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THE ROLE OF PROBIOTICS, PREBIOTICS, AND HUMAN MILK OLIGOSACCHARIDES
IN INFANT FORMULAE

BY

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DISSERTATION

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ABSTRACT

It is recommended that infants are breast fed exclusively for the first six months of life; however, less than 15% of infants in the United States are meeting this recommendation and only 44% of infants receive human milk to any extent at six months of age (Gartner et al., 2005; Li et al., 2005; Eidelman et al., 2012). Nearly 40% of infants receive formula as their sole source of nutrition by one month of age (Li et al., 2005). Probiotics and/or prebiotics are increasingly supplemented in infant formulae and are generally regarded as safe in healthy infants without concern for growth or adverse effects (Holscher et al., 2012a; Holscher et al., 2012b). Additionally, human milk oligosaccharides have been shown to enhance intestinal maturation *in vitro*. Thus, we aimed to assess 1) the effects of the probiotic *Bifidobacterium animalis* subspecies *lactis*, 2) the prebiotics galacto- and fructo-oligosaccharides, and 3) the individual and combined effects of specific human milk oligosaccharides as means to narrow the compositional gap between human milk and infant formula. The central hypothesis of this research was that specific ingredients would enhance gastrointestinal health and development.

Addition of probiotics to infant formula may positively affect immune function in non-exclusively breastfed infants. Probiotics are live microorganisms that, when administered orally in adequate amounts, survive the digestion process and confer a benefit to the host (FAO/WHO, 2001). Our first study investigated the effects of an infant formula containing the probiotic *Bifidobacterium animalis* subspecies *lactis* (*B. lactis*) on intestinal immunity and inflammation (Holscher et al., 2012a). Six-week-old healthy, full-term infants (n=172) were enrolled in a prospective, randomized, double-blind, controlled clinical trial with 2 groups studied in parallel to a breastfed comparison group. Formula-fed (FF) infants were randomized to partially

hydrolyzed whey formula (CON), or the same formula containing 10^6 CFU *B.lactis*/g (PRO) for 6 weeks. Fecal secretory IgA (sIgA), calprotectin, lactate, and stool pH were assessed at baseline, 2, and 6 weeks. Anti-poliovirus-specific IgA and anti-rotavirus-specific IgA in stool were assessed at 2 and 6 weeks. Among vaginally-delivered FF infants (n=39), PRO consumption increased ($p<0.05$) fecal sIgA, compared to CON. Anti-poliovirus-specific IgA concentration increased ($p<0.05$) in all infants consuming PRO; whereas anti-rotavirus-specific IgA tended to increase ($p=0.056$) with PRO consumption in cesarean-delivered infants (n=14). Anthropometrics and tolerance did not differ significantly between FF infants. In conclusion, infants consuming formula with *B. lactis* produced feces with detectable presence of *B. lactis* and augmented sIgA concentration. Furthermore, cesarean-delivered infants consuming *B. lactis* had heightened immune response, as evidenced by increased anti-rotavirus- and anti-poliovirus-specific IgA following immunization. These results demonstrate that negative immune-related effects of not breast feeding and cesarean delivery can be mitigated by including *B. lactis* in infant formula and thereby provide infants a safe, dietary, immune-modulating bacterial introduction.

As infant formula containing prebiotics may beneficially impact gastrointestinal tolerance and composition of the commensal microbiota, we assessed gastrointestinal tolerance and fecal microbiota, pH, and short-chain fatty acid (SCFA) concentrations of infants consuming formula with or without prebiotics in a second trial utilizing the a similar model as that discussed above (Holscher et al., 2012b). Prebiotics are selectively fermented ingredients that result in specific changes, in the composition and/or activity of the GI microbiota, thus conferring benefit(s) to the host (Gibson et al., 2010). FF were randomized to consume a partially hydrolyzed whey formula with (PRE) or without (CON) 4g/L of galacto-oligosaccharides and fructo-oligosaccharides (9:1)

for 6 weeks. Fecal bacteria, pH, and SCFA were assessed at baseline, 3, and 6 weeks. Caregivers of subjects recorded stool characteristics and behavior for 2 days before the 3- and 6-week visits. Feces from infants fed PRE had a higher absolute number ($p=0.0083$) and proportion ($p=0.0219$) of bifidobacteria than feces of CON-fed infants and did not differ from feces of BF. BF had a higher proportion of bifidobacteria than CON ($p=0.0219$) and a lower number of *Clostridium difficile* than FF ($p=0.0087$). Feces from FF infants had higher concentrations of acetate ($p<0.001$), butyrate ($p<0.001$), propionate ($p<0.001$) and total SCFAs ($p=0.0230$) than BF; however, fecal pH was lower ($p=0.0161$) in PRE and BF than CON. Prebiotic supplementation did not alter stool patterns, tolerance, or growth. To conclude, infant formula containing the studied oligosaccharides is well-tolerated, increased abundance and proportion of bifidobacteria, and reduced fecal pH in healthy infants.

Human milk oligosaccharides (HMOs) are abundant in human milk (≥ 15 g/L) and associated with enhanced intestinal maturation *in vitro* (Kuntz et al., 2008; Kuntz et al., 2009). Therefore, we aimed to: 1) determine the time course of impact of a model HMO, and; 2) assess the induction of ‘epithelial differentiation’ by three individual HMOs using an *in vitro* epithelial model of the crypt-villus axis. In study 1, pre-confluent HT-29, pre-confluent Caco-2Bbe, confluent Caco-2Bbe, and post-confluent Caco-2Bbe cells were cultured individually and randomized to 1 of 5 treatments: lacto-N-neotetraose (LNnT) at 0, 100, 200 or 400 mg/L or energy control (0 mg/L plus lactose + N-acetylglucosamine) for 24, 48 or 72 hours of incubation to assess effects on proliferation and differentiation. In study 2, pre-confluent HT-29, pre-confluent Caco-2Bbe, and post-confluent Caco-2Bbe cells were randomized to treatments with one of three HMOs at the following levels for 72 hours of incubation: a) LNnT at 0, 20, 200 or 2000 mg/L or energy control (0 mg/L plus lactose + N-acetylglucosamine); b) 2'-fucosyllactose

(2'FL) at 0, 20, 200 or 2000 mg/L or energy control (0 mg/L plus lactose + fucose); c) 6'sialyllactose (6'SL) at 0, 40, 400, 4000 mg/L or energy control (0 mg/L plus lactose + sialic acid). Dependent variables in study 2 included proliferation, differentiation, cell cycle analysis, apoptosis, disaccharidase activity, and absorption and barrier function via modified Ussing chambers. In study 1, compared to 0 mg/L control, LNnT decreased cell proliferation in pre-confluent HT-29 cells at 48 ($p<0.05$) and 72 hours ($p<0.01$) and pre-confluent CaCo-2Bbe at 72 hours ($p < 0.01$). Alkaline phosphatase (AP) activity increased over time and was highest in post-confluent CaCo-2Bbe cells ($p<0.001$); however, was not affected by treatment at any time point. In study 2, HT-29 cell proliferation was reduced ($p<0.05$) with 200 and 2000 mg/L LNnT, 2000 mg/L 2'FL, and 400 and 4000 mg/L 6'SL. CaCo-2Bbe cell proliferation was reduced ($p<0.05$) with 2000 mg/L LNnT, 2000 mg/L 2'FL and 4000 mg/L 6'SL. Reduced proliferation was associated with cell cycle arrest in the G2/M phase in pre-confluent HT-29 cells treated with 200 ($p=0.027$) and 2000 mg/L ($p=0.0033$) of LNnT or 2000 mg/L 2'FL ($p=0.021$) and pre-confluent Caco-2Bbe cells treated with 4000 mg/L 6'SL. Interestingly, in pre-confluent HT-29 cells, treatment with 4000 mg/L 6'SL resulted in S phase arrest ($p<0.001$), whereas there was a trend ($p=0.058$) for cell cycle arrest in the G2/M phase with 400 mg/L 6'SL. Differentiation was increased 31% ($p=0.030$) in HT-29 cells and sucrase activity increased 54% ($p=0.036$) in post-confluent CaCo-2Bbe cells with 2000 mg/L 2'FL. Transepithelial resistance increased 21% ($p=0.002$) in post-confluent Caco-2Bbe cells with 200 mg/L LNnT. In summary, inhibition of proliferation was associated with enhanced epithelial differentiation. Well-differentiated cells had greater digestive and barrier function when treated with 2'FL and LNnT, respectively. Further study is needed to assess whether synergist effects occur following concomitant administration of HMOs.

Thus, we evaluated the effects of concomitant administration of 3'-sialyllactose (3'SL), 2'FL, and 6'SL utilizing the same model of the crypt-villus axis discussed above. Cells were randomized to 1 of 18 treatments for 72 hours of incubation: individual HMO treatments at low and high doses (3'SL at 0.2 and 1.0 g/L, 6'SL at 0.4 and 1.0 g/L, 2'FL at 0.2 and 2.0 g/L), concomitant treatments of the previous HMOs at both low and high doses, and controls (0 g/L, media, 4 g/L total HMO, and lipopolysaccharide (LPS)). Pre-confluent HT-29 cells had reduced proliferation ($p < 0.05$) with individual high dose treatments of 6'SL and 2'FL, concomitant administration of the high doses of the HMOs ($p < 0.05$), and total HMO ($p < 0.001$). Pre-confluent CaCo-2Bbe cells were less sensitive to reduction in proliferation where only high dose concomitant treatments containing 2'FL ($p < 0.01$) significantly reduced proliferation in addition to the total HMO ($p < 0.001$). Interestingly, 2'FL did not enhance differentiation in HT-29 cells as we had previously shown; however, it did increase ($p < 0.05$) differentiation in pre-confluent CaCo-2Bbe cells. HMO treatments did not enhance AP activity, a general marker of differentiation, nor did it increase disaccharidase activity in post-confluent CaCo-2Bbe cells. Disaccharidase activity was only affected by time ($p < 0.001$) in that lactase activity was higher at 12 days as compared to 21 days and the opposite was found for sucrase activity. Apoptosis and necrosis were both significantly decreased ($p < 0.001$) in post-confluent CaCo-2Bbe cells treated with 4.0 g/L HMO as compared to 0 g/L control. In summary, HMO treatment consistently inhibits proliferation with some associated enhancement of epithelial differentiation. Although total pooled HMO reduced apoptosis and necrosis, it is less clear if this is associated with functional outcomes. Further study is needed to determine if these effects translate *in vivo*; however, results of our *in vitro* model of the crypt-villus axis suggest addition of HMOs may promote intestinal maturation.

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CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

Exclusive breastfeeding is recommended for infants until six months of age; however, less than 15% of infants in the United States receive human milk exclusively during this time period and less than half of infants (44.3%) are breast fed to any extent at six months of age (Gartner et al., 2005; Li et al., 2005; Eidelman et al., 2012). By one month of age, nearly 40% of infants are receiving formula as their only source of nutrition (Li et al., 2005).

The gastrointestinal (GI) tract undergoes numerous structural and functional changes after birth as it adapts to extrauterine life (Catassi et al., 1995; Carver and Barness, 1996). In addition to these changes, colonization of the infant's GI tract is also occurring, with the mode of delivery and feeding method impacting the types and numbers of bacteria that will initially take residence (Bettelheim et al., 1974; Weng and Walker, 2006). Human milk is considered the gold standard of nutrition for infants as it contains a number of functional nutritive and non-nutritive components which aid in the maturation and protection of the neonatal GI tract, as well as the composition of the microbiota (Carver and Barness, 1996; Walker, 2000). Furthermore, breastfed infants have been shown to have decreased risk for a number of diseases including diarrhea, lower respiratory tract infections, urinary tract infections, bacteremia, and necrotizing enterocolitis (NEC) (Istre et al., 1985; Lucas and Cole, 1990; Dewey et al., 1995; Lopez-Alarcon et al., 1997; Schanler et al., 1999; Bachrach et al., 2003; Marild et al., 2004; Morrow et al., 2004; Newburg et al., 2005; Sisk et al., 2007).

The health benefits associated with human milk are numerous. However, as a large proportion of U.S. infants lack exposure to human milk during the neonatal developmental

period, it is essential that further research is done to narrow the compositional gap between human milk and infant formula and identify ingredients which can be added to formulae to impart some of the benefits of breast feeding on to formula-fed infants.

Probiotics and/or prebiotics are increasingly supplemented in infant formulae and are generally regarded as safe in healthy infants without concern for growth or adverse effects (Braegger et al., 2011). The most commonly studied probiotics include bifidobacteria and lactobacillus. Our research investigating the effects of *Bifidobacterium animalis* subspecies *lactis* within infant formula can be found in Chapter 3. Combinations of galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) are among the most commonly studied prebiotics. Our work, detailed in Chapter 4, also contributes to this body of literature. Benefits associated with supplementation of infant formula with either probiotics or prebiotics include increased fecal bifidobacteria, enhanced immunity, and decreased incidence of some infections and allergies.

Recently, human milk oligosaccharides (HMOs) have been added to the list of human milk components that may contribute to neonatal intestinal development (Kuntz et al., 2008; Kuntz et al., 2009). Additionally, HMOs have been associated with the enhancement of commensal bacteria within the GI microbiota as well as decreased incidence of several infectious diseases in infants (Newburg et al., 2005; Sela and Mills, 2010). As such, HMOs have become an obvious choice for potential ingredients worthy of investigation for addition to infant formulae. Chapters 5 and 6 will detail our work on the effects of HMOs on intestinal cell maturation *in vitro*.

PROBIOTICS

Probiotics are live microorganisms that, when consumed in adequate amounts survive digestion and provide benefit to the host (FAO/WHO, 2001). Supplementation of infant formula with probiotics has been suggested as a means to modify the intestinal microbiota in ways that may benefit the health of the developing infant (Saavedra, 2001). Specifically, the establishment of the infant's microbiota is thought to have an integral role in immune development. It is well established that bifidobacteria dominate the microbiota of breastfed infants (Yoshioka et al., 1983; Matsuki et al., 1999; Harmsen et al., 2000). Further, the dominance of bifidobacteria is thought to contribute to some of the health benefits breastfed infants possess as compared to formula-fed infants including lower incidence of infection and gastrointestinal disturbances (Weizman et al., 2005).

Of particular interest with regard to immunomodulatory effects of probiotics is the stimulatory effect bifidobacteria have been shown to have on the production of secretory immunoglobulin A (sIgA) (Fukushima et al., 1998; Mullie et al., 2004; Rautava et al., 2006). SIgA is the predominant mucosal immunoglobulin within the GI tract and plays an integral role in the innate mucosal response to microbial and dietary antigens. Human milk provides exogenous sIgA to infants, and significantly higher concentrations of fecal sIgA have been reported in breastfed versus formula-fed infants during the first six months of life (Koutras and Vigorita, 1989; Kohler et al., 2002).

One of the most commonly studied probiotic bacteria is *Bifidobacterium animalis* subspecies *lactis* (*B. lactis*). Supplementation of infant formula with *B. lactis* has been shown in a number of studies to support normal growth in infants (Langhendries et al., 1995; Ziegler et al., 2003; Saavedra et al., 2004; Braegger et al., 2011). Further, supplementation with *B. lactis* has

also been associated with a number of positive clinical outcomes including reduction in antibiotic use, febrile episodes, number of severe illness days, otitis media infection, and GI infections (Phuapradit et al., 1999; Saavedra et al., 2004; Weizman et al., 2005; Rautava et al., 2009). Additionally, consumption of *B. lactis* has been reported to improve allergy-related skin conditions (Isolauri et al., 2000; Sistek et al., 2006).

Research has shown that supplementation of *B. lactis* also beneficially impacts immune defense by increasing fecal sIgA levels (Fukushima et al., 1998; Mohan et al., 2008; Holscher et al., 2012a). In a small clinical trial by Fukushima and colleagues, toddlers ages 15 to 31 months receiving formula containing *B. lactis* for three weeks had significantly higher levels of fecal sIgA and anti-poliovirus specific IgA (Fukushima et al., 1998). Preterm infants were reported to have increased fecal sIgA concentrations following *B. lactis* supplementation during the first three weeks of life (Mohan et al., 2008). Fecal sIgA levels have also been investigated in healthy full-term infants with conflicting results. A trial by Bakker-Zierikzee and colleagues (2006) in which *B. lactis* containing formula was initiated soon after birth did not find an effect of supplementation on fecal sIgA levels through 32 weeks of life. In contrast, our laboratory group found that supplementation of *B. lactis* over a six-week period beginning at 6 weeks of age resulted in a greater increase in fecal sIgA concentrations when compared to control infants. Furthermore, infants in the probiotic group also had greater concentrations of vaccine specific antibodies following rotavirus and poliovirus immunizations (Holscher et al., 2012a). These results suggest that one benefit of a balanced GI microbiota influenced by probiotic supplementation is to provide stimulatory support to the mucosal immune system.

