after stagnant incubation for 6 h. (C) Motility agar plates centrally inoculated and imaged after 24 h, flagellation as detected by crystal violet staining at 1000x total magnification and TEM, bar = 4 μm.
Figure 4.7. Autoaggregation competition assays with harvested filaments from CS29544. (A) CS29544 wild-type flagella TEM image, bar = 4 μm. Arrows indicate bundles of flagella. (B) Time course of autoaggregation in BHI broth over 6 h. Stationary phase CS29544 cultures combined with harvested FliC filaments (0.1 – 20 μg/mL), bovine serum albumin (BSA, 20 μg/mL) and PSB (no protein control). Experiment is mean ± standard error of three independent replicates.
Figure 4.8. *In silico* analysis of the CS29544 FliC protein. (A) Theoretical secondary structure of FliC from I-TASSER. Conserved regions: N-terminus in blue, C-terminus in green. Hypervariable region: hydrophobic peak in red, hydrophobic valley in yellow. (B) Hydrophobicity index along the linear amino acid sequence of FliC. Hydrophobic peak highlighted in red, hydrophobic valley highlighted in yellow.
Figure 4.9. Biofilm formation on polyvinyl chloride of CS29544 and select gene knockout and complementation strains. Cellular population are mean ± standard error of three independent replicates. Gene knockouts: ΔmotAB and ΔfliC and FliC complements: ΔfliC /fliC. Values with no letters in common are significantly different (p < 0.05).
4.8 References


CHAPTER FIVE

A PIGLET MODEL FOR STUDYING CANDIDA ALBICANS COLONIZATION OF THE HUMAN ORO-GASTROINTESTINAL TRACT

5.1 Abstract

Pigs from a variety of sources were surveyed for oro-gastrointestinal (oro-GIT) carriage of Candida albicans. Candida albicans-positive animals were readily located, but we also identified C. albicans-free pigs. We hypothesized that pigs could be stably colonized with a C. albicans strain of choice, simply by feeding yeast cells. Piglets were farrowed routinely and remained with the sow for 4 days to acquire a normal microbiota. Piglets were then placed in an artificial rearing environment and fed sow milk replacer. Piglets were inoculated orally with one of three different C. albicans strains. Piglets were weighed daily, and culture swabs were collected to detect C. albicans orally, rectally, and in the piglet’s environment. Stable C. albicans colonization over the course of the study did not affect piglet growth. Necropsy revealed mucosally associated C. albicans throughout the oro-GIT with the highest abundance in the esophagus. Uninoculated control piglets remained C. albicans-negative. These data establish the piglet as a model to study C. albicans colonization of the human oro-GIT. Similarities between oro-GIT colonization in humans and pigs, as well as the ease of working with the piglet model, suggest its adaptability for use among investigators interested in understanding C. albicans-host commensal interactions.

5.2 Introduction

Normally, *Candida albicans* is a commensal fungus, harmlessly colonizing the human oro-gastrointestinal tract (oro-GIT; reviewed in Koh, 2013). Disruption of the host’s immune defenses, compromise of GIT integrity, or alterations in the GIT microbiota, can cause commensal fungal cells to turn into a deadly disease-causing inoculum. Extraintestinal translocation of *C. albicans* is a key part of this process.

While considerable effort has been devoted to understanding *C. albicans* pathogenesis, study of *C. albicans* colonization has come into vogue more recently. Mouse models are used most frequently for these analyses. Koh (2013) presented a thorough and insightful review of murine models for studying GIT colonization. Currently, the most commonly used model involves antibacterial treatment of adult mice to alter the GIT microbiota composition, introduction of *C. albicans* by drinking water or oral gavage, and monitoring fungal persistence in fecal pellets. While this model is easily adapted to many laboratories, the murine GIT is naturally resistant to *C. albicans* colonization, leading to concerns about the artificiality of this approach.

Pigs are frequently used as human biomedical models. Andrutis *et al.* (2000) discussed selection of pig models for the study of bacterial, viral and parasitic enteric pathogens, because of similar pathologies produced in pigs and humans. Andrutis *et al.* (2000) used gnotobiotic piglets as a model for *C. albicans* GIT colonization and dissemination. Perhaps gnotobiotic piglets were sought because of the conventional idea that *C. albicans* is associated with all domestic animals (reviewed in Wrobel *et al.*, 2008), thereby requiring extra effort to derive a *C. albicans*-negative animal. Pigs were delivered by Cesarean section and housed in microbiological isolators to prevent introduction of normal microbiota. Immunosuppressive
treatments caused disseminated disease that was similar to humans. While impressive, this
gnotobiotic piglet model was labor-intensive, making it difficult to adapt for widespread use.