PREBIOTICS

Prebiotics are selectively fermented food ingredients that promote specific changes in composition and/or activity of bacteria already present within the GI microbiota thus promoting host health and well-being (Gibson et al., 2010). Oligosaccharides which are resistant to digestion by human enzymes arrive intact to the colon and stimulate both the number and concentration of fecal bifidobacteria (Moro et al., 2002). It is hypothesized that this predominance of bifidobacteria in the microbiota contributes to some of the health benefits associated with breastfeeding.

Infant formulae are commonly supplemented with the prebiotics GOS and FOS which are derived from plants or produced by technological means (Kunz and Rudloff, 2006). These prebiotic oligosaccharides are composed of the monosaccharides fructose, galactose, and glucose (Kunz and Rudloff, 2006). GOS consist of galactose chains linked by β 1-6 linkages bound to a terminal glucose by α 1-4 linkage and fructose monosaccharides are linked in FOS via β 1-2 linkages (**Figure 1.1 and 1.2**) (Tunland and Meyer, 2002; Kunz and Rudloff, 2006).

GOS and FOS have been reported in a number of trials in healthy infants to be safe without concern for adequate growth or adverse effects (Moro et al., 2002; Schmelzle et al., 2003; Haarman and Knol, 2005; Knol et al., 2005a; Knol et al., 2005b; Waligora-Dupriet et al., 2007; Ziegler et al., 2007; Costalos et al., 2008; Braegger et al., 2011; Holscher et al., 2012b). Additionally, supplementation of infant formula with varying blends and concentrations of GOS and FOS has been shown to increase fecal bifidobacteria in healthy term infants (Moro et al., 2002; Schmelzle et al., 2003; Boehm et al., 2004; Bakker-Zierikzee et al., 2005; Haarman and Knol, 2005; Knol et al., 2005a; Knol et al., 2005b; Holscher et al., 2012b). Further, infant trials have found outcomes such as decreased incidence of infection and atopic dermatitis to be

associated with infants receiving formula supplemented with GOS/FOS (Moro et al., 2006; Arslanoglu et al., 2008; Gruber et al., 2010).

Additional benefits of supplementation of the non-digestible prebiotic oligosaccharides GOS/FOS include fermentation by the GI microbiota which results in production of short chain fatty acids (SCFA). SCFA have a number of functions including energy salvage, stimulation of sodium and water absorption, reduction of colonic pH, and inhibition of overgrowth of pathogenic bacteria within the GI microbiota (Wang and Gibson, 1993). Supplementation of GOS/FOS to infant formula has been reported in several clinical trials to increase fecal SCFA and decrease fecal pH (Bakker-Zierikzee et al., 2005; Knol et al., 2005b; Bakker-Zierikzee et al., 2006; Holscher et al., 2012b).

In summary, the growing literature supports the safety and tolerance of infant formula containing prebiotics and suggests that supplementation of GOS/FOS results in an increased proportion of bifidobacteria and an associated lower fecal pH and enhanced fermentation profile.

HUMAN MILK OLIGOSACCHARIDES

Human milk plays an integral role in the development of the GI tract of infants. Composed of a magnitude of nutrients and bioactive components which are uniquely adapted to meet the needs of the growing infant, human milk stimulates structural and functional maturation of the GI tract (Donovan, 2006). HMOs are one such abundant component of human milk which contributes to the maturation of the neonatal intestine (Kuntz et al., 2008; Donovan, 2009). Human milk contains nearly 200 unique oligosaccharides, a proportion of up to 15 grams per liter which varies between women and temporally (Coppa et al., 1999; Ninonuevo et al., 2006; Wu et al., 2010). Moreover, HMOs are the third most abundant solid component

following lactose (70 g/L) and lipids (40 g/L) and narrowing surpassing protein (8g/L) (Coppa et al., 1999; Chaturvedi et al., 2001a; Ninonuevo et al., 2006; German et al., 2008b; Thurl et al., 2010; Wu et al., 2010). Human milk is unique in its oligosaccharide composition and complexity. Milk from animals is almost completely lacking in free complex oligosaccharides (Kunz et al., 2000). Infant formula is devoid of HMOs, only plant derived or synthetic FOS and GOS are currently supplemented (Tungland and Meyer, 2002; Kunz and Rudloff, 2006).

A number of functions have been associated with HMOs including enhanced immunity and prebiotic effects (Newburg et al., 2005; LoCascio et al., 2007). Additionally, recent studies have shown that HMO may impact maturation of the neonatal GI tract (Kuntz et al., 2008; Kuntz et al., 2009).

Human Milk Oligosaccharide Structures

HMOs, synthesized in the mammary gland, range in length from simple three sugar units up to more complex thirty-two sugar units (Ninonuevo et al., 2006; Wu et al., 2010). They are composed of the five monosaccharides: D-glucose, D-galactose, N-acetylglucosamine, L-fucose, and sialic acid (N-acetylneuraminic acid) (**Figure 1.3**) (Kunz et al., 2000). HMOs consist of neutral and acidic fractions which are determined primarily by the presence of sialic acid groups which produce an anionic charge. The core structures of HMOs consist of N-acetylglucosamine monomers linked to galactose monomers via β 1-3 or β 1-6 linkages creating the backbone with lactose at the reducing end (Kunz et al., 2000). Attachment of fucose and/or sialic acid further diversify the oligosaccharides by their linkages at varying positions within the core region and along the core elongation chain (Kunz et al., 2000). Sialic acid added to galactose on the reducing end lactose by α 2-3 and/or α -2-6 linkages forms 3'-sialyllactose and

6'-sialyllactose (**Figure 1.4**), respectively; additionally, fucose added to lactose in α 1-2 or α 1-3 linkages forms 2'-fucosyllactose (**Figure 1.5**) and 3'-fucosyllactose, respectively (Bode, 2009; Wu et al., 2010). These short-chain oligosaccharides are termed trisaccharides (Bode, 2009). Lacto-N-neotetraose, a type II tetrasaccharide, is made up galactose β 1-4 linked to N-acetylglucosamine linked to the galactose of the lactose molecule by a β 1-3 linkage (**Figure 1.6**) (Kunz et al., 2000). In the more complex milk oligosaccharides, up to 15 N-acetyllactosamine repeating units are added to lactose to elongate the oligosaccharide; sialic acid may be added to the lactose or polylactosamine backbone in α 2-3 and/or α 2-6 linkages or fucose may be added in α 1-2, α 1-3 and/or α 1-4 linkages (Kunz et al., 2000; Bode, 2006).

Genetic differences also influence oligosaccharide structures and variation within populations. The addition of fucose monosaccharides is related to the varying levels of structural complexity because of the presence of three different fucosyltransferases that are genetically determined (Thurl et al., 1997). The oligosaccharides present in human milk vary between women based on genetics. Oligosaccharides closely follow the Lewis blood group classification which is based off of a Lewis gene coding for an α 1-3/4 fucosyltransferase and a secretor gene coding for an α 1-2 fucosyltransferase (Thurl et al., 1997; Stahl et al., 2001). The most prevalent, α 1-2-fucosyltransferase, is found in approximately 70% of the population who are Lewis blood group Le(a-b+) while the second most prominent transferase, α 1-4-fucosyltransferase, is present in approximately 20% of the population who are classified as Le(a+b-) and attaches fucose to a subterminal N-acetylglucosamine of type 1 chains through α 1-4 linkages (Thurl et al., 1997; Stahl et al., 2001). Women who possess the α 1-2 fucosyltransferase have 2'fucosyllactose present in their breast milk and are classified as secretors; women possessing the α 1-4 fucosyltransferase lack 2'fucosyllactose and are classified

as nonsecretors (Thurl et al., 1997; Stahl et al., 2001). The remaining 10% of the population have milk samples which are void of α 1-4 linked fucose residues and are classified as Lewis blood types Le(a-b-) (Thurl et al., 1997; Stahl et al., 2001).

Several different sialyltransferases, which add sialic acid residues to the lactose backbone, are also present in addition to the different fucosyltransferase thus adding to the complexity of oligosaccharide structures within human milk (Kunz et al., 2000). The varying degrees and combinations of fucosylation and/or sialylation result in approximately 200 distant HMOs, with the majority being fucosylated or neutral oligosaccharide (Ninonuevo et al., 2006). The two major groups of HMOs are classified as neutral oligosaccharides and anionic oligosaccharides (Ninonuevo et al., 2006; Wu et al., 2010). Neutral oligosaccharides are made up of galactose, n-acetylglucosamine, lactose and fucose, while anionic oligosaccharides are made up of the same oligosaccharides with the addition of sialic acid (Ninonuevo et al., 2006).

Variations in Human Milk Oligosaccharides

HMOs vary among individuals and over the course of lactation with the general temporal trend being a gradual decline in oligosaccharide concentrations (Miller et al., 1994; Coppa et al., 1999; Chaturvedi et al., 2001a; Thurl et al., 2010). The variation among individuals can be categorized into four groups based on associated with the Lewis blood type and secretor status genes and thus the resulting structure of the terminal group of the oligosaccharides and oligosaccharide patterns (Thurl et al., 1997). As most studies quantifying HMO focus on either neutral or acidic fractions but not both, it has been difficult to gain a true understanding of oligosaccharide composition and trends. However, a recent study by Thurl and colleagues

provided analysis of both fractions thus providing a more complete picture of the total oligosaccharide content of human milk (Thurl et al., 2010).

Thurl and co-workers (2010) investigated the concentrations of major neutral and acidic oligosaccharides in 30 German women from postpartum days 3 to 90. They reported that the neutral and acidic oligosaccharide fractions of human milk are primarily made up of fourteen neutral oligosaccharides, of which concentrations vary significantly between secretors and non-secretors, and six major acidic oligosaccharides which do not significantly differ based on secretor categorization (Thurl et al., 2010). **Table 1.1 and Table 1.2** summarize the average concentrations (g/L) of the most prevalent neutral and acidic oligosaccharide fractions present in each of the three groups. The sum of the 14 neutral oligosaccharides and 6 acidic oligosaccharides quantified in this study are reflective of the total neutral and total acidic oligosaccharide fractions that have been individually reported in other trials (Miller et al., 1994; Coppa et al., 1999; Ninonuevo et al., 2008).

Women in the study conducted by Thurl et al. (2010) were categorized based on their Lewis blood groups which were reflective of overall European populations proportions: Le(a-b+) represented 73% of the subjects (group 1), Le(a+b-) represented 17% of the subjects (group 2), 10% were Le(a-b-) (group 3), and none of the subjects were Le(a+b+). As predicted by the Lewis blood group classifications, milk samples from Le(a-b+) donors (secretors) contained all fourteen of the neutral oligosaccharides which included the presence of α 1,2-, α 1,3-, and α 1,4-fucosylated oligosaccharides. Milk samples from the non-secretors (Lea+b-) lacked α 1-2-fucosyloligosaccharides. Women in group 3, secretors with Le(a-b-), had milk samples that contained α 1,2- and α 1,3- fucosylated oligosaccharides but lacked α 1,4- fucosylated oligosaccharides. The six major acidic oligosaccharides did not significantly vary between the

three groups. In assessing the temporal trend in oligosaccharide concentrations from day 3 to 90 postpartum, they found that acidic oligosaccharides decreased from approximately 3 g/L to 1 g/L in all groups while neutral oligosaccharides decreased in both secretor group, from approximately 11 g/L to 9 g/L but remained relatively constant at approximately 5 g/L in the non-secretor group (**Figure 1.7**) (Thurl et al., 2010).

Milk Oligosaccharides

Human milk and animal milk is generally quite different in terms of oligosaccharide composition, concentration, and complexity. In domestic animal milk, compositional differences exist in both the neutral and acidic fractions. Neutral oligosaccharide fractions of animal milk rarely contain fucosylated oligosaccharides which are the most dominate type in human milk. Instead linkages to galactose or N-acetylglucosamine are dominant in animals. Sialic acid is present in animal milk as both N-acetylneuraminic acid and N-glycolylneuraminic acid while human milk only contains N-acetylneuraminic acid (Boehm and Stahl, 2007). In bovine milk, sialyllactose is the dominate oligosaccharide, most notably 3'sialyllactose (Kunz et al., 2000). Concentrations of oligosaccharides in animal milk are also much lower than the 5-15 g/L found in human milk. The oligosaccharide concentrations of the most relevant domestic animals are 10- to 100- fold less than human milk (Boehm and Stahl, 2007). Bovine milk contains only eight neutral and ten sialylated oligosaccharides as compared to approximately 200 total HMOs (Gopal and Gill, 2000; Wu et al., 2010). Milk from common experimental animals such as mice and pigs, as well as milk from farm animals such as goats, sheep and cows is nearly completely lacking in free complex oligosaccharides; only milk from elephants is comparable to human milk in terms of complex oligosaccharides content (Kunz et al., 2000; Kelder et al., 2001; Osthoff et

al., 2008). Although not identical, one notable structural similarity between human and animal milk oligosaccharide cores structures is the β -glycosidically bound galactose which protects the molecule from digestion as it passes through the infant GI tract (Chaturvedi et al., 2001b; Boehm and Stahl, 2007).

Oligosaccharides in Infant Formulae

As the majority of infant formula is composed primarily of bovine milk, it contains only trace amounts of less complex milk oligosaccharides. To mimic the prebiotic effects of HMOs, infant formulae are commonly supplemented with the prebiotic oligosaccharides (PBOs) GOS and FOS which are derived from plants or produced by technological means (Kunz and Rudloff, 2006). PBOs are composed of the monosaccharides galactose, fructose, glucose, xylose and arabinose of which only glucose and galactose are found in HMOs (Kunz and Rudloff, 2006). In addition to the differences in monosaccharides which compose the oligosaccharide chains, the linkage specificity also varies between HMOs and PBOs. Fructose monosaccharides are linked in FOS via β 1-2 linkages and GOS consist of galactose chains linked by β 1-6 linkages bound to a terminal glucose by α 1-4 linkage. Monosaccharide linkages in HMOs consist of a variety of α linkages as well as β 1-3 and β 1-4 linkages (Tungland and Meyer, 2002; Kunz and Rudloff, 2006).

Prebiotic Effects of Human Milk Oligosaccharides

HMOs are resistant to digestion and arrive intact to the infant colon where they provide fermentation substrates to the commensal microbiota and aid in selective colonization and development of a protective microbiota (Chaturvedi et al., 2001b; Zivkovic et al., 2011).

Bifidobacteria make up a large portion of the microbiota of the breastfed infant, as compared to their formula fed counterparts, with prominent species including *Bifidobacterium longum*, *Bifidobacterium breve*, and/or *Bifidobacterium bifidum* (Matsuki et al., 1999; Harmsen et al., 2000). A number of health benefits have been associated with bifidobacteria in the developing infant including reduced incidence of NEC, reduced rotavirus infections, reduced susceptibility to antibiotic related GI disturbances, and modulation of the immune system (Arunachalam et al., 2000; Picard et al., 2005).

B. bifidum and *B. longum* subsp. *infantis* have been shown to thrive on HMOs as an energy source while *B. longum* subsp. *longum* and *B. breve* grow moderately well (**Table 1.3**) (LoCascio et al., 2007). The modes of utilization of HMOs as an energy source vary between these bacteria. *B. longum* subsp. *infantis* ATCC15697 has been shown to prefer HMOs with chain lengths less than seven while *B. longum* subsp. *longum* DJO10A selectively consumes Lacto-N-neotetraose (LNnT) (LoCascio et al., 2007; Ward et al., 2007; Zivkovic et al., 2011). *B. breve* ATCC15700 consumes LNnT as well as monosaccharides which suggest that cross-feeding may be occurring between these bacteria within the GI tract. The consumption of HMOs by these bacterium seems to be selective of the major HMOs produced by the lactating mammary gland as the majority of oligosaccharides have chain lengths less than 7 monosaccharides and LNnT is also abundantly available (LoCascio et al., 2007; Ward et al., 2007; Zivkovic et al., 2011).

Bacterial fucosidase and sialidase activities relevant to fucosylated and sialylated oligosaccharide catabolism provides further insight to the potential activities related to HMO fermentation within the GI tract. *B. longum* subsp. *infantis* has high sialidase activity as well as fucosidase activity, likely internalizing the HMO molecules prior to catabolizing them (LoCascio

et al., 2007). *B. bifidum*, on the other hand, has been shown to export fucosidases which cleave the fucose molecule prior to the bacterium internalizing the remaining oligosaccharide (LoCascio et al., 2007; Ward et al., 2007). *B. breve* is also thought to internalize LNT, a HMO lacking fucose or sialic residues, for catabolism as well as utilize free monosaccharides cleaved from HMOs (LoCascio et al., 2007; Ward et al., 2007; Zivkovic et al., 2011).

Interestingly, from these data it seems likely that HMOs do not directly nourish the growing neonate, but instead enrich the GI microbiota providing substrates for bacterial fermentation and subsequently benefiting the growth of commensals while hindering the growth of pathogens.

Anti-adhesion Effects of Human Milk Oligosaccharides

A lower incidence of diseases in breastfed infants was documented as early as the 1930s, when several fold reductions in diarrheal diseases and respiratory infections were seen in breastfed infants as compared to their formula-fed counterparts (Grulee et al., 1934). A number of enteric pathogens including *Escherichia coli*, *Campylobacter jejuni* and *Helicobacter pylori*, Noroviruses, and Rotavirus target specific intestinal epithelial cell surface glycan epitopes for adherence during the first stage of pathogenesis. HMOs, which also possess common intestinal epithelia epitopes, have become an area of interest for deducing the mechanisms behind lower incidences of disease in breastfed infants (Morrow et al., 2004; Newburg et al., 2004; Newburg et al., 2005). Decoy binding of oligosaccharide epitopes in place of binding of intestinal epithelia epitopes by pathogens is a potential mechanism by which infection and diarrheal diseases are reduced in human milk fed infants as compared to formula-fed infants (Newburg et al., 2004; Morrow et al., 2005; Newburg et al., 2005)

Human milk which contains α 1,2-fucosylated oligosaccharides has been associated with anti-adhesive effects in several *in vivo* and *in vitro* studies (Morrow et al., 2004). An *in vivo* study of 2- to 4-day-old suckling mice infected with *E. coli* stable toxin (STa) showed that mice fed the STa with human milk had increased survival time as compared to control; additional analyses revealed that the separated neutral oligosaccharide fraction contained α 1,2-fucosylated oligosaccharides (Crane et al., 1994; Newburg et al., 2004). An *in vitro* study using T84 cells was conducted to determine the mechanism by which ST infection was inhibited—results of the trial showed that the fucosylated oligosaccharide bound the extracellular guanylate cyclase domain and thus prevented binding of the ST and the associated infection (Crane et al., 1994).

The oligosaccharide 2'fucosyllactose (2'FL) has also demonstrated anti-adhesive effects *in vivo*. A reduced incidence of campylobacter infection has been shown in mice fed milk from dams transfected with the human fucosyltransferase I gene. The transfected dams' milk contained increased H-2 epitopes, the known target epitope of campylobacter. Pups receiving the enhanced dam milk had enhanced protection from infection as compared to control (Ruiz-Palacios et al., 1990).

In breastfed infants, an inverse correlation has been found between risk of campylobacter diarrhea and 2'FL breast milk sample concentrations suggesting a protective effect of 2'FL against campylobacter in human infants (Morrow et al., 2004). Morrow and colleagues (2004) also reported an inverse correlation between another fucosylated human milk oligosaccharide, lactodiflucohexaose-1 (LDFH-1) and norovirus associated diarrhea in breastfed infants, likely linked to the presence of a Lewis b moiety, as well as an overall inverse correlation between total concentration of α 1,2-linked fucosyloligosaccharides in breast milk samples and moderate-to severe diarrhea from all causes (Morrow et al., 2004).

In summary, a number of different anti-adhesive mechanisms are associated with fucosylated oligosaccharides allowing them to inhibit attachment of *E. coli*, *Campylobacter* and Norvirus thus providing a potential benefit of reduced diarrheal illness in infants.

Glycome-modifying Effects of Human Milk Oligosaccharides

In addition to HMOs anti-adhesive effects on diarrheal pathogens, milk oligosaccharides have also been associated with glycome-modifying effects *in vitro*, suggesting that modifications in adhesion of pathogens and commensal bacteria may result due to the changes in expression of intestinal epithelial cell surface glycans (**Figure 1.8**) (Bode, 2009). An *in vitro* study conducted in CaCo-2 cells showed that incubation with 3'sialyllactose reduced adhesion of enteropathogenic *E. coli* (EPEC) by 90% as compared to controls (Angeloni et al., 2005). These glycan modifying effects are hypothesized to be an additional means by which HMOs provide protective effects to the breastfed infant.

Intestinal Epithelial Cell Effects of Human Milk Oligosaccharides

Kuntz and colleagues (2008) hypothesized that neutral and acidic HMO fractions or individual oligosaccharides would affect cell dynamics in non-transformed fetal intestinal crypt cell line, HIEC, and in transformed human intestinal cell lines, HT-29 and CaCo-2. The *in vitro* model consisted of HIEC cells to model undifferentiated intestinal stem-like crypt cells, HT-29 cells to model normal crypt colonocytes, and CaCo-2 cells to model intestinal epithelial cells. Under pre-confluent conditions, these cells types create a model of the proliferating cells of the crypt/villus axis and thus simulate the development within the fetal and neonatal intestine (Kuntz et al., 2008).

They reported that neutral and acid HMO fractions induced growth inhibition in all cell types in a dose dependent manner after 72 hours of incubation with the most sensitivity in HT-29 and CaCo-2 cells. HT-29 cells experienced growth inhibition by several individual neutral and acidic HMOs including lacto-N-tetraose (LNT) at 1.4 mg/ml and 6'SL at 1.5 mg/ml, however 2'FL at 2 mg/ml did not significantly affect proliferation. CaCo-2 and HEIC cells were not affected by LNT or 2'FL but showed a significant decrease with 6'SL as compared to control. Alkaline phosphatase (AP) activity, a marker of cellular differentiation, was assessed following a 72 hour incubation with neutral or acidic fractions and it was found that AP activity significantly increased as compared to controls in HT-29 and HEIC cells treated with 15 mg/L of the neutral fraction as well as with 7.5 mg/L and 15 mg/L of the acidic fraction. CaCo-2 cells, a more differentiated cell type, were not significantly affected by neutral or acidic HMO fractions or individual HMOs. Treatment with individual HMOs at 1 mg/ml resulted in an increase in AP activity as compared to control in HT-29 and HIEC cells treated with 6'SL. Cellular caspase-3 activity, a measure of apoptosis, increased in both HT-29 cells and HEIC cells in the 15 mg/L neutral HMO fraction group, however the acidic fraction did not significantly affect any of the cell types at any level of treatment as compared to control. In summary, this trial by Kuntz and coworkers demonstrated that neutral and acidic HMO fractions induce growth inhibition in intestinal cell lines by two different mechanisms 1) suppression of cell cycle progression through induction of differentiation and/or 2) induction of apoptosis (Kuntz et al., 2008).

To determine potential mechanisms behind the growth inhibition of the intestinal cell lines exposed to neutral and acid HMO fractions in the study discussed above, Kuntz and colleagues used flow cytometry to assess cell cycle events and quantitative real-time RT-PCR to examine effects on cell cycle genes (Kuntz et al., 2009). They found that both neutral and acidic

fractions induced G2/M arrest in a dose dependent manner in all cell types (HT-29, CaCo-2, and HEIC). RT-PCR results showed an increase in mRNA expression levels of the cell cycle gene cyclin B/GAP-DH as compared to control in all three cell types after 72 hours of incubation with both neutral and acidic HMO fractions at a concentration of 15 mg/L. Additionally, cyclin A/GAP-DH increased in HT-29 and HIEC cells following incubation with the acidic fraction but not with the neutral fraction, while there was no effect in CaCo-2 cells. Further, it was found that the block in cell cycle progression was related to CDKI levels, specifically an increase in p21^{cip1} and p27^{kip1} in both neutral and acidic HMO treatment groups for HT-29 and HEIC cells. CaCo-2 cells had increased levels of p21^{cip1} but not p27^{kip1}. These differences were independent of p53 levels, as no differences were detected for any cell type within any treatment group. Lastly, it was found that both acidic and neutral HMOs induced phosphorylation of the epidermal growth factor receptor (EGFR) and increased extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 phosphorylation. These results suggest that HMO associated growth inhibition is related to altered cell cycle progression with concurrent effects on regulator genes and stimulation of mitogen-activated protein kinase (MAP-kinase) signaling (Kuntz et al., 2009).

SUMMARY

In summary, the beneficial attributes of probiotics, prebiotics, and human milk oligosaccharides make them attractive ingredients for infant formula supplementation. As previously discussed, each has been shown to enhance commensal bacteria within the GI microbiota. Further, the enhancement of the commensal resident bacteria stimulates immune factors such as sIgA, as well as provides colonization resistance against pathogens. In addition to all these effects, HMOs may also have a role in maturation of the GI tract. Thus, additional

research examining the effects of probiotics, prebiotics, and HMOs *in vivo* and *in vitro* becomes increasingly important. The information gained would provide a greater understanding of the role of each in GI health and development.

FIGURES AND TABLES

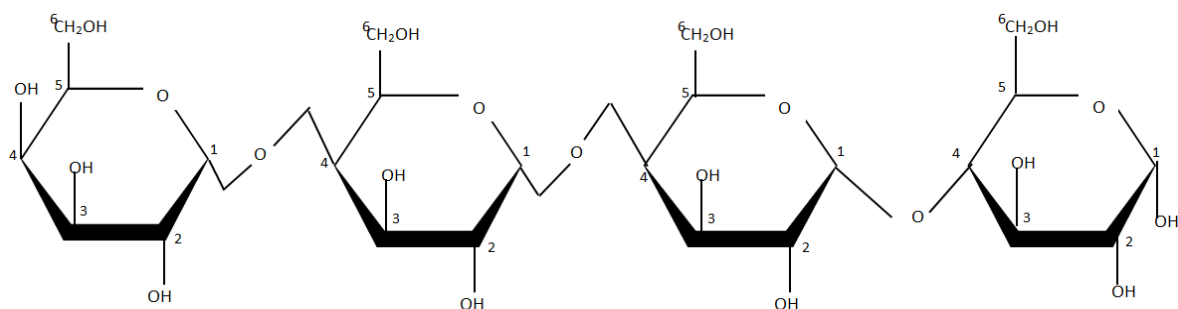


Figure 1.1. Galacto-oligosaccharide

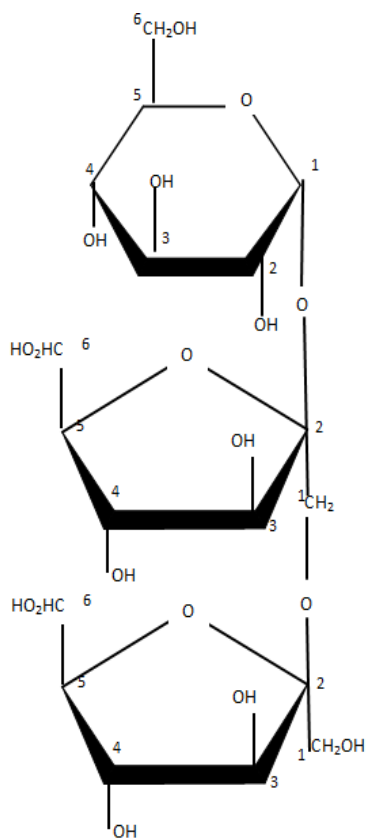


Figure 1.2. Fructo-oligosaccharide

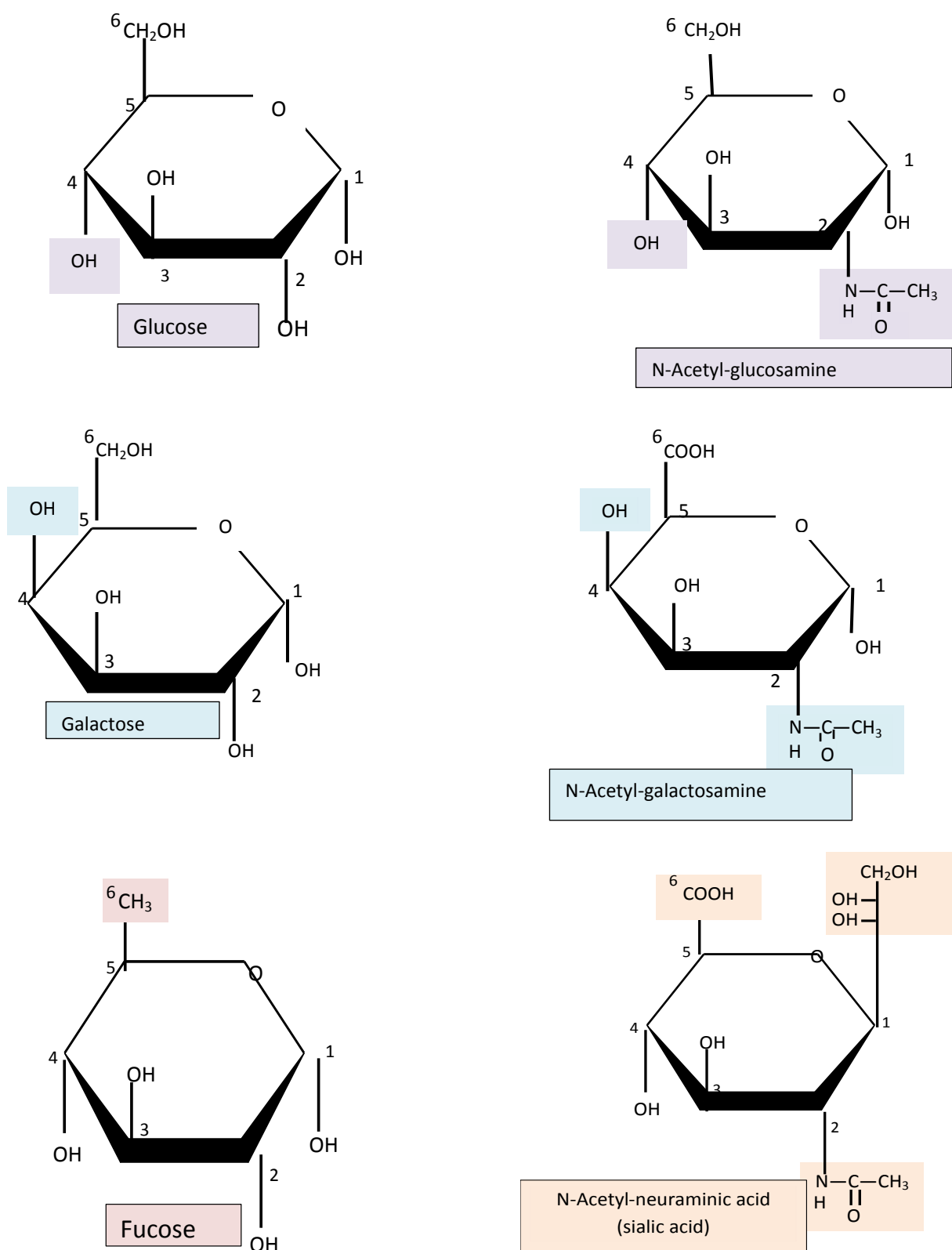


Figure 1.3. Monosaccharides used to synthesize human milk oligosaccharides. Adapted from Kunz, 2006.