Work described here investigates use of routinely farrowed piglets as a model for C. albicans colonization. Study parameters were selected to promote acquisition of a normal microbiota. Non-intensive housing methods were designed, with the intention of facilitating broad use of the model among investigators who study C. albicans-host interactions associated with oro-GIT colonization.

5.3 Materials and Methods

5.3.1 Detection of yeasts in the pig microbiota

Nine pigs, from 7 different farms, were submitted for necropsy to the Veterinary Diagnostic Laboratory (VDL) at the University of Illinois and sampled to detect yeasts. Swabs from the oral cavity, large colon, and rectum were plated on Sabouraud agar (per liter: 40 g glucose, 10 g peptone, 20 g Bacto agar) with chloramphenicol (20 μg ml⁻¹; Sab + Cml), and incubated for 24 h at 37 ºC. Pure cultures were derived for colonies with yeast-like cellular morphology. Each isolate was streaked to CHROMagar Candida (www.chromagar.com) and tested for germ tube formation by inoculation into RPMI 1640 medium (Gibco catalog no. 11875-085). Yeasts were identified by PCR of genomic DNA using primers ITS4 (5’ TCCTCCGCTTATTGATATGC 3’) and ITS5 (5’ GGAAGTAAAAGTCGTAACAAGG 3’) that amplify the internal transcribed spacer region of the nuclear-encoded rRNA genes (White et al., 1990). DNA sequences were aligned against the non-redundant nucleotide database using BLAST. C. albicans isolates were analyzed using multilocus sequence typing (MLST) as described previously (Bougnoux et al., 2003; Wrobel et al., 2008). Oral and rectal swabs were
also collected from normally healthy pigs on the University of Illinois Veterinary Research Farm (VRF). A total of 34 pigs were sampled from a herd of approximately 200. Fourteen pigs were from the nursery unit (15-40 lbs), 10 were growers (40-100 lbs), and 10 were from the finishing unit (100-280 lbs). Swabs were plated on Sab + Cml and yeasts identified as described above. Animal experiments were conducted with the approval of the University of Illinois Institutional Animal Care and Use Committee.

5.3.2 Farrowing and artificial rearing of piglets

VRF piglets are an outbred population that includes a mix of the Yorkshire, Landrace, and Duroc breeds. Piglets were farrowed normally and housed with the sow in farrowing crates to ensure intake of colostrum. On day 4, piglets were moved to an artificial rearing environment. Piglets were handled minimally: they were not subjected to any of the normal processing of VRF piglets that typically includes ear notching, removal of needle teeth, tail docking, iron dextran injection (100 mg; Aspen Veterinary Resources), Penicillin G injection (300,000 units; Butler Schein), and castration of males. Approximately equal numbers of male and female piglets were used in the experiments.

Piglets were moved to 4 x 4 foot raised pens constructed from painted steel hog panels, lined with solid plastic sheeting to prevent animal-to-animal contact. Pens had plastic slotted flooring over a flush pan. All material was easily disinfected. Piglets had 24-h access to water via a nipple-triggered drinking cup. Rooms were ventilated to outdoor air by a variable-speed, 12-inch exhaust fan. Room temperature was controlled by use of a room heater or an air conditioning unit. Heat lamps were lowered over each pen, when additional heat was required.
Housing met or exceeded the standards and recommendations of the Guide for the Care and Use of Laboratory Animals (8th edition).

Piglets were fed Ralco-Birthright Acidified Baby Pig Milk Replacer (Ralco Nutrition, Inc.; 5 cups per gallon of water; 360 ml kg⁻¹ body weight, up to 2000 ml daily). In the initial study, milk replacer was provided hourly using a timer and pump system. The pig’s bowl, stock bottles, and tubing were washed and bleached daily, and always returned to the same pig’s feeding system to prevent cross-contamination between animals. Subsequent work showed that milk replacer can be provided in a plastic baby pig waterer (see Miller Little Giant item number 291-25). Half of the total daily volume of milk replacer was provided twice daily. Plastic waterers were washed and bleached daily, then returned to the same pen. Research workers wore clean coveralls and boots. Gloves were changed between handling animals and materials in different pens. A bleach bootwash outside of each room was used to control spread of C. albicans in the animal facility.

5.3.3 Inoculation of piglets with C. albicans and assessment of colonization

Piglets and their environment were confirmed C. albicans-free before each study. Oral swabs sampled liberally over all oral surfaces. For rectal swabs, the swab tip was inserted into the pig’s rectum and withdrawn, often covered in fecal material. Environmental swabs were wet in the piglet’s drinking cup, then rubbed thoroughly across all pen surfaces. Swabs were plated on Sab + Cml as described above.

After 1 day of acclimation, pigs were inoculated orally with 10⁹ C. albicans cells. One of three strains was used: SC5314 (the most commonly studied isolate, for which the genome sequence is known; Jones et al., 2004), 216 (an oral isolate from a normally healthy human;
Wrobel et al., 2008), or 3317 (collected from the oral cavity a VDL necropsy pig evaluated for lameness; see above). *C. albicans* strains were taken from -80 °C storage and streaked to YPD plates (per liter: 10 g yeast extract, 20 g peptone, 20 g glucose, 20 g Bacto agar). Plates were incubated at 37 °C for 24 h then stored at 4 °C for ≤ 1 week. An isolated, representative colony was inoculated into 20 ml YPD and incubated for 16 h at 30 °C and 200 rpm shaking. Yeasts were collected by centrifugation, washed twice in Dulbecco’s phosphate buffered saline without calcium or magnesium (DPBS), and counted using a hemocytometer. The yeast inoculum was resuspended in 1 ml DPBS. Piglets readily drank the inoculum or DPBS control from a needleless tuberculin syringe.

Oral, rectal, and environmental swabs were collected 8 h following inoculation, and once every 24 h afterward. Swabs were plated on Sab + Cml. Plates were incubated for 24 h at 37 °C and *C. albicans* colonies counted. A 7-category scale was used to report the plating results: 0 (no growth), 1 (1-25 colonies), 2 (26-99 colonies), 3 (100-499 colonies), 4 (500-1000 colonies), 5 (>1000 colonies, with distinct colony growth still visible), and 6 (lawn of growth). Piglets were weighed daily to monitor growth.

Two independent experiments were conducted. In the first, *C. albicans* isolates were tested using one pig per strain. A DPBS-inoculated pig was maintained in the same room to control for unintentional *C. albicans* transfer. Pigs were monitored for 21 days, including 15 days on milk replacer and 6 days on solid feed (pelleted Diet 111 from the University of Illinois Department of Animal Sciences feed mill; per ton: 625.2 lbs corn, 500 lbs whey, 404.8 lbs soybean meal, 200 lbs lactose, 150 lbs appetein, 60 lbs fat, 22.8 lbs lime, 12.6 lbs dicalcium phosphate, 8 lbs zinc oxide, 7 lbs swine trace minerals, 4 lbs Vitamix ADEK, 2.8 lbs DL-methionine, 2 lbs iodized salt, and 0.8 lbs lysine). In the second experiment, *C. albicans* isolates
were tested using 2 pigs per strain. Pigs were housed individually, with complete experimental replicates in separate rooms, and monitored for 13 (1st group) or 14 days (2nd group) while fed milk replacer. An uninoculated control pig was placed in each room. Because duplicate animals were used for each treatment, results from the second experiment are featured here. However, results were reproducible across both experiments.

Pigs were anesthetized with an intramuscular injection of telazol:ketamine:xylazine (4.4 mg kg\(^{-1}\)), then euthanized by intracardiac overdose of sodium pentobarbital. Oral and rectal swabs were plated on Sab + Cml. Tissue sections were collected from the esophagus, stomach, duodenum, proximal jejunum, mid jejunum, distal jejunum, ileum, cecum, spiral colon, and large colon. \(C.\) \textit{albicans} associated with the GIT mucosa was evaluated by collecting scrapings from the lumen of each tissue using the edge of a sterile glass microscope slide. A portion of the mucosa was weighed and homogenized in DPBS. Serial 10-fold dilutions were plated on Sab + Cml and incubated for 24 h at 37 °C to count colony forming units (CFUs). Another portion of the mucosa was baked overnight in a vacuum oven at 80 °C. The ratio of dry weight to wet weight was calculated, and then multiplied by the mass of the original mucosal sample that was homogenized and plated. This final dry weight equivalent was used as a divisor to calculate CFU g\(^{-1}\) dry weight. Reported results were rounded to the nearest order of magnitude.

In addition to the piglets housed in the artificial rearing environment, a piglet of similar age was selected from the VRF farrowing unit and necropsied as a control. All samples from this animal were negative for \(C.\) \textit{albicans} (data not shown).
5.4 Results

5.4.1 Yeasts isolated from pigs

To determine how readily *C. albicans* could be isolated from pigs, swabs were collected from pigs that were submitted to the VDL, and also from pigs at the VRF (see Materials and methods). VDL submissions provided the means to sample pigs from a variety of local sources. While some pigs were submitted because of infectious disease (enteritis, pneumonia), others were normally healthy (used for teaching purposes), or evaluated for conditions, such as lameness. Eight of the 9 VDL pigs were yeast-positive as detected by our culture technique; *C. albicans* was isolated from 4 of them. There was no association between *C. albicans* isolation and pig health. *Candida tropicalis* (2 pigs), *Trichosporon asahii* (1 pig), and *Candida catenulata* (1 pig) were also isolated from the *C. albicans*-positive pigs. *Issatchenkia orientalis* (2 pigs), *C. catenulata* (1 pig), *C. tropicalis* (2 pigs) and *Candida rugosa* (1 pig) were isolated from the *C. albicans*-negative pigs.