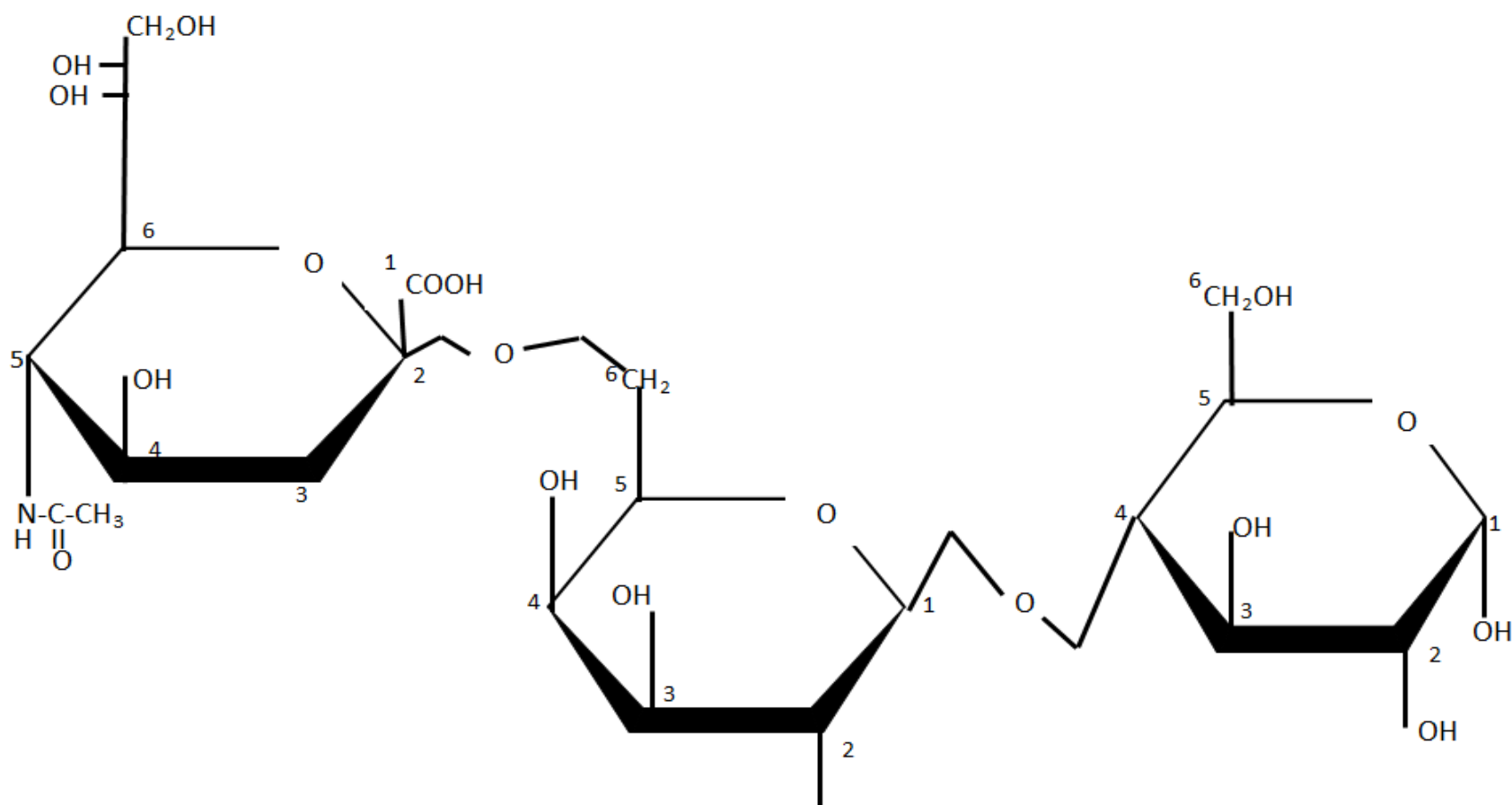


Figure 1.4. 6'-Sialyllactose (6'SL)

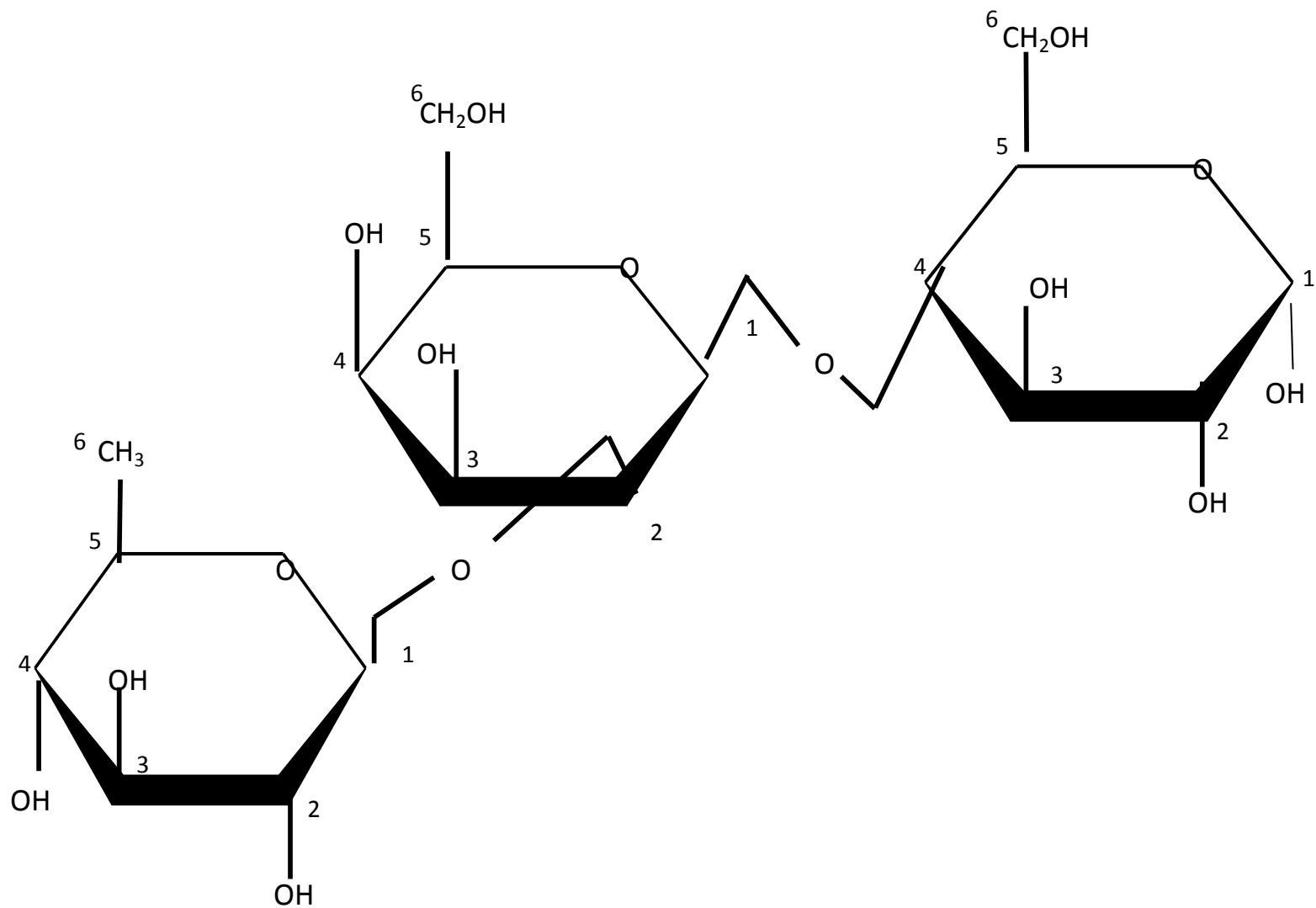


Figure 1.5. 2'-Fucosyllactose (2'FL)

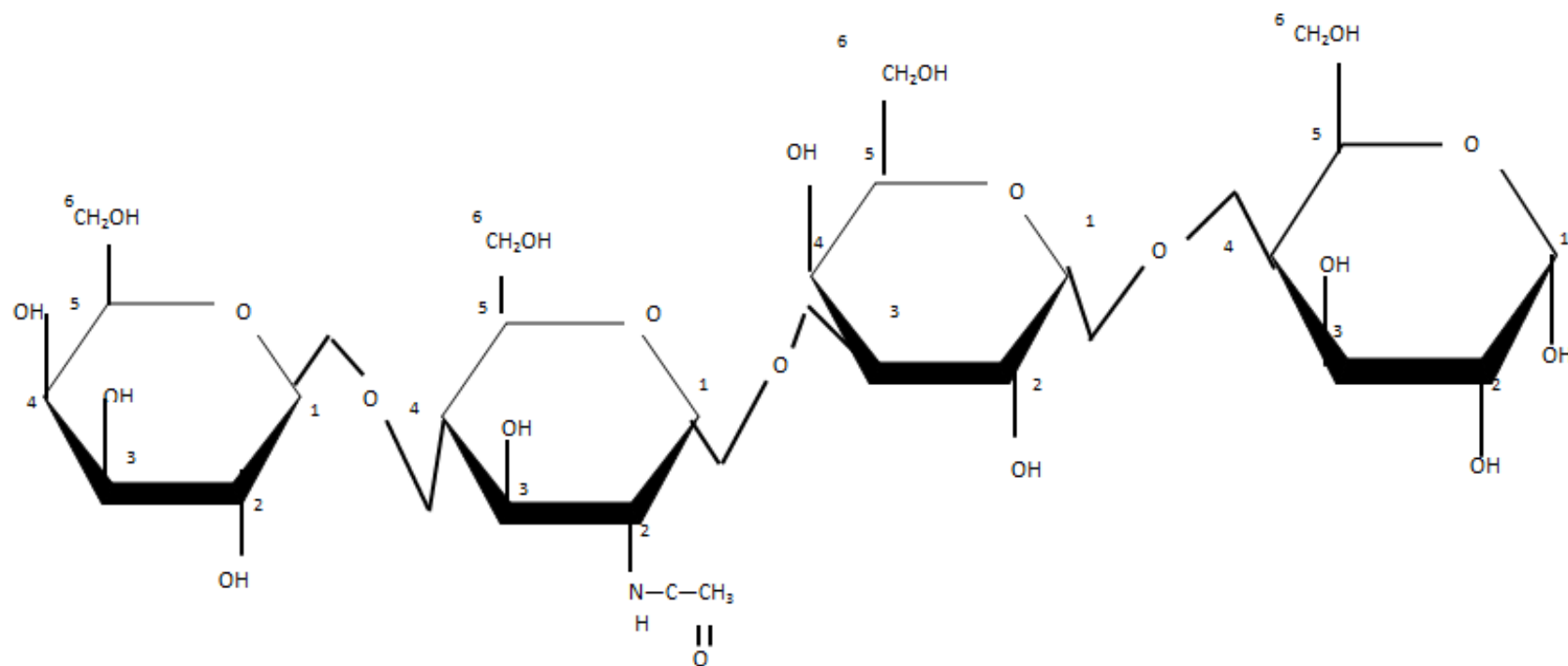


Figure 1.6. Lacto-N-neotraose (LNnT)

	2'FL	3-FL	LDFT	LNFP I	LNFP II	LNFP III	LNDFH I	LNDFH II	2'-F-LNH	3'-F'LNH	2',3'-DF-LNH
Group One	3.13	0.42	0.41	1.58	0.22	0.38	1.28	0.2	0.21	0.16	0.33
Group Two	ND	1.79	ND	ND	1.25	0.38	ND	0.45	ND	0.35	ND
Group Three	4.57	0.15	0.17	3.18	ND	0.31	ND	ND	0.26	0.13	0.58

Table 1.1. Average neutral human milk oligosaccharide concentration (g/L) over the course of 90 days of lactation. Adapted from Thurl 2010.

	3'-SL	LSTa	DSLNT	LSTb	6'SL	LSTc
Group One	0.27	0.04	0.34	0.08	1.22	0.29
Group Two	0.24	0.04	0.42	0.11	1.14	0.21
Group Three	0.31	0.06	0.41	0.08	1.31	0.31

Table 1.2. Average acidic human milk oligosaccharide concentration (g/L) over the course of 90 days of lactation. Adapted from Thurl, 2010.

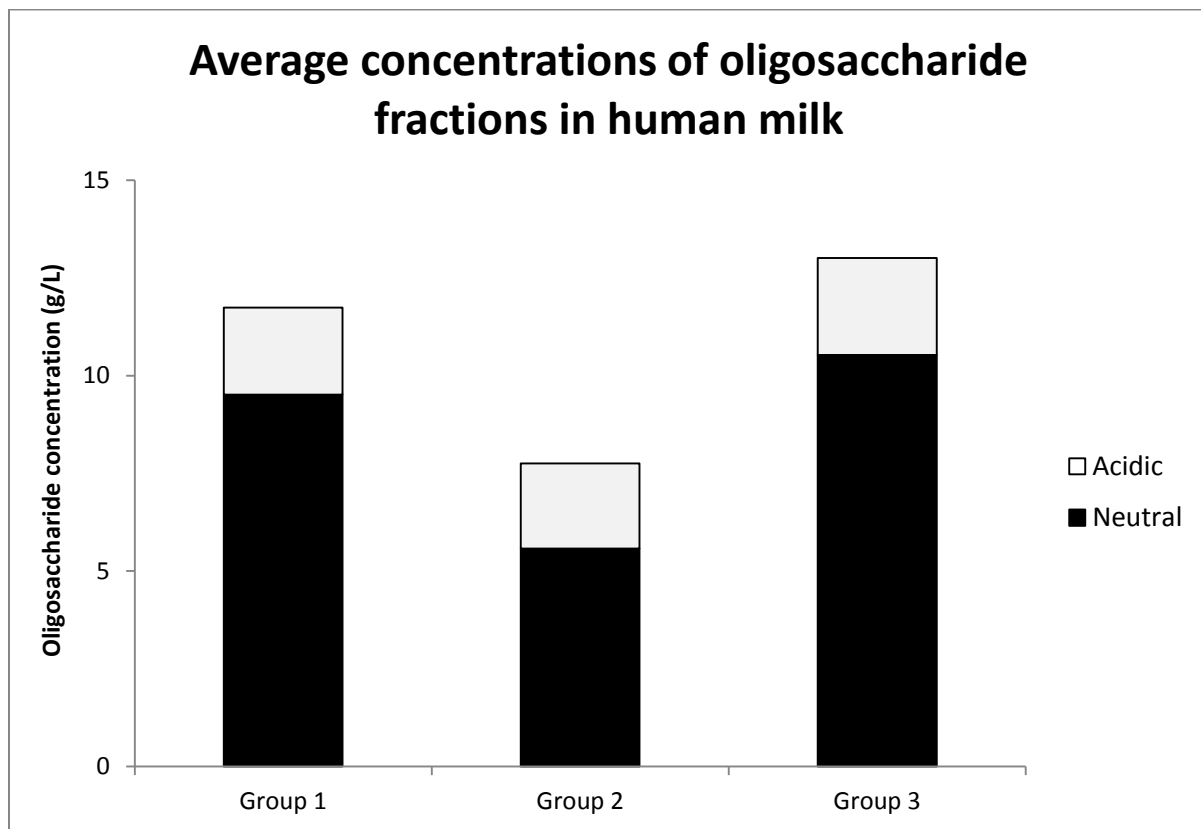


Figure 1.7. Average total oligosaccharide content over course of 90 days. Average concentration of oligosaccharide fractions vary between groups of women classified based on Lewis blood groups and the presence or absence of the secretor gene. Figure adapted from Thurl, 2010.

	<i>B. bifidum</i>	<i>B. longum</i> subsp. <i>infantis</i>	<i>B. longum</i> subsp. <i>longum</i>	<i>B. breve</i>
HMO growth	High	High	Moderate	moderate
HMOs degraded	LNT, LNnT	LNT, chains < 7	LNnT	LNT, LNnT and monosaccharides
HMO units & monosaccharide growth	Lacto-N-biose (LNB), Glc, Gal	LNB, Glc, Gal, Glucosamine, Fucose, sialic acid	LNB, glucose, galactose	LNB, Glc, Gal, Glucosamine, Fucose, sialic acid
Extracellular activities & enzymes	•β-galactosidase(s) •lacto-N-biosidase •1,2a- & 1-3/4a-fucosidases •endo-a-N-acetyl-galactosaminase		•lacto-N-biosidase •endo-a-N-acetyl-galactosaminase •lacks sialidase and fucosidases	
Intracellular activities & enzymes	Internalizes free LNB after fucose cleaved	Internalizes HMO, intracellular glycosidase, sialidases & fucosidases		Internalizes LNnT and free monosaccharides from HMO

Table 1.3. Prebiotic Effects of HMOs. (LoCascio et al., 2007; Ward et al., 2007; Zivkovic et al., 2011)

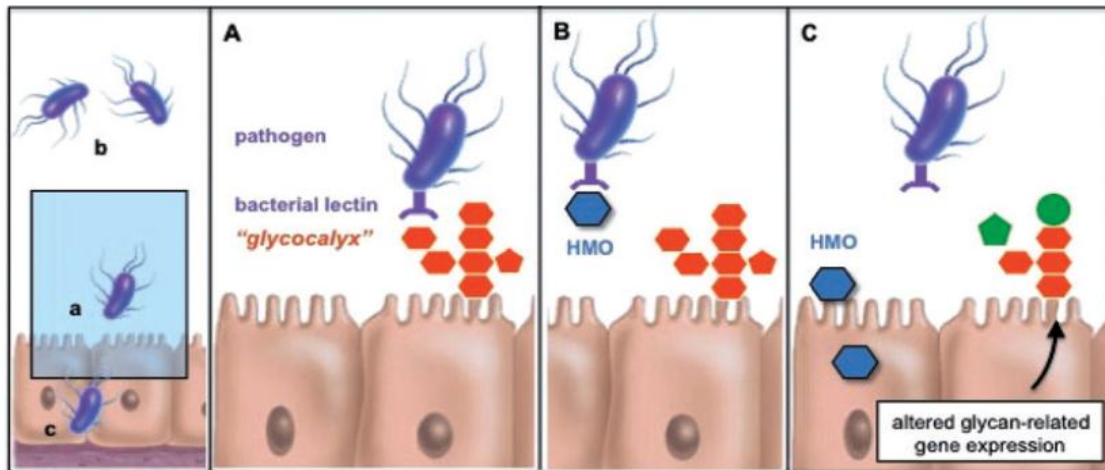


Figure 1.8. Human milk oligosaccharides provide anti-adhesive and glycome modifying effects. A) Bacteria bind lectins, glycan-binding proteins, during the initial stages of pathogenesis. B) HMOs may provide decoy binding sites and prevent adhesion of pathogens. C) HMOs may also alter epithelial glycans preventing recognition of pathogen binding sites. (Bode, 2009). Figure reprinted with permission from John Wiley and Sons.