MLST analysis of the *C. albicans* pig isolates showed that all were from clade 8, and clustered closely with strains commonly found in central Illinois wildlife (Wrobel et al., 2008). One isolate had an identical diploid sequence type (DST 785) with a deer isolate (strain DA047). In all 4 *C. albicans* pig strains, genotype designations for the *ACC1, ADP1, MPI1, SYA1*, and *ZWF1b* loci were identical (7, 6, 3, 6, and 37 respectively), matching 8 of the 11 wildlife isolates described by Wrobel et al. (2008).

The ease of identifying *C. albicans*-positive pigs from the VDL contrasted sharply with results from VRF pigs. Of 34 randomly selected VRF pigs, only 9 were yeast-positive (3 nursery pigs, 2 growers, and 4 finishers). Importantly, none of the pigs were positive for *C. albicans*. A single yeast species each was isolated from 8 of the 9 pigs, including *C. catenulata* (3 pigs),
Trichosporon montevideense (2 pigs), and Candida glabrata, C. tropicalis, and Debaryomyces hansenii (1 pig each). One pig had two different yeasts: C. catenulata and D. hansenii.

5.4.2 Stable C. albicans colonization of piglets

Lack of C. albicans isolation from VRF pigs, but frequent isolation among pigs from other farms suggested that pigs may be colonized naturally with C. albicans, as long as an encounter occurs. We hypothesized that the C. albicans-negative status of the VRF pigs was due to biosecurity practices that prevent direct or indirect contact with pigs with wildlife and/or their feces. To test this hypothesis, normally farrowed VRF piglets were removed from the sow at 4 days, housed individually in an artificial rearing environment, and fed commercial sow-milk replacer. Following acclimation, pigs were fed a C. albicans inoculum to test the stability of C. albicans colonization and its effect on pig growth and health. Pigs were assigned randomly to one of four experimental groups: an uninoculated control, or inoculation with one of three C. albicans strains.

The mean total change in piglet body weight over the course of the study (Δkg) was 5.0 kg (Figure 5.1). There was no significant difference between Δkg values for control and C. albicans-inoculated piglets (p = 0.10 using the Generalized Linear Model with Tukey’s mean separation). Colony counts from plating oral, rectal and environmental swabs showed that C. albicans passed through the piglets within 24 h (Figure 5.2). After that time, C. albicans was detected readily from rectal swabs of most animals, but at a colony count considerably lower than observed for the oral swabs. Oral swab counts remained high throughout the experiment, with greater numbers recorded for strains 216 and 3317 (both strains of oral origin) compared to SC5314, a human bloodstream isolate from a disseminated candidiasis patient (Gillum et al.,
1984). *C. albicans* in the environment was low, but detectable over the course of the experiment. In all instances, swabs from the uninoculated control piglets were *C. albicans*-negative.

A veterinary pathologist conducted a necropsy of each animal. Gross examination of the entire oro-GIT revealed no evidence of hyperkeratosis, edema, swelling, hemorrhage, erosion, or ulceration; consistent with the conclusion that *C. albicans* was part of the pig commensal microbiota. Limited microscopic examination of GIT tissue revealed yeast cells in the lumen or associated loosely with the mucosal surface. Examination of CFU g\(^{-1}\) mucosa dry weight showed *C. albicans* colonization up to \(10^7\) CFU g\(^{-1}\) in the esophagus (Figure 5.3). In general, CFU g\(^{-1}\) values decreased in the lower GIT. Overall, necropsy data paralleled swab data. For example, pig 3317 (1) had few CFU from rectal swabs and necropsy specimens from the lower GIT. *C. albicans* was detected throughout the GIT in inoculated animals, but not in the negative controls, where colony counts were zero. *C. albicans* colonies recovered from each animal were tested using MLST to verify that the recovered strains were the same as the inoculation strain (data not shown). These data demonstrated that simply feeding *C. albicans* to a normally farrowed, untreated piglet resulted in stable colonization with the *C. albicans* strain of choice.

### 5.5 Discussion

Our culture-based survey of pigs from various farms established that *C. albicans* can exist as a commensal of normally healthy swine. Identification of a local source of *C. albicans*-negative pigs suggested their utility in creating a model of *C. albicans* commensalism. This model demonstrated stable colonization without the need for antimicrobial treatment that is a hallmark of the most commonly used murine model. The pig model allows *C. albicans*-host interactions to be studied in the presence of the normal GIT microbiota. While the overall cost
of experimental pigs is higher than mice, our work shows that this work can be conducted in a non-intensive environment, without requirements for specialized equipment or skills. These features suggest that the model is easily adaptable for use by other investigators.

The yeasts recovered from pigs in our study are among those documented in domestic swine. Urubschurov et al. (2008) identified 17 cultivatable yeasts from the GIT of piglets reared on both a commercial and research farm in Germany. All of the yeasts we identified were on their list, with the exception of *C. rugosa* and *D. hansenii*, suggesting broad commonalities between GIT yeasts in swine. Importantly, none of the German pigs had *C. albicans* supporting our hypothesis that the *C. albicans* status of a herd is largely the result of management practices.

The literature documents the presence of *C. albicans* in commercial swine herds, even as the cause of disease. Zlotowski et al. (2006) described necropsy findings from two pigs infected with porcine circovirus 2 (PCV2) that causes postweaning multisystemic wasting syndrome. Each pig had oral or esophageal mucocutaneous lesions from which *C. albicans* was isolated. PCV2 infection is associated with compromised immune function (Lee et al., 2010), characterized by decreased counts of circulating B and T lymphocytes and depletion of lymphocytes in lymphoid tissues (Segalés et al., 2001; Darwich et al., 2002). These results suggest further intriguing parallels between the pig model and human disease: lymphocyte depletion and immunosuppression caused by human immunodeficiency virus infection frequently leads to oro-esophageal candidiasis, which is an initial hallmark of AIDS (Powderly et al., 1999).

An unexpected finding of our work is the large number of *C. albicans* CFU associated with the esophageal mucosa. Cole et al. (1996) noted that the esophagus is the most common site for human gastrointestinal candidiasis, and the gastroesophageal junction is the most
frequently colonized site in the human GIT. These results provide even stronger connections between our piglet model and humans, and suggest the potential for *C. albicans* esophageal growth to serve as an inoculum that continually seeds the lower GIT.

Future efforts will further evaluate the utility of our piglet model for studying *C. albicans*-host interactions in the context of colonization and disease. Immunosuppressive treatments could be tested for their effect on extraintestinal *C. albicans* translocation. The normal microbiota could be evaluated for its role in protection against disseminated disease. The presented model may also prove useful for testing potential treatments or nutritional interventions that could reduce *C. albicans* colonization.

5.6 Acknowledgements

We thank Dennis Birkey and Ted Shearer of the Veterinary Research Farm for their support of the study. We thank Dennis French for assistance with inoculating pigs with *C. albicans*. We thank Dimitri Kashtanov, Max Van Tassell, and Dexter Chen for their assistance with processing tissue samples. The University of Illinois Campus Research Board provided funding for this work.
5.7 Figures

**Figure 5.1.** Pig growth (increase in kg body weight) over the study timeline. Purple circles = strain SC514; red squares = strain 216; blue diamonds = uninoculated control; green triangles = strain 3317.
Figure 5.2. Heatmap depiction of *C. albicans* colonies grown from swabs of each pig’s oral cavity, rectum, and environment over the study timeline. A scale of 0 to 6 (defined in the text; colored scale bar shown) described the number of CFU grown from each swab on an agar plate. Darker squares indicate higher CFU counts, while beige squares indicate a negative result. *C. albicans* strain and individual pig numbers are shown at the right of the figure. Control pigs were not inoculated with *C. albicans* and remained negative throughout the experiment. Because group (1) pigs were necropsied one day later than group (2) pigs, an additional observation was available (day 14). White squares were used as placeholders for the group (2) pigs.
Figure 5.3. Heatmap depiction of CFU g⁻¹ mucosal dry weight from each pig. Pig numbers are included at the bottom of the figure. The location from which mucosa was collected is shown at the right. The scale bar indicates CFU g⁻¹ mucosal dry weight on a logarithmic scale. Darker colors indicate higher values.
5.8 References


CHAPTER SIX

CONCLUSIONS AND FUTURE DIRECTIONS

Consumption of prebiotics can beneficially modulate the gut microbiota. For some neonates, this modulation may be critical to their health. In Chapter 2, I demonstrated that galactooligosaccharides and polydextrose supplementation in neonatal piglet formula could significantly increase the lactobacilli population in the ascending colon of neonatal piglets. Further analysis revealed that this increase was not a result of direct fermentation by the lactobacilli isolates. Unfortunately, prebiotic utilization by specific population in vivo is not straightforward. Many individual members of a community may respond to prebiotic supplementation and indirectly lead to the increase or decrease of specific members. I hypothesize that commensal microbes specializing in glycan catabolism, such as Bacteroides, excrete enzymes extracellularly. These enzymes cleave glycans indiscriminately making smaller molecular substrates available to the community. It is tempting to speculate that the observed increase in lactobacilli occurred by this hypothesis.