CHAPTER 2

RATIONALE AND OBJECTIVES OF RESEARCH

RATIONALE OF RESEARCH

Although human milk is recommended to be fed exclusively to developing infants for the first six months of life, less than half of infants are meeting this recommendation (Eidelman et al., 2012). As health outcomes are generally better in infants receiving human milk, further research is necessary to identify key ingredients that will aid in narrowing the compositional gap between human milk and infant formula and thus provide benefit to formula-fed infants. Ingredients of interest include probiotics, prebiotics, and human milk oligosaccharides (HMOs). The information gained from additional study of these ingredients may allow for a narrowing of this compositional gap and improvements to formula-fed infants health outcomes.

AIMS AND SCOPE OF RESEARCH

The overall **objective** of this thesis research is to examine the effects of probiotics, prebiotics, and human milk oligosaccharides as means to narrow the compositional gap between human milk and infant formula. Thus, we **aimed** to assess 1) the effects of the probiotic *Bifidobacterium animalis* subspecies *lactis* (*B.lactis*), 2) the prebiotics galacto- and fructo-oligosaccharides, and 3) the individual and combined effects of human milk oligosaccharides within *in vivo* and *in vitro* models. The **central hypothesis** of this research is that specific ingredients will enhance gastrointestinal health and development. The specific aim will be investigated using the following hypotheses:

Hypothesis 1: Consumption of an infant formula containing *B. lactis* for 6 weeks will positively impact the intestinal-associated immune system and resident microbiota of formula-fed infants. **Chapter 3** establishes that infant formula containing *B. lactis* is well tolerated and stimulates intestinal immunity as evidenced by enhanced fecal secretory immunoglobulins A (sIgA) and vaccine-specific IgA following immunization. Furthermore, it demonstrates that some of the negative immune-related effects of not breast feeding and cesarean delivery can be mitigated by supplementing infant formula with *B. lactis* thereby providing a safe, dietary, immune-modulating bacterial introduction to infants.

Hypothesis 2: Consumption of the galacto- and fructo-oligosaccharide-containing formula for six weeks will be well-tolerated and increase the proportion of fecal bifidobacteria to total bacteria in formula-fed infants. **Chapter 4** establishes that infant formula containing galacto- and fructo-oligosaccharides is well-tolerated by formula fed infants, increases the abundance and proportion of fecal bifidobacteria, and reduces fecal pH.

Hypothesis 3: Three individual HMOs, 2'-fucosyllactose (2'FL), lacto-N-neotetraose (LNnT), and 6'-sialyllactose (6'SL), will induce epithelial differentiation in an *in vitro* epithelial model of the crypt-villus axis consisting of pre-confluent HT-29 cells, pre-confluent CaCo-2Bbe cells, and post-confluent CaCo-2Bbe cells. **Chapter 5** investigates the interaction between individual HMOs and the transformed intestinal epithelial cell lines, HT-29 and Caco-2Bbe. Using an *in vitro* model, it establishes that all three HMOs inhibit cellular proliferation, with 2'FL also providing stimulation for enhancement of epithelial cell differentiation. Furthermore, it demonstrates that well differentiated cells have greater digestive and barrier function when treated with 2'FL and LNnT, respectively.

Hypothesis 4: Concomitant administration of the HMOs, 2'-fucosyllactose (2'FL), 3'-sialyllactose (3'SL) , and 6'-sialyllactose (6'SL), will induce epithelial differentiation and enhance maturation of the small intestine *in vitro* in an epithelial model of the crypt-villus axis consisting of pre-confluent HT-29 cells, pre-confluent CaCo-2Bbe cells, and post-confluent CaCo-2Bbe cells. **Chapter 6** explores the effects of administration of concomitant HMOs on transformed intestinal epithelial cell lines, HT-29 and Caco-2Bbe. Within the *in vitro* model, it establishes that there is an additive effect of HMO on cellular proliferation and an associated enhancement of differentiation in pre-confluent Caco-2Bbe cells.

Chapter 7 summarizes the results of these studies and discusses the insights gained from the research. It also explores future directions of the research with particular emphasis on the need to investigate the physiological relevance of the *in vitro* effects of HMOs within preclinical animal models so that we may better understand their effects on intestinal maturation, digestive and absorption capabilities, and barrier function within the gastrointestinal tract.

CHAPTER 3

***BIFIDOBACTERIUM LACTIS* ENHANCES INTESTINAL ANTIBODY RESPONSE IN FORMULA-FED INFANTS: A RANDOMIZED, DOUBLE-BLIND, CONTROLLED TRIAL¹**

ABSTRACT

Background: Addition of probiotics to infant formula may positively affect immune function in non-exclusively breastfed infants. This study aimed to investigate the effect of infant starter formula containing the probiotic *Bifidobacterium animalis* subspecies *lactis* (*B. lactis*) on intestinal immunity and inflammation. **Methods:** Six-week-old healthy, full-term infants (n=172) were enrolled in a prospective, randomized, double-blind, controlled clinical trial with 2 groups studied in parallel to a breastfed comparison group. Formula-fed (FF) infants were randomized to partially hydrolyzed whey formula (CON), or the same formula containing 10⁶ CFU *B. lactis*/g (PRO) for 6 weeks. Fecal secretory IgA (sIgA), calprotectin, lactate, and stool pH were assessed at baseline, 2, and 6 weeks. Anti-poliovirus-specific IgA and anti-rotavirus-specific IgA were assessed at 2 and 6 weeks. **Results:** Among vaginally-delivered FF infants, PRO consumption increased (p<0.05) fecal sIgA, compared to CON. Anti-poliovirus-specific IgA concentration increased (p<0.05) in all infants consuming PRO; whereas anti-rotavirus-specific IgA tended to increase (p=0.056) with PRO consumption in cesarean-delivered infants. Anthropometrics and tolerance did not differ significantly between FF infants. **Conclusions:** Infants consuming formula with *B. lactis* produced feces with detectable presence of *B. lactis*

¹ The final, definitive version of this paper has been published in the Journal of Parenteral and Enteral Nutrition, 36, Supplement 1, January 2012 by SAGE Publications, Inc., All rights reserved. © 2012 A.S.P.E.N. Holscher, H.D., Czerkies, L.A., Cekola, P., Litov, R., Benbow, M., Santema, S., Alexander, D.D., Perez, V., Sun, S. & Saavedra, J.M., & Tappenden, K.A. 2012a, "*Bifidobacterium lactis* Bb12 Enhances Intestinal Antibody Response in Formula-Fed Infants", Journal of Parenteral and Enteral Nutrition, vol. 36, no. 1 suppl, pp. 106S-117S. DOI: 10.1177/0148607111430817

and augmented sIgA concentration. Further, cesarean-delivered infants consuming *B. lactis* had heightened immune response, as evidenced by increased anti-rotavirus- and anti-poliovirus-specific IgA following immunization. These results demonstrate that negative immune-related effects of not breast feeding and cesarean delivery can be mitigated by including Bb12 in infant formula and thereby providing infants a safe, dietary, immune-modulating bacterial introduction.

CLINICAL RELEVANCY STATEMENT

Breast milk, the ideal source of nutrition for infants, is also a source of secretory immunoglobulin (Ig) A, as well as bacteria, which enhance both passive and active infant immunity. These benefits are not provided by routine use infant formula. The addition of probiotics to infant formula is reported to increase fecal secretory IgA. This study demonstrates that supplementation of infant formula with *Bifidobacterium animalis* subspecies *lactis* (*B. lactis*) results in detectable fecal levels of *B. lactis* and increased concentrations of secretory-, anti-rotavirus-specific, and anti-poliovirus-specific IgA. Therefore, including *B. lactis* in infant formula provides infants a safe, dietary, immune-modulating bacterial experience.

INTRODUCTION

While it is known that breastfeeding is best, infant formulas are often used to supplement breast milk, and mothers of some infants may choose not to breastfeed. Attempts to optimize infant formula have been undertaken to impart some of the benefits of breast milk to the formula-fed infant. One such strategy is to positively influence the composition and activity of the intestinal microbiota of the formula-fed infant to more closely reflect that of the breastfed infant.

An infant starter formula containing the probiotic *Bifidobacterium lactis* is currently being marketed in the US and is available in many other parts of the world.

Probiotics are live microorganisms that, when administered orally in adequate amounts survive the digestion process and confer a beneficial effect on the host (FAO/WHO, 2001). Saavedra proposed probiotics as a concept by which the use of these nonpathogenic agents “constitutes a purposeful attempt to modify the relation with our immediate microbial environment in ways that may benefit our health” (Saavedra, 2001). The microbial environment of the intestinal microbiota is an integral part of the immune system. In particular, it has been established that the microbiota of the breastfed infant is dominated by bifidobacteria early in life, and this has been thought to contribute to the many health benefits breastfed infants possess (Yoshioka et al., 1983). The lower incidence of infection and gastrointestinal disturbances observed in breastfed infants compared to their formula-fed counterparts may be related to differences in their respective microbiota. One such benefit of a balanced intestinal microbiota influenced by probiotics is to provide stimulatory support to the mucosal immune system.

Non-pathogenic bacteria are not considered to be a probiotic until a clinical benefit to the host has been demonstrated. Amongst those probiotic bacteria that have been better studied is *B. lactis*. Safe administration of *B. lactis* to pediatric populations has been reported widely in the literature, with no adverse effects on growth noted (Langhendries et al., 1995; Ziegler et al., 2003; Saavedra et al., 2004; Braegger et al., 2011). Further, *B. lactis* consumption is reported to beneficially impact immune function, by increasing fecal IgA levels (Fukushima et al., 1998; Mohan et al., 2008). Associated outcomes including reduction in antibiotic use, reduction in febrile episodes, reduction in number of severe illness days, reduction in otitis media infection, and improvements in allergy-related skin conditions have also been seen (Isolauri et al., 2000;

Saavedra et al., 2004; Weizman et al., 2005; Sisteck et al., 2006; Rautava et al., 2009). In a small group of toddlers (15-31 months) fed a *B. lactis*-containing formula, significantly higher levels of fecal sIgA and anti-poliovirus IgA were observed after 3 weeks of feeding (Fukushima et al., 1998). Separately, Mohan demonstrated increased fecal sIgA concentration in a study of premature infants fed a *B. lactis*-containing formula for the first 3 weeks of life (Mohan et al., 2008). Fecal IgA levels of full-term infants exclusively fed a *B. lactis*-containing formula has only been reported in one other study to date, however the current trial differs in the timing of probiotic initiation and the composition of the formula vehicle (Bakker-Zierikzee et al., 2006).

Secretory immunoglobulin A (sIgA) is the predominant immunoglobulin in mucosal surfaces and provides key protection in the innate mucosal immune response to dietary and microbial antigens. While breast milk contains sIgA, infant formula does not. Significantly higher levels of fecal sIgA in samples from breastfed infants compared to formula-fed infants have been reported through the first 6 months of life (Koutras and Vigorita, 1989; Kohler et al., 2002). Probiotics have been shown to increase fecal sIgA levels in a pediatric population after 3 weeks of feeding (Fukushima et al., 1998). Further investigations to determine ways to increase levels of fecal sIgA in formula-fed infants could lead to improvements in infant formula.

The primary outcome of the this trial was to compare the fecal sIgA concentration of six-week-old healthy infants following 6 weeks of feeding of a partially hydrolyzed whey-based starter formula (control) or the same formula containing *Bifidobacterium animalis* subspecies *lactis* (*B. lactis*). Secondary outcomes, which quantified markers of immunity and inflammation, were fecal sIgA concentration at 8 weeks of age (following 2 weeks of feeding) and fecal abundance of *B. lactis*, anti-rotavirus-specific IgA, anti-poliovirus-specific IgA, calprotectin, lactate, and stool pH after 2 and 6 weeks of formula feeding. We hypothesized that consumption

of an infant formula containing Bb12 for 6 weeks would positively impact the intestinal-associated immune system and resident microbiota of formula-fed infants.

METHODS

Subjects and Study Design

The study was conducted from September 2007 to March 2008 at 5 study sites (Pedia Research in KY/IN, Southwest Children's Research Associates in San Antonio, Texas and Scott & White Memorial Hospital in Temple & Killeen, TX) within the United States. Newborn infants (2–6 weeks of life) were recruited. All subjects were required to live within 45 minutes of a study center and have a working freezer in their home. Infants receiving antibiotic therapy in the 30 days prior to enrollment, or breastfed subjects whose mothers had received antibiotic therapy within the same period, were not eligible for the study. Other criteria for exclusion from the study were: known or suspected cow-milk allergy; congenital illness or malformation that affects infant feeding and/or growth; significant pre-natal and/or post-natal disease; immunocompromise; any readmission to hospital (except for hyperbilirubinemia) prior to enrollment; diarrhea requiring treatment in the 7 days prior to enrollment, or; receipt of medication other than multivitamin supplement. Parents of all participants were required to sign a written informed consent prior to enrollment. The studies were approved by the relevant IRB of each site recruiting subjects and the University of Illinois, where all laboratory analyses of samples occurred. The procedures followed were in accordance with the ethical standards of the responsible institutional or regional committee on human experimentation.

Formula-fed (FF) infants were randomized to receive either CON or PRO at 6 weeks of age and consume the assigned formula exclusively for the following 6 weeks. The control

formula was a commercially available partially hydrolyzed whey infant formula (CON; Nestlé Good Start ® Nestlé, Florham Park, NJ), whereas the test formula (PRO) was identical to CON with the exception of the addition of 10^6 CFU *B. lactis* /g powder. Both formulas provided 20 kcal/oz with 2.2 g protein/100 kcal from 100% partially hydrolyzed whey. Randomization of FF subjects was stratified by delivery (vaginal versus cesarean-section). The self-selected breastfed reference group was exclusively breastfed and followed for 6 weeks starting at 6 weeks of age.

Weight of each subject was taken at baseline (V0) and after 2 (V1) and 6 weeks (V2) of feeding at study visits. Caregivers were instructed to complete daily records for 2 days prior to the visits. Using paper diaries, caregivers of all subjects recorded the same data regarding formula intake volumes, stool patterns and tolerance-related behavior. Caregivers of breastfed subjects documented the length of each feeding or the amount of expressed breast milk given at each feeding, in addition to stool patterns and tolerance-related behavior. All subjects were asked to receive immunizations according to the schedule outlined by the Department of Health and Human Services, which included receipt of rotavirus (Rotateq, Merck, Whitehouse Station, NJ) and inactivated poliovirus (Ipol, Sanofi Pasteur SA, Swiftwater, PA) vaccines at 8 weeks of age. Stool sample for Visit 1 was collected prior to vaccinations.

Within 72 hours of each study visit, stool samples were collected at home in 30 ml polypropylene fecal collection tubes (Globe Scientific, Paramus, NJ) within 30 minutes of passing. Caregivers brought the fresh sample in a cooler with ice packs to the site within 30 minutes of collection or the tube with the sample was placed in a home freezer for 2-72 hours prior to transporting the sample to a study site in the manner outlined above. Upon arrival at the study site, samples were promptly placed at -20°C for a maximum of 2 weeks before shipment on ice to the University of Illinois where samples were stored at -80°C until analysis.

Fecal Homogenates

Fecal homogenates were prepared by thawing frozen fecal samples on ice then creating a 10% wt:vol mixture in PBS followed by homogenization with a homogenizer (Model PCU-11; Brinkman Instruments, Westbury, NY) for 1 minute. Aliquots of fecal homogenates for quantification of sIgA, anti-polio-specific IgA and anti-rotavirus-specific IgA were stored at -80°C until analysis.

Secretory IgA

Fecal secretory IgA (sIgA) was determined from the fecal samples that were collected at baseline, 2 and 6 weeks of feeding. Analysis of sIgA was performed as described before by Scholtens and colleagues, with minor modifications (Bakker-Zierikzee et al., 2006; Scholtens et al., 2008). Modifications included a decreased concentration of the secondary antibody, biotin-conjugated mouse anti-human IgA1/IgA2 monoclonal antibody (BD Pharmingen 555884, Franklin Lakes, NJ), to a concentration of 200 ng/ml and use of 1-step Ultra TMB-ELISA (Pierce 34028, Rockford, IL) for 3 minutes prior to addition of 1.8 M H₂SO₄ to stop the enzymatic color development. The plates were read at 450 nm in a plate reader. Fecal sIgA concentrations were determined using serially diluted purified human IgA isolated from colostrum (Sigma I-1010, St. Louis, MO) to generate a standard curve. Results are expressed as change from baseline (mg/sIgA per gram feces).

Anti-poliovirus- and Anti-rotavirus-Specific IgA

Measurements of anti-poliovirus-specific IgA and anti-rotavirus-specific IgA from fecal samples collected prior to vaccination (V1) and following vaccination (V2) were conducted in a similar manner as the sIgA protocol with modifications in the original coating of the plate, preparation of the standard curve, sample dilutions and incubation with 1-Step Ultra TMB.