Similarly, pathogenic microorganisms can benefit from liberated glycans in vivo. In Chapter 3, I observed a significant amount of growth of NEC-associated pathogenic Enterobacteriaceae in the presence of a variety of prebiotics and mono- and disaccharides that compose human milk oligosaccharides. Conversely, no Enterobacteriaceae tested was able to utilize 2’-fucosyllactose, 6’-sialyllactose, and Lacto-N-neotetraose. To more appropriately analyze prebiotic and human milk oligosaccharide utilization, I isolated an Enterobacteriaceae consortium from piglet feces. This community was capable of fermenting several prebiotics and the human milk oligosaccharide, Lacto-N-neotetraose. Further characterization of the Enterobacteriaceae inoculum before and after fermentation could determine the microbes
associated with the catabolism of the added glycans. This knowledge could be used to understand which pathogenic Enterobacteriaceae in the gastrointestinal tract may proliferate in the presence of prebiotics or human milk oligosaccharides.

The research presented in Chapters 2 & 3 highlights the challenges researchers experience when feeding fermentable substrates to microbial communities. Future feeding trials should focus on distinguishing which microbes are directly or indirectly responding to prebiotic supplementation. These interactions can be explored through analysis of the metagenome. With metagenomics, it is possible to identify which gene pathways are up- or downregulated in response to prebiotics supplementation. A time course evaluation of an ex vivo fecal community could begin to uncover the intricate time-dependent shifts in the microbial population. Connecting this information with alterations in gene content can begin to answer the questions of which microbes provide the enzymatic capacity to the system. These studies can assist identification of the indirect factors that lead to the observed increase in lactobacilli following galactooligosaccharides and polydextrose supplementation.

In Chapter 4, I demonstrated that autoaggregation in Cronobacter sakazakii ATCC 29544 is mediated by direct interactions between the flagella of neighboring bacteria. It is not known if autoaggregation in C. sakazakii ATCC 29544 contributes to its ability to form biofilms or colonize the gastrointestinal tract. Based on the breadth of literature, determination of its impact on biofilm formation would be quite straightforward. I would suggest testing biofilm formation on stainless steel and a variety of plastics, including polyvinyl chloride and polyurethane enteral feeding tubes. This knowledge can assist the development of novel methods to disrupt or prevent autoaggregation promoted biofilm formation. Furthermore, animal models can be utilized to analyze if autoaggregation in C. sakazakii ATCC 29544 is required for colonization of

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the gastrointestinal tract. I would propose that neonatal piglets would be an appropriate model to answer these questions. I can use my experience from Chapter 5 to design and develop a colonization or infection *C. sakazakii* piglet model. With this model, I can assess whether autoaggregating *C. sakazakii* ATCC 29544 have a competitive advantage over nonautoaggregating variants. For a broader impact, these studies can provide needed information into the pathogenesis of *C. sakazakii*. Additionally, a surveillance study of autoaggregation in several strains of *C. sakazakii* can identify if this colonization strategy is utilized by all strains of *C. sakazakii* or if it’s unique to *C. sakazakii* ATCC 29544 and closely related strains.
APPENDIX A

CHAPTER TWO SUPPLEMENTAL TABLES AND FIGURES

Table A.1. Carbohydrate utilization of the lactobacilli isolated from piglets fed formula (FORM), formula supplemented with GOS and PDX (F+GP).\(^1\)

<table>
<thead>
<tr>
<th></th>
<th>FORM</th>
<th></th>
<th>F+GP</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>GOS</td>
<td>PDX</td>
<td>Glucose</td>
</tr>
<tr>
<td>Ileum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Utilization(^2)</td>
<td>2.3</td>
<td>4.7</td>
<td>51.2</td>
<td>7.3</td>
</tr>
<tr>
<td>Moderate Utilization</td>
<td>4.7</td>
<td>14.0</td>
<td>48.8</td>
<td>2.4</td>
</tr>
<tr>
<td>High Utilization</td>
<td>93.0</td>
<td>81.4</td>
<td>0.0</td>
<td>90.2</td>
</tr>
<tr>
<td>Relative Utilization to Glucose(^3)</td>
<td>100</td>
<td>87.5</td>
<td>0.0</td>
<td>100</td>
</tr>
<tr>
<td>Ascending Colon</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Utilization</td>
<td>10.7</td>
<td>10.7</td>
<td>89.3</td>
<td>10.9</td>
</tr>
<tr>
<td>Moderate Utilization</td>
<td>0.0</td>
<td>12.5</td>
<td>10.7</td>
<td>0.0</td>
</tr>
<tr>
<td>High Utilization</td>
<td>89.3</td>
<td>76.8</td>
<td>0.0</td>
<td>89.1</td>
</tr>
<tr>
<td>Relative Utilization to Glucose</td>
<td>100</td>
<td>86</td>
<td>0.0</td>
<td>100</td>
</tr>
</tbody>
</table>

\(^1\) Data presented as percent utilizers.
\(^2\) Utilization defined as low: OD<0.4, moderate: 0.4>OD<0.7 and high: OD>0.7.
\(^3\) Percent of isolates capable of utilization of glucose \textit{in vitro} under the chosen conditions.
Table A.2. Genera of ascending colon contents of 21d old piglets fed formula (FORM), formula supplemented with GOS and PDX (F+GP).\(^1\)

<table>
<thead>
<tr>
<th>Genera</th>
<th>FORM Mean</th>
<th>SEM</th>
<th>F+GP Mean</th>
<th>SEM</th>
<th>ANOVA p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Catabacter</strong></td>
<td>0.07</td>
<td>0.02</td>
<td>0.01</td>
<td>0.01</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td><strong>Hydrogenanaerobacterium</strong></td>
<td>0.97</td>
<td>0.23</td>
<td>0.25</td>
<td>0.11</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Lactobacillus</strong></td>
<td>0.06</td>
<td>0.02</td>
<td>0.71</td>
<td>0.44</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>Oscillospira</strong></td>
<td>6.45</td>
<td>2.18</td>
<td>1.59</td>
<td>0.57</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>Parabacteroides</strong></td>
<td>8.67</td>
<td>2.22</td>
<td>18.33</td>
<td>3.98</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>Ruminococcus</strong></td>
<td>9.54</td>
<td>2.37</td>
<td>4.13</td>
<td>1.06</td>
<td>0.03</td>
</tr>
</tbody>
</table>

\(^1\) Genera included in analysis had a False Discovery Rate (FDR) < 0.1, data expressed as percent relative abundance.
Table A.3. Lactobacilli detected in ileum and ascending colon of 21d old piglets fed formula (FORM), formula supplemented with GOS and PDX (F+GP) or sow-reared (SOW) as determined by 16S amplicon sequencing\(^1,2\)

<table>
<thead>
<tr>
<th></th>
<th>Ileum</th>
<th>Ascending Colon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FORM</td>
<td>F+GP</td>
</tr>
<tr>
<td>\textit{L. agilis}</td>
<td>0.7</td>
<td>0.5</td>
</tr>
<tr>
<td>\textit{L. coleohominis}(^3)</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>\textit{L. curvatus}(^3)</td>
<td>0.4</td>
<td>0.8</td>
</tr>
<tr>
<td>\textit{L. equicursoris}(^3)</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>\textit{L. gasseri}(^3)</td>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td>\textit{L. mucosae}</td>
<td>43.6</td>
<td>33.4</td>
</tr>
<tr>
<td>\textit{L. plantarum}</td>
<td>15.4</td>
<td>0.9</td>
</tr>
<tr>
<td>\textit{L. reuteri}</td>
<td>1.6</td>
<td>7.6</td>
</tr>
<tr>
<td>\textit{L. ruminis}(^3)</td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>\textit{L. taiwanensis}(^3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{Lactobacillus} sp.</td>
<td>4.4</td>
<td>2.1</td>
</tr>
</tbody>
</table>