Plates were coated with 1:100 dilutions of either IPOL vaccine or oral Rotateq vaccine in a 20mM NaHCO₃ coating buffer (pH 9.5) for poliovirus-specific or rotavirus-specific IgA, respectively (Mullie et al., 2004). As standards are not commercially available for either anti-poliovirus- or anti-rotavirus-specific IgA, the respective standard curves were generated by pooling separately all V2 fecal samples from infants receiving the polio vaccine and/or the rotavirus vaccine to create a relative measurement of antibodies present. The total pooled samples used to relatively quantify the amount of anti-poliovirus or anti-rotavirus-specific IgA were serially diluted and included on each plate. Fecal homogenates were diluted between 10 and 200 times. Incubation time with 1-Step Ultra TMB (3,3',5,5'-tetramethylbenzidine) was increased to 30 minutes at room temperature before stopping the enzymatic color reaction with stop solution. Anti-polio-specific and anti-rotavirus-specific IgA concentrations were determined from their respective standard curves and expressed as change in units per gram feces from V1 to V2 as all vaccinations took place after collection of the V1 sample.

Fecal Calprotectin, Lactate, and pH

Fecal calprotectin and lactate were measured using commercial kits, PhiCal Fecal Calprotectin Immunoassay (Genova Diagnostic, Asheville, NC) and Lactate Assay Kit (BioVision K607, San Francisco, CA), respectively. Fecal pH value was immediately obtained upon thawing sample by submerging the pH probe (Futura Refillable Probe, Beckman Instrument, Inc., Fullerton, California) into the fecal matter and waiting until the pH meter (UP-5, Denver Instrument, Denver, Colorado) reading stabilized.

Stool DNA Extraction and Quantitative Polymerase Chain Reaction of Bb12

DNA from stool samples was isolated using the QIamp DNA stool mini kit (Qiagen, Valencia, CA) with minor modifications previously described by Solano-Aguilar and colleagues

(Solano-Aguilar et al., 2008). Briefly, 1.4 ml of lysis buffer was added to 180-220 mg of feces followed by immediate mixing for one minute. Homogenate was then incubated at 95°C for 5 minutes to facilitate increased DNA yield, followed by removal of inhibitors and proteinase K digestion. DNA was then bound to a column, washed, and eluted.

The amount of Bb12 copy numbers in fecal samples was determined using a primer-probe set specific for the *tuf* gene as previously described by Solano-Aguilar and colleagues, 2008.(Solano-Aguilar et al., 2008) Primers and probes were purchased from Applied Biosystems (ABI, Foster City, CA) as follows: *tuf* Forward GTG TCG AGC GCG GCA A; Reverse CTC GCA CTC ATC CAT CTG CTT; BGB probe ATC AAC ACG AAC GTC GAG A. Commercially-available Taqman Gene Expression assays (Applied Biosystems, Carlsbad, CA) supplied minor groove binding probes and primers for amplification and analysis of the *tuf* gene of Bb12.

Dr. Gloria Solano-Aguilar (The Henry A. Wallace Beltsville Agricultural Research Center, Beltsville, MD) generously donated a genomic *tuf* gene fraction (136.52 nmoles/L; 8.21×10^{10} copies/ μ l) for use to generate the standard curve. The size of the fragment (120 bp) and its molecular mass were quantitatively determined on the DNAChip (Agilent, Waldbronn, Germany) using Bioanalyzer (Agilent Technologies, Wilmington, DE).

Bb12 *tuf* gene abundance was measured with a Taqman ABI 7900 (Applied Biosystems). Data were analyzed with ABI Sequence Detection System software (Version 2.2.1, Applied Biosystems) using standard curve methodology. In this method, the threshold cycles from the Bb12 *tuf* gene were compared with a standard curve generated from a series of dilutions of the *tuf* gene standard. The calculated concentration of the Bb12 copy numbers was normalized to

fecal mass (mg). Each sample was assayed in triplicate. Both positive (DNA extracted from the PRO formula) and negative controls were included on each plate.

Statistical Analysis

The statistical power analysis and the number of study subjects were based on the primary outcome, level of fecal sIgA, to investigate whether infants consuming infant formula containing Bb12 have a higher level of fecal sIgA compared to infants receiving the control formula. The number of infants was determined by having sufficient statistical power to test the differences in changes in fecal IgA from baseline between the probiotic formula and the control formula, using two- tailed t tests at a level of significance of $p=0.05$, i.e., Type I error = 0.05.

Fukushima and colleagues found that the mean fecal sIgA at baseline was 1.49 ± 0.32 mg/g and rose to 4.29 ± 1.09 mg/g after 8 days of feeding with probiotic formula.(Fukushima et al., 1998) We assumed that infants fed with control formula would not experience a change in level of fecal IgA from baseline values to values at 8 days of follow-up. Therefore, we calculated the sample size per group for a statistical power of 0.80 for detecting a difference of 2.8 mg/g or greater. The standard deviation of the fecal IgA was 0.8 mg/g at baseline in Fukushima's study and increased to 2.88 mg/g after 8 days of exposure to probiotic-containing formula. To detect a group difference in changes from baseline, the sample size necessary to achieve a power of 0.80 was at least 36 infants per group. As such, 90 subjects (45 for each formula group) were recruited to compensate for an anticipated attrition of 20% over the study period. A separate group of exclusively breastfed infants were included as a reference. In anticipation of a similar attrition in this group, 45 breastfed infants were recruited in the expectation that 36 of them would complete the study.

Data were analyzed using SAS (Version 9.1; SAS Institute). The generalized linear model included main effects for treatment and delivery and an interaction of treatment and delivery. Treatment means were compared using Student's t-test. Significance of difference was set at $P < 0.05$. Change in fecal SIgA from baseline was assessed using per protocol and intent-to-treat approaches. All other analyses were performed only on subjects completing the study per protocol. Histograms and q-q plots were used to visually assess normality, log transformations were utilized as needed. Values reported are means $\pm$ standard error of the mean (SEM).

The secondary outcomes of gastrointestinal tolerance, weight gain, and adverse events were analyzed according to per protocol (PP) and intention-to-treat (ITT) designs. To compare the differences in tolerance data and body weight data between the two formula groups and a breastfed group, a general linear model analysis was performed followed by a Tukey-Kramer procedure to control the overall type 1 error rate (Tukey, 1953; Kramer, 1956). Mixed effects models were applied to serial values at baseline (V0), 2 weeks of feeding (V1), and 6 weeks of feeding (V2). The analysis was performed with route of delivery and study center as covariates.

RESULTS

Of 172 recruited infants, 21 FF infants and 6 breastfed infants did not complete the baseline visit at 6 weeks of age (**Figure 3.1**). Infants included in the intent to treat analysis included fifty-two infants whose mothers selected to exclusively breastfed (39 vaginally delivered and 13 by cesarean-section), and 93 FF infants were randomized to CON (43 subjects; 27 vaginally delivered and 16 by cesarean-section) or PRO (50 subjects; 34 vaginally delivered and 16 by cesarean-section). Forty breastfed and 75 FF infants completed the study per protocol

(PP) with 41 randomized to PRO (25 vaginally-delivered and 16 by cesarean-section) and 34 to CON (19 vaginally-delivered and 15 by cesarean-section). Nine subjects in each of the FF groups (18 total; 5 drop-out before study completion; 4 lost-to-follow-up; 6 received non-study formula; 1 received cereal; 1 investigator withdrew subject; 1 study formula unrelated serious adverse event (hospitalization for respiratory syncytial virus)) and 12 breastfed infants (6 not exclusively breastfed; 3 incorrect timing of stool collection; 1 false inclusion; 2 drop-out before study completion) were excluded from the per protocol analysis due to study withdrawal or non-compliance.

Measures of birth weight, length, gestational age, or route of delivery did not differ between feeding groups at baseline. These data as well as subjects' pre-study feeding regimen are presented in **Table 3.1**. The average formula intake for infants assigned to the PRO was 24 ounces per day (1.0×10^8 CFU Bb12) at 8 weeks of age and 25.6 ounces (1.1×10^8 CFU Bb12) per day at 12 weeks of age. The average formula intake for infants assigned to the CON formula was 23 ounces per day at 8 weeks of age and 23.8 ounces per day at 12 weeks of age. There was no difference in intake between PRO and CON ($p=0.2829$). Breastfed infants were fed an average of 7 times per day.

Stool samples from 145 infants were analyzed as intent-to-treat (ITT; primary outcome only) while stool samples from 115 infants were included in PP analysis. Tolerance outcomes and growth are reported for all recruited subjects on an ITT basis. Data on fecal samples are reported on ITT and per protocol subjects for fecal sIgA and PP infants only for all other outcomes assessed on stool samples.

Fecal Bb12 abundance was assessed by quantifying *tuf* gene copies and was found to be present in 93% and 88% of PRO fecal samples at V1 and V2, respectively. There was no

difference ($p=0.12$) in Bb12 *tuf* gene copy numbers in PRO (26.6 ± 15.0), CON (1.5 ± 1.4), or BF (50.1 ± 22.3) samples at baseline, prior to study formula initiation (**Figure 3.2**). As expected, Bb12 *tuf* gene copy number abundance was significantly greater ($p=0.0095$) in PRO vs CON at V1 (3290 ± 1210 vs. 26 ± 11 respectively) and V2 (5105 ± 1909 vs. 39 ± 18 respectively), thereby validating compliance regarding assigned formula intake within the study population. Breast fed infants were found to have 231.9 ± 46.3 Bb12 *tuf* gene copy numbers at V1 and 203.7 ± 63.3 at V2.

Breast fed infants had significantly higher ($p<0.001$) concentrations of fecal sIgA in stool samples as compared to FF infants at all time points (**Figure 3.3**). Vaginally-delivered PRO infants had increased ($p=0.046$; 1.053 ± 1.017 mg/g) fecal sIgA concentration from baseline to 6 weeks of feeding (V0 to V2) compared to CON (-1.809 ± 1.341 mg/g change from baseline) in the ITT groups (**Figure 3.4a**). Additionally, the change from V0 to V1 also tended ($p=0.063$) to be significant in PRO ITT-vaginally delivered infants ($1.77 + 1.52$ mg/g change from baseline) as compared to CON ($-1.82 + 1.9$ mg/g change from baseline) as shown in **Figure 3.4a**. Fecal sIgA concentration was not affected by formula composition in the cesarean-delivered ITT (**Figure 3.4b**).

Within per protocol (PP) infants, PP vaginally-delivered PRO infants demonstrated a trend toward increased fecal sIgA from baseline to visit 2 similar to the ITT-vaginally-delivered infants ($p=0.052$; PRO: 1.80 ± 1.9 mg/g change from baseline; CON: -2.58 ± 1.83 mg/g change from baseline). There was no difference in fecal sIgA in PP-cesarean-delivered infants, data not shown.

In cesarean-delivered infants, PRO (54.9 ± 30.0 U/g change) tended to increase ($p=0.056$) anti-rotavirus-specific IgA concentrations from V1 to V2, however no change was

observed with CON (11.5 ± 17.0 U/g change) (**Figure 3.5**). There was also a trend ($p=0.07$) for probiotic supplementation to increase anti-rotavirus-specific IgA concentration from V1 to V2 when all three groups were analyzed without stratification by delivery method (PRO: 20.2 ± 20.5 U/g change; CON: 6.0 ± 23.1 U/g change; BF: -59.1 ± 31.6 U/g change).

From V1 to V2, the increase in anti-poliovirus-specific IgA concentration was higher in all PRO (48.2 ± 40.2 U/g change) versus CON (-18.0 ± 20.0 U/g change) stool samples ($p=0.031$), including those from cesarean-delivered infants ($p=0.026$; PRO: 51.6 ± 26.2 U/g change; CON: 19.4 ± 19 U/g change) as shown in **Figure 3.6**. A similar trend ($p=0.10$) in poliovirus-specific IgA concentration is noted in vaginally-delivered infants consuming PRO; however, statistical significance was not achieved due to high variability (PRO: 45.9 ± 66.6 U/g change; CON: -50.7 ± 31.8 U/g change) (**Figure 3.6**). Again, there was also a trend ($p=0.075$) for probiotic supplementation to increase anti-poliovirus-specific IgA concentration from V1 to V2 when all three groups were analyzed without stratification by delivery method (PRO: 48.2 ± 40.2 U/g change; CON: -18.0 ± 20.0 U/g change; BF: -16.5 ± 20.3 U/g change).

Fecal calprotectin concentration increased from V0 to V1 in the all PRO group ($P=0.043$) as compared to CON (**Figure 3.7**). This same difference was also seen in infants delivered by cesarean section ($p=0.039$) but not by those delivered vaginally ($p=0.21$). At V2 there was no difference ($p=0.16$) between PRO (177.9 ± 34.3 $\mu\text{g/g}$ feces) and CON (149.9 ± 15.3 $\mu\text{g/g}$ feces) infants fecal calprotectin levels regardless of delivery method. The change in fecal calprotectin from V0 to V1 and V2 to V0 did not differ between BF and FF (data not shown). However, BF infants did have significantly higher calprotectin levels than CON and PRO infants at V0 ($p=0.0002$; BF: 307.8 ± 34.9 $\mu\text{g/g}$; CON: 195.2 ± 19.4 ; PRO: 165.6 ± 19.9), V1 ($p=0.028$; BF:

260.3 $\pm$ 36.7 μ g/g; CON: 153.9 $\pm$ 17.2; PRO: 202.3 $\pm$ 29.3), and V2 (p=0.0024 BF: 319.2 $\pm$ 52.3 μ g/g; CON: 149.9 $\pm$ 15.2; PRO: 177.9 $\pm$ 34.3) as shown in **Figure 3.7**.

Fecal pH was not affected by treatment. However, infants in the breastfed reference group had significantly lower fecal pH at all time points as shown in **Figure 3.8** (V0 p=0.0155; PRO: 6.5 $\pm$ 0.09; CON: 6.5 $\pm$ 0.1; BF: 6.1 $\pm$ 0.1; V1 p=0.0135; PRO: 6.4 $\pm$ 0.07; CON: 6.4 $\pm$ 0.1; BF: 6.1 $\pm$ 0.1; V2 p=0.0255; PRO: 6.2 $\pm$ 0.1; CON: 6.3 $\pm$ 0.08; BF: 5.9 $\pm$ 0.1.

Additionally, there were not differences between formula groups when stratified by delivery method.

Lactate concentration (**Figure 3.9**) was found to be significantly higher (p=0.013) in BF infants as compared to FF infants at V1 (PRO 4.7 $\pm$ 0.9; CON: 3.5 $\pm$ 0.6; BF: 8.4 $\pm$ 1.8). When stratified for delivery method, vaginally delivered PRO infants had decreased (p=0.033) lactate concentrations from V0 to V2 (-0.40 $\pm$ 0.1) as compared to CON (0.23 $\pm$ 0.2). The opposite trend (p=0.086) was seen in cesarean-delivered PRO infants, where lactate concentrations increased from V0 to V2 PRO (0.02 $\pm$ 0.2) as compared to CON (-0.62 $\pm$ 0.9). Lactate concentrations did not differ between formula groups when left unstratified for delivery.

There were no differences in stool frequency, color, or consistency between the two formula groups. The breastfed group had a significantly greater average daily number of bowel movements (3.4 per day at V1 and 2.4 per day at V2) than each of the two formula groups (1.3 to 1.4 per day at V1 and V2). Breastfed infants had yellow stools 85% of the time, and these proportions were significantly higher than in either of the two formula groups (18.5% in PRO versus 9% in CON). Approximately 84% of the time, infants assigned to formula had soft stool which was significantly higher than breastfed infants. However, breastfed infants had significantly higher proportions of liquid stool than either formula group (p<0.0001). There

were no differences among the groups in gas, spit-up, or vomiting. Mean weight percentiles generated from the World Health Organization growth charts did not differ among PRE, CON, and BF. Breastfed infant girls had significantly higher weight percentiles than infant girls fed with either formula.

DISCUSSION

B. lactis was detected within the collected stool samples following ingestion of the probiotic (*B. lactis*) containing study formula. The use of a primer-probe specific for the *tuf* gene region allowed for highly sensitive and specific quantification of *B. lactis*. The qPCR assay has been shown to have sensitivity to 10 copies of the *tuf* gene and specificity adequate to differentiate between even the most closely related *B. animalis* subspecies *animalis* as well as *B. breve*, *B. longum*, *B. suis* (Solano-Aguilar et al., 2008). Our results demonstrated that Bb12 was present in significantly greater levels in PRO infants as compared to CON and BF. Although Bb12 was detected in the majority of fecal samples after 2 weeks (88%) and 6 weeks (93%) of probiotics feeding it was not detected in 100% of the fecal samples. This likely relates to insufficient *tuf* gene copy numbers present in the tested fecal sample when quantities were limited. Additionally, it is possible that unreactive samples were related to non-compliant use of study formula.

Secretory IgA (sIgA) is a predominant mucosal immunoglobulin and plays an integral role in protection from antigens, toxins, and potential pathogens (Forchielli and Walker, 2005). Fecal levels of sIgA are a good measure of levels of sIgA available in the colon and can be assessed non-invasively. Not surprisingly, we observed a significantly higher concentration of total fecal sIgA in breast fed infants than formula fed infants due to the exogenous contribution

of sIgA through the mother's breast milk. Within the formula fed groups, we found that the probiotic formula containing Bb12 significantly increased fecal sIgA from baseline in vaginally delivered PRO infants by ITT analysis after 6 weeks of feeding and had a very strong trend toward increasing sIgA from baseline in vaginally delivered PRO per protocol infants after 6 weeks of feeding. No treatment effect of *B. lactis* was found on sIgA levels in cesarean-section delivered infants or when treatment groups were left unstratified for delivery method. A potential explanation for the treatment effect within vaginally delivered infants PRO infants and not in cesarean delivered PRO infants may be related to the composition of the infants' microbiota. Microbiota varies based on delivery method, and it is well established that infants born by vaginal delivery have higher proportion of bifidobacteria than their cesarean delivered counterparts (Yoshioka et al., 1983; Harmsen et al., 2000; Penders et al., 2006; Biasucci et al., 2008; Huurre et al., 2008). Additionally, studies have found that bifidobacteria stimulate the production of sIgA (Fukushima et al., 1998; Mullie et al., 2004; Rautava et al., 2006). Thus, the addition of *B. lactis* to an already bifidobacteria dominant microbiota in vaginally delivered infants may have enhanced the effects of the presence of the probiotic and thus resulted in larger change from baseline in secretory IgA during the treatment period.

Alternatively, the addition of *B. lactis* may have contributed to further enhancement of bifidobacteria as was the case in preterm infants supplemented with *B. lactis* from the first day of birth until 21 days where a trend was found between bifidobacteria cell counts and fecal sIgA ($r=0.344$, $p=0.08$) (Mohan et al., 2006). As these effects were only seen in vaginally delivered infants and not cesarean delivered infants, inadequate power may have been an issue within the smaller subsample of infants (14 infants in both PRO and CON in both the ITT and PP groups) stratified for cesarean section delivery. As discussed above, this trial was powered to detect a

change in sIgA regardless of delivery method. The effects of *B. lactis* supplementation on fecal SIgA in infants have only been investigated in a limited number of trials to date. *B. lactis* supplementation in healthy children and preterm infants has been shown to enhance fecal SIgA (Fukushima et al., 1998; Mohan et al., 2008). Bakker-Zierikzee and colleagues investigated Bb12 supplementation in healthy full-term infants; however, they did not detect a significant effect of Bb12 supplementation on fecal SIgA levels. Our differing results may be due to inadequate power of the Bakker-Zierikzee and colleagues' study as the post-hoc power analysis suggested a five-fold increase in each group was needed to reach adequate sensitivity (Bakker-Zierikzee et al., 2006). Additionally, statistical analysis on data in the Bakker-Zierikzee trial was done only on infants comprising the 10th to 90th percentile due to high variation between subjects instead of utilizing logarithmic transformations to normalize data, and delivery method was not accounted for in their statistical modeling. In the present study, log transformations were utilized to normalize data due to high variation, and analysis was stratified by delivery method thus allowing for detection of the significant increase in fecal SIgA from baseline in PRO infants that were vaginally delivered.

Similar to our findings, *Bifidobacterium longum-infantis* and *Bifidobacterium breve* have been shown to increase fecal SIgA and anti-poliovirus-specific IgA in healthy full term infants after 4 months of feeding (Mullie et al., 2004). To the best of our knowledge, this is the first trial to investigate the immune modulating effects of *B. lactis*, or any probiotic, on fecal anti-rotavirus or anti-polio-specific IgA in healthy full term infants. Few studies have investigated immune effects related to the interaction of infant delivery method and probiotic feeding. One trial found that probiotic supplementation consisting of 4 bacteria strains (*Lactobacillus rhamnosus* GG, *L. rhamnosus* LC705, *Bifidobacterium breve* Bb99, and *Propionibacterium freudenreichii* ssp.

Shermanii) provided protection from IgE-associated allergy in caesarean delivery children until age 5 while those born by vaginal delivery were not afforded the same protective effects (Kuitunen et al., 2009). Our results similarly support enhanced protective effects of probiotics in infants born by caesarean delivery. As our study contained *B. lactis* and the other two trials each included *Bifidobacteria* spp. it is possible that the microbiota's of the infants in all three trials were afforded immunological benefits associated with an enriched *Bifidobacterium* population as an effect of the respective bifidobacteria added as probiotics. As discussed above, it is well established that the dominant bacteria within vaginally delivered infants is bifidobacteria (Yoshioka et al., 1983; Harmsen et al., 2000; Penders et al., 2006; Biasucci et al., 2008; Huurre et al., 2008). As shown in our trial and the trial by Kuitunen and colleagues, further immunological benefit may be gained by a *Bifidobacterium* probiotic to cesarean delivered infants as it may be contributing to a shift the commensal bacterial populations to be more similar to the vaginally-delivered infant (Kuitunen et al., 2009).

At birth, the immune system of infants is skewed toward T-helper (Th)-2 responses (Prescott et al., 1998). According to the “hygiene hypothesis”, exposure to bacteria is important in changing the immune response to be more skewed toward Th-1 responses, which in turn has been linked to reducing propensity to allergic diseases (Yazdanbakhsh et al., 2002; Bufford and Gern, 2005; Rook and Brunet, 2005; Weng and Walker, 2006). As infants born by cesarean section have been shown to have increased incidences of allergic diseases such as allergic rhinoconjunctivitis and asthma one could speculate that this correlation may also be related to the hygiene hypothesis (Renz-Polster et al., 2005). By extension, the sterile delivery methods of cesarean section delivery may provide a lesser initial exposure to bacteria and hinder the infant's progression toward a more skewed Th1 response. In the present study, cesarean delivered PRO

infants saw a significant increase in both anti-poliovirus-specific and anti-rotavirus-specific fecal IgA after 6 weeks of feeding. Thus, it is possible that Bb12 provided immune-modulating bacterial exposure that was more pronounced in the cesarean-delivered infants. Additionally, all per protocol PRO infants experienced an increase in anti-poliovirus-specific fecal IgA suggesting that the addition of Bb12 to infant formula may be helpful in mitigating some of the negative immune-related effects associated with formula feeding.

Calprotectin, a zinc and calcium binding protein which constitutes over half of the soluble cytosolic protein within human neutrophil granulocytes, is a non-invasive marker of gastrointestinal inflammation (Steinbakk et al., 1990; Carroll et al., 2003; Kapel et al., 2010). Calprotectin has been measured in a number of studies to evaluate inflammation and immune status of the gastrointestinal tracts of infants (Golden et al., 2002; Olafsdottir et al., 2002; Rugtveit and Fagerhol, 2002; Carroll et al., 2003; Campeotto et al., 2004; Mohan et al., 2006; Dorosko et al., 2008; Rhoads et al., 2009; Calabrese et al., 2010; Kapel et al., 2010). Existing literature reports conflicting data regarding fecal calprotectin in breastfed and formula-fed infants. Our findings are in agreement with some published studies in full term infants monitored during the pre-weaning period showing calprotectin concentrations were significantly higher in BF infants as compared to FF infants (Dorosko et al., 2008; Calabrese et al., 2010). Few studies have evaluated the effects of probiotic supplementation on calprotectin levels (Mohan et al., 2008; Kukkonen et al., 2010). One study in healthy full-term infants assessed the effects of a probiotic capsule containing 4 bacteria strains (*Lactobacillus rhamnosus* GG, *L. rhamnosus* LC705, *Bifidobacterium breve* Bb99, and *Propionibacterium freudenreichii* ssp. *Shermanii*) on calprotectin and fecal SIgA levels at 3 and 6 months of age. This trial found a positive correlation between fecal SIgA and calprotectin and reported a correlation between

higher fecal SIgA levels and lower IgE associated atopic diseases at 2 yrs of age (Kukkonen et al., 2010). Kukkonen and colleagues concluded from this trial that these findings were indicative of immune maturation with associated reduction in risk for atopic diseases during the first two years of life. Further, they proposed that this slight immune activation may be a potential mechanism by which probiotics stimulate immune maturation. Another trial which investigated the effect of probiotic feeding (*B. lactis*) on preterm infant fecal calprotectin levels found the opposite effect in that calprotectin levels were attenuated in the infants consuming Bb12. Our results suggest that the effect of probiotic supplementation varies over time, with an initial increase in calprotectin levels followed by a decrease to levels of non-supplemented infants by 6 weeks of feeding. The results of our trial and that of Kukkonen and colleagues support the hypothesis that *B. lactis* aids in priming of the gastrointestinal tract of full term infants to enhance maturation and attenuate the inflammatory responses to bacterial and dietary antigens. Clearly, more research is needed to investigate the effects of probiotic supplementation on calprotectin in infancy.

Fecal pH was lower at each time point in BF versus FF infants; however there were no differences between FF groups even when stratified for delivery method. In coordination with the lower pH, fecal lactate concentrations were higher in BF infants as compared to FF infants, but again no effect was seen as a result of the addition of Bb12 to infant formula when infants were not separated by delivery method. When infants within the formula groups were stratified for delivery method, fecal lactate concentrations decreased from baseline to visit 2 (6 weeks of study formula) within the vaginally delivered infants with the opposite trend occurring within the cesarean-delivered infants. As these small changes in fecal lactate concentration were not mirrored by a significant change in fecal pH, the biological relevance of this is likely minimal.

Our results differ from Mohan and colleagues where fecal pH was shown to decrease in preterm infants fed *B. lactis* and the decreased pH was mirrored by an increase in fecal lactate concentration (Mohan et al., 2008). Trials in full-term infants have not investigated the effects of Bb12 supplementation on fecal lactate levels.

CONCLUSIONS

By virtue of containing bioactive substances such as SIgA, microbes and human milk oligosaccharides, breast feeding is superior to formula feeding in maintaining high intestinal levels of protection in infants during the first 3 months of life. Breastfed infants' total fecal SIgA levels decrease over time; however, they remain significantly higher than formula-fed infants at all time points. Infants fed formula supplemented with *B. lactis* had significantly higher levels of *B. lactis* and infants born by vaginal delivery had increased concentrations of SIgA. Additionally, infants born by cesarean-section who received the probiotic formula had enhanced immune response to poliovirus and rotavirus vaccines as evidenced by an increase in anti-rotavirus- and anti-poliovirus-specific IgA. These results demonstrate that negative immune-related effects from absence of breast feeding and cesarean-section delivery may be mitigated by including *Bifidobacterium animalis* subspecies *lactis* *B. lactis* in infant formula and thereby providing infants a safe, dietary, immune-modulating bacterial experience. Anti-viral specific mucosal antibodies in this study were evident after only a single dose of vaccines. This enhancement in antibody responses could be of further clinical significance as related to levels of protection achieved from oral viral vaccines in non-breast fed infants. Further research is needed to investigate the degree and potential for immune-related benefits of probiotics, and their relation to infant microbiota, and mode of delivery.

ACKNOWLEDGEMENTS

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TABLES AND FIGURES

	BF		CON		PRO	
	Vaginally Delivered (n=39)	Delivery by C- section (n=13)	Vaginally Delivered (n=27)	Delivery by C- section (n=16)	Vaginally Delivered (n=34)	Delivery by C- section (n=16)
Birth weight (g)	3355±79	3552±126	3176±82	3379±78	3277±75	3278±149
Birth length (cm)	50.8±0.5	50.4±0.6	50.3±0.6	48.6±0.4	50.3±0.5	49.5±0.5
Gestational age (weeks)	39±0.1	39±0.3	38±0.2	38±0.2	39±0.2	39±0.2
Pre-study feeding	9 subjects received some formula for 1 day	4 subjects received some formula for 1.3±0.3 days	7 subjects received breastmilk for 11.3±4.5 days	6 subjects received breastmilk for 10.3±5.3 days	5 subjects received breastmilk for 13.1±4.9 days	3 subjects received breastmilk for 9.3±4.0 days

Table 3.1. Baseline characteristics of Intention-To-Treat (ITT) Population. BF: breastfed; CON: control infant formula group; PRO: probiotic infant formula group. Data are expressed as means + SEM.

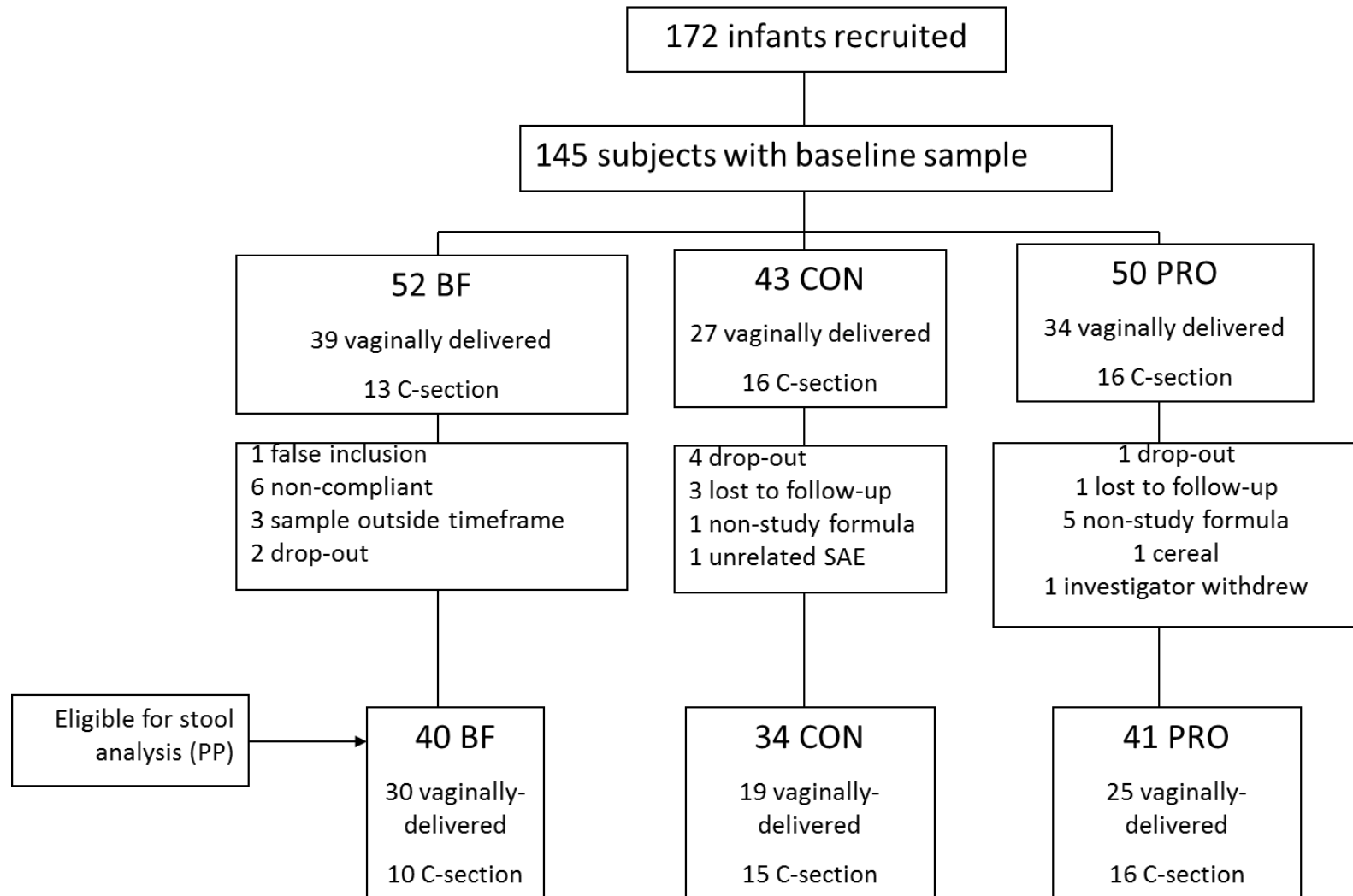


Figure 3.1. Schematic of subject retention and inclusion in stool per protocol analysis (PP). BF: breastfed; CON: control infant formula group; PRO: probiotic infant formula group; SAE: serious adverse event.