\(^1\) Data presented as percent relative abundance.
\(^2\) Non-detected lactobacilli represented by blank cells.
\(^3\) Aggregated as Minor \textit{Lactobacillus} sp in Figure 2.4.
Figure A.1. Ileal microbial communities of 21-d-old piglets fed either a bovine milk-based formula (FORM), formula supplemented with GOS and PDX (F+GP) or were sow-reared (SOW). Heat map illustrates the relative abundances of top 20 genera detected in the ileal contents. Hierarchical clustering of unweighted Unifrac distances is represented by the dendrogram.
Figure A.2. Ascending colon microbial communities of 21-d-old piglets fed either a bovine milk-based formula (FORM), formula supplemented with GOS and PDX (F+GP) or were sow-reared (SOW). Heat map illustrates the relative abundances of top 35 genera detected in ascending colon contents. Hierarchical clustering of unweighted Unifrac distances is represented by the dendrogram.
Figure B.1. Lag time, hr, achieved on 1% fermentable substrate (glucose, lactose or maltodextrin) or 1% fermentable substrate supplemented with 1% HMO (2’-fucosyllactose (2’-FL), 6’-sialyllactose (6’-SL), lacto-N-neotetraose (LNnT)) in ZMB-1 media. Blank is corrected by subtraction of wells containing no cells and no carbohydrate. Statistical significance determined by ANOVA using Dunnett’s test; *p<0.1, **p<0.05, ***p<0.005. Average of three or more independent experiments.
Figure B.2. Doubling time, hr, achieved on 1% fermentable substrate (glucose, lactose or maltodextrin) or 1% fermentable substrate supplemented with 1% HMO (2’-fucosyllactose (2’-FL), 6’-sialyllactose (6’-SL), lacto-\(\beta\)-neotetraose (L\(\beta\)NnT)) in ZMB-1 media. Blank is corrected by subtraction of wells containing no cells and no carbohydrate. Statistical significance determined by ANOVA using Dunnett’s test; *p<0.1, **p<0.05, ***p<0.005. Average of three or more independent experiments.
Figure B.3. Max deltaOD, 600nm, achieved on 1% fermentable substrate (glucose, lactose or maltodextrin) or 1% fermentable substrate supplemented with 1% HMO (2’-fucosyllactose (2’-FL), 6’-sialyllactose (6’-SL), lacto-N-neotetraose (LNnT)) in ZMB-1 media. Blank is corrected by subtraction of wells containing no cells and no carbohydrate. Statistical significance determined by ANOVA using Dunnett’s test; *p<0.1, **p<0.05, ***p<0.005. Average of three or more independent experiments.
APPENDIX C

CHAPTER FOUR SUPPLEMENTAL VIDEOS

**Video C.1.** Autoaggregation of stationary phase *Cronobacter sakazakii* and nonautoaggregating clonal variant cultures captured by time lapse video. Autoaggregation tube assays in BHI broth, vortexed and held statically at room temperature for 1 h. Still images were taken every 10 s by a stationary DSLR camera with an intervalometer. Images (360 frames) were stitched together to create a video file with 24 frames/s.

**Video C.2.** Autoaggregation of stationary phase *Cronobacter sakazakii* cultures in the presence of harvested wild-type flagella captured by time lapse video. Autoaggregation tube assays in BHI broth containing FliC filaments (5, 10 & 20 μg/mL), bovine serum albumin (BSA, 20μg/mL), and protein storage buffer (PSB, no protein control), vortexed and held statically at room temperature for 6 h. Still images were taken every 20 s by a stationary DSLR camera with an intervalometer. Images (1080 frames) were stitched together to create a video file with 72 frames/s.
Figure D.1. This research article was the result of collaboration that explored which phosphotransferase system transporters were involved in the utilization of lactose in Lactobacillus gasseri. Three putative lactose transporters (PTS 6, 8 & 9) were identified through transcript expression analysis and removal of all three transporters resulted in a L. gasseri strain unable to utilize lactose. This project was initially submitted with characterization of only PTS 6 & 8 and was returned for additional experiments. My primary contributions were to assist construction of the PTS 9 knockout in L. gasseri (wild-type, 6, 8, 6/8 double gene replacement), transcript analysis confirming upregulation of PTS 9 in PTS 6 & 8 deficient strains, conduct bacterial growth experiments with select carbohydrates, including lactose, determination of bacterial growth parameters with an in-house MATLAB® script, and revision of the manuscript for re-submission.

Figure D.2. This research article was the result of a collaboration that evaluated the changes in the fecal microbiota composition of overweight and obese men following consumption of soymilk for three months. Soymilk consumption was associated with a beneficial shift (decrease) in Firmicutes to Bacteroidetes ratio. Additionally, *Lactobacillus* abundance decreased with consumption of soymilk; whereas, *Bacteroides-Prevotella* abundance increased with consumption of all milks. My primary contributions involved developing procedures for collection and processing of fecal samples, extraction of DNA from feces for microbial analysis, quantification of bacterial groups by qPCR, data analysis and figure preparation, and composition of the manuscript.

Figure D.3. This research article was the result of a collaboration that investigated the potential hydrolytic capability of rat cecal microbes *ex vivo*. Detectable isothiocyanates were observed following consumption of broccoli for one or two weeks as compared to no broccoli controls. Additionally, isothiocyanate formation was not detected in glucosinolate-free broccoli demonstrating that isothiocyanates formation was not promoted by other broccoli components, i.e. fiber. My contributions included designing and conducting the *ex vivo* experiments, analysis of data and assistance with the preparation of the manuscript.

Figure D.4. This research article was the result of a collaboration that developed and implemented an open-source regression script in the Python language. Experiments utilizing a previous MATLAB® script were included in Chapter 3 and the cited poster listed below. My main contributions were assistance in the development of the scripts, providing test bacterial growth data sets, data analysis of script output, preparation of the poster and manuscript.