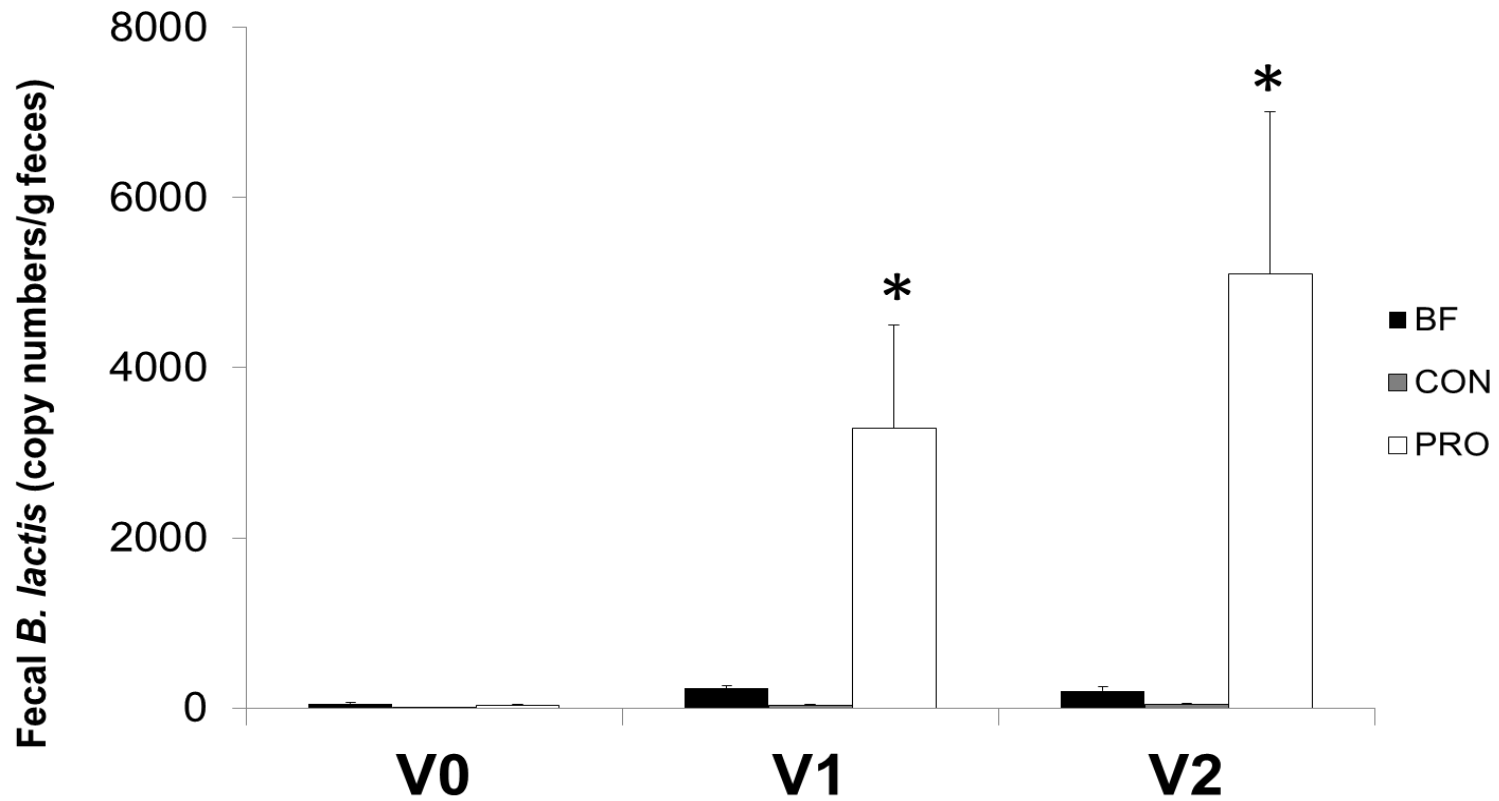


Figure 3.2. Effect of probiotic supplementation on fecal *B. lactis* copy numbers per gram feces in all infants. There was no difference at baseline (V0) in copy numbers between groups ($p=0.12$). Probiotics increase *B. lactis* copy numbers in formula-fed infants after 2 week and 6 weeks of study formula consumption. PRO infants had significantly greater ($p=0.0095$) *B. lactis* at V1 and V2 as compared to CON at V1 and V2. Data are expressed as means + SEM. BF: breast-fed reference group; CON: control infant formula group; PRO: probiotic infant formula group. V0: baseline sample at 6 weeks of age; V1: 8 weeks of age; V2: 12 weeks of age.

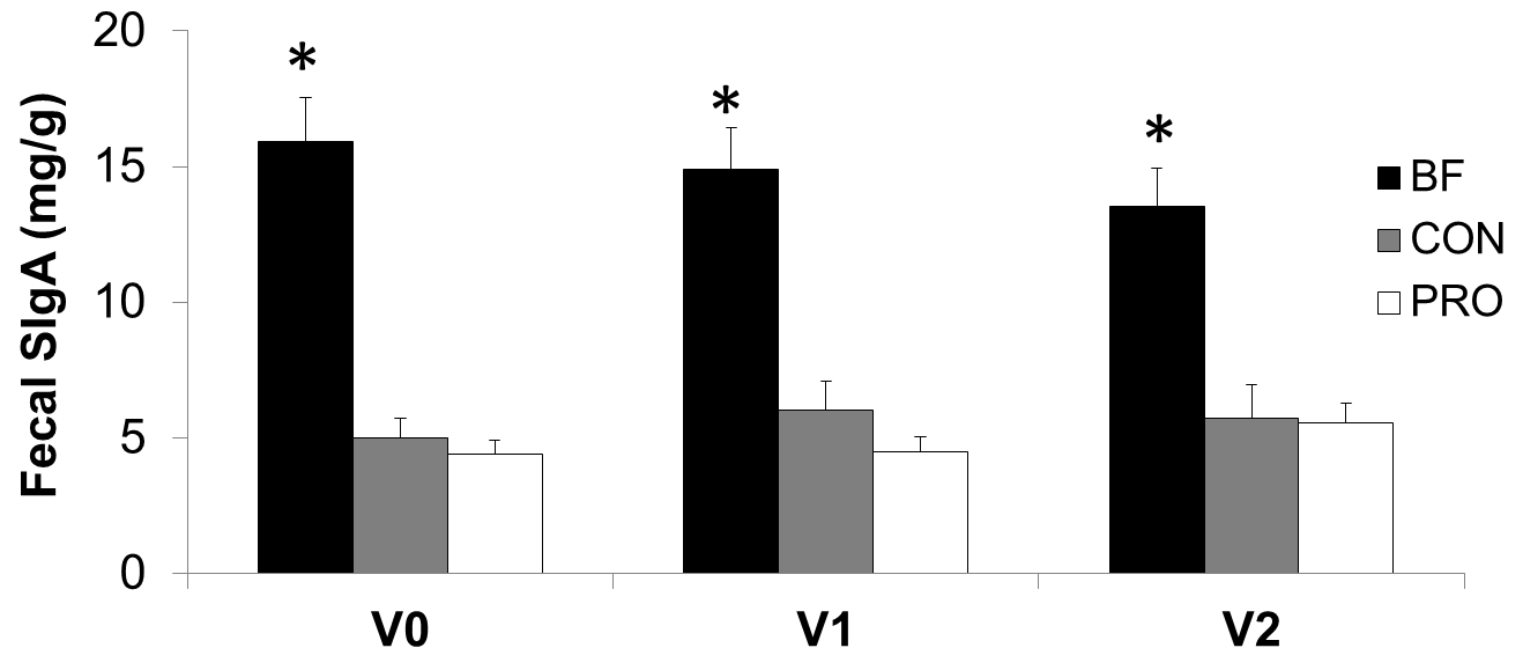


Figure 3.3. Effect of probiotic supplementation on fecal sIgA concentration (in milligram per gram feces) of infants at baseline (V0), after 2 weeks (V1) and after 6 weeks (V2). Breastfed infants have higher fecal sIgA ($p < 0.001$) than formula-fed infants at all time points. Data are expressed as means + SEM. BF: breastfed CON: control infant formula group; PRO: probiotic infant formula group

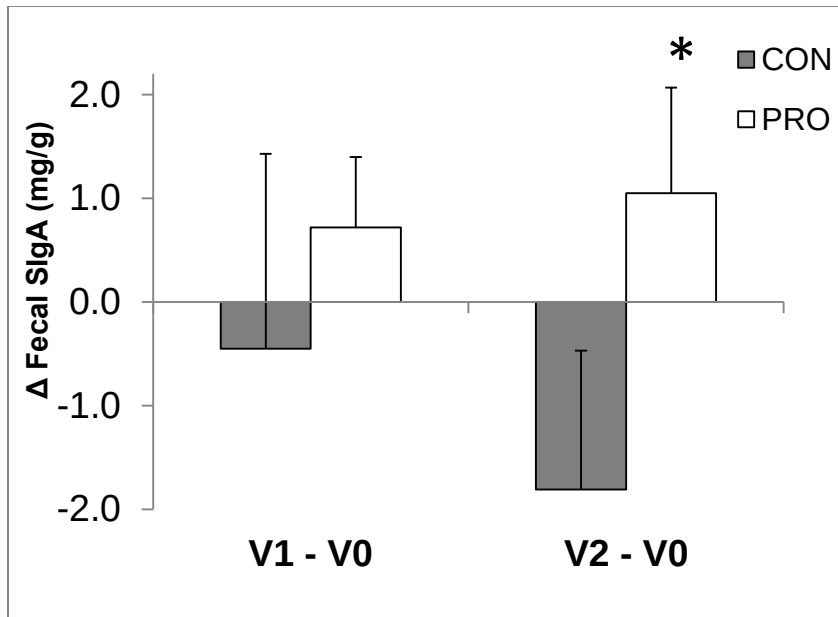


Figure 3.4 a.

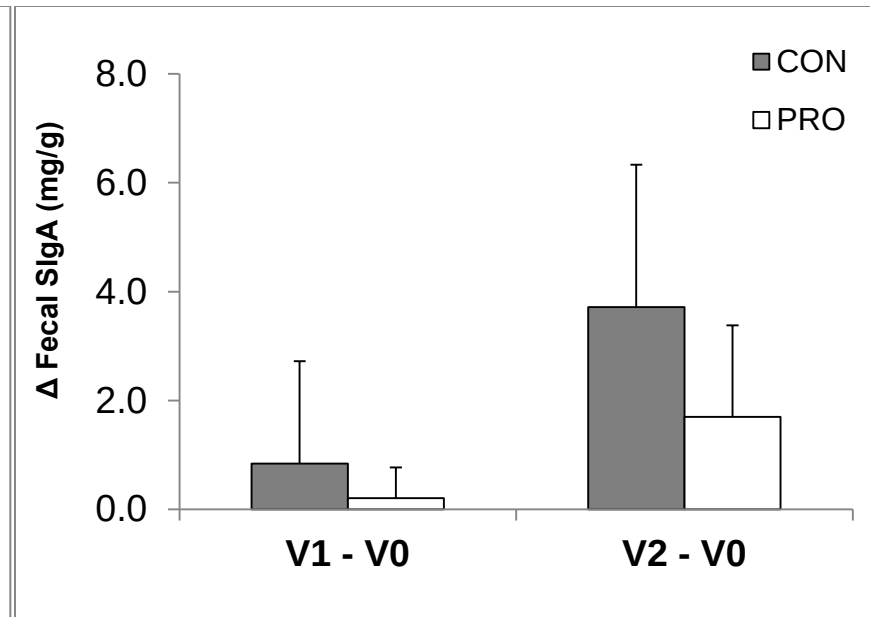


Figure 3.4 b.

Figure 3.4. Within the intent to treat design probiotics increased fecal sIgA in vaginally delivered formula-fed infants after 6 weeks of study formula consumption (3.4a). Data are expressed as means + SEM. CON: control infant formula group; PRO: probiotic infant formula group. Vaginally-delivered PRO infants had a significantly greater ($p=0.046$) increase in fecal sIgA concentration from V0 (baseline at 6 weeks of age) to V2 (12 weeks of age) compared to CON. Bb12 supplementation also tended ($p=0.063$) to enhance the change of fecal sIgA at V1 (8 weeks of age) in PRO ITT-vaginally delivered infants as compared to CON. There was no treatment effect in ITT-cesarean section infants in change from baseline at V1 ($p=0.24$) or V2 ($p=0.26$) (3.4b).

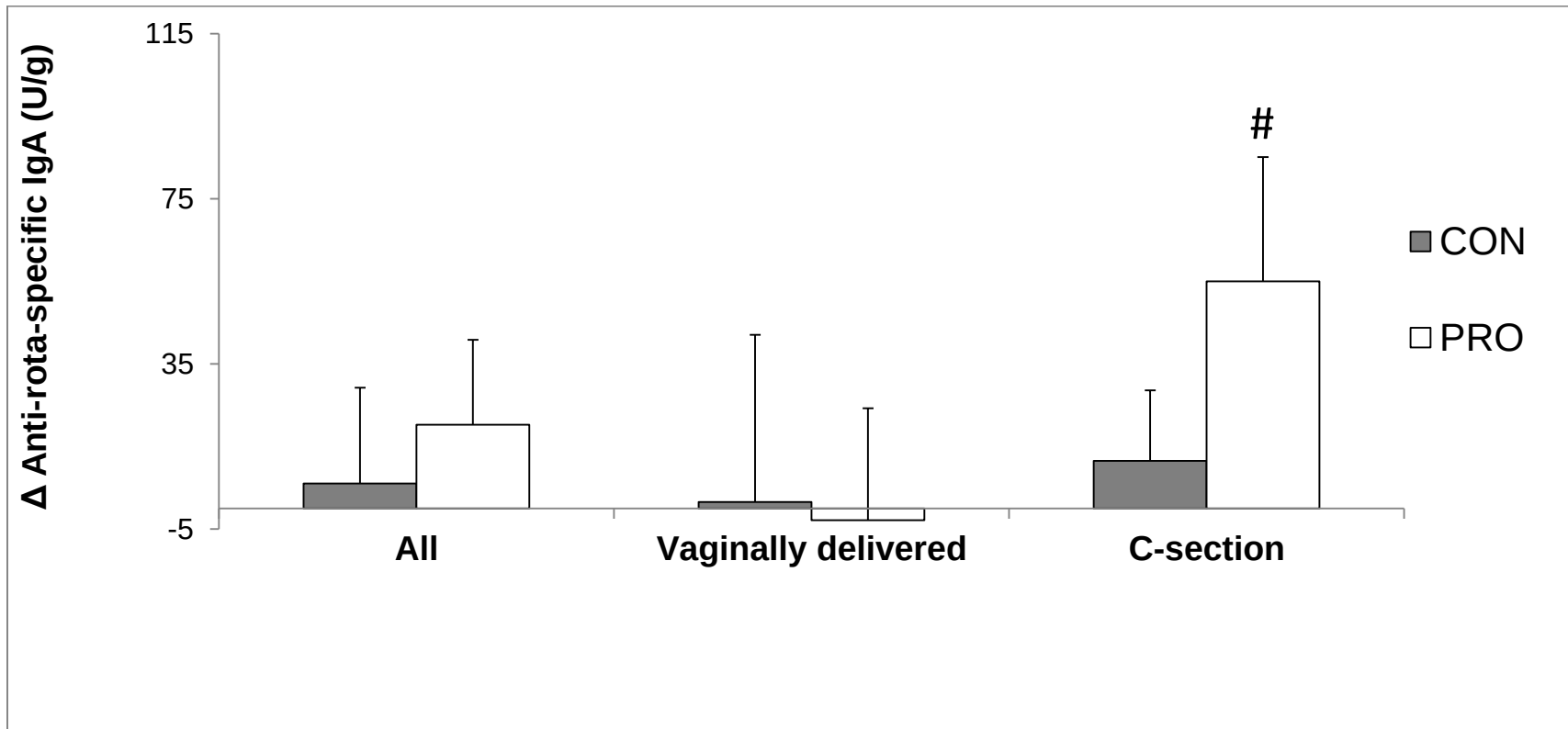


Figure 3.5. Probiotics increase anti-rotavirus-specific IgA in infants delivered by C-section. Data are expressed as means + SEM. CON: control infant formula group; PRO: probiotic infant formula group. PRO infants delivered by cesarean-section had greater ($p=0.056$) changes in anti-rotavirus-specific IgA concentrations from V1 (8 weeks of age prior to receiving vaccination) to V2 (12 weeks of age after receiving vaccination).



Figure 3.6. Fecal anti-poliovirus-specific IgA concentration increased in infants consuming probiotic formula. Data are expressed as means + SEM. CON: control infant formula group; PRO: probiotic infant formula group. From V1 (8 weeks of age prior to receiving vaccination) to V2 (12 weeks of age after receiving vaccination), the increase in anti-poliovirus-specific IgA concentration was higher ($p=0.031$) for PRO vs CON. When looking at infants based on delivery method, PRO infants delivered by C-section had significantly greater ($p=0.026$) changes in fecal anti-poliovirus-specific IgA concentration as compared to CON infants delivered by C-section. A similar numerical increase in change in anti-poliovirus-specific IgA concentration was noted in vaginally-delivered PRO infants as compared to CON infants; however, statistical significance was not achieved due to high variability.

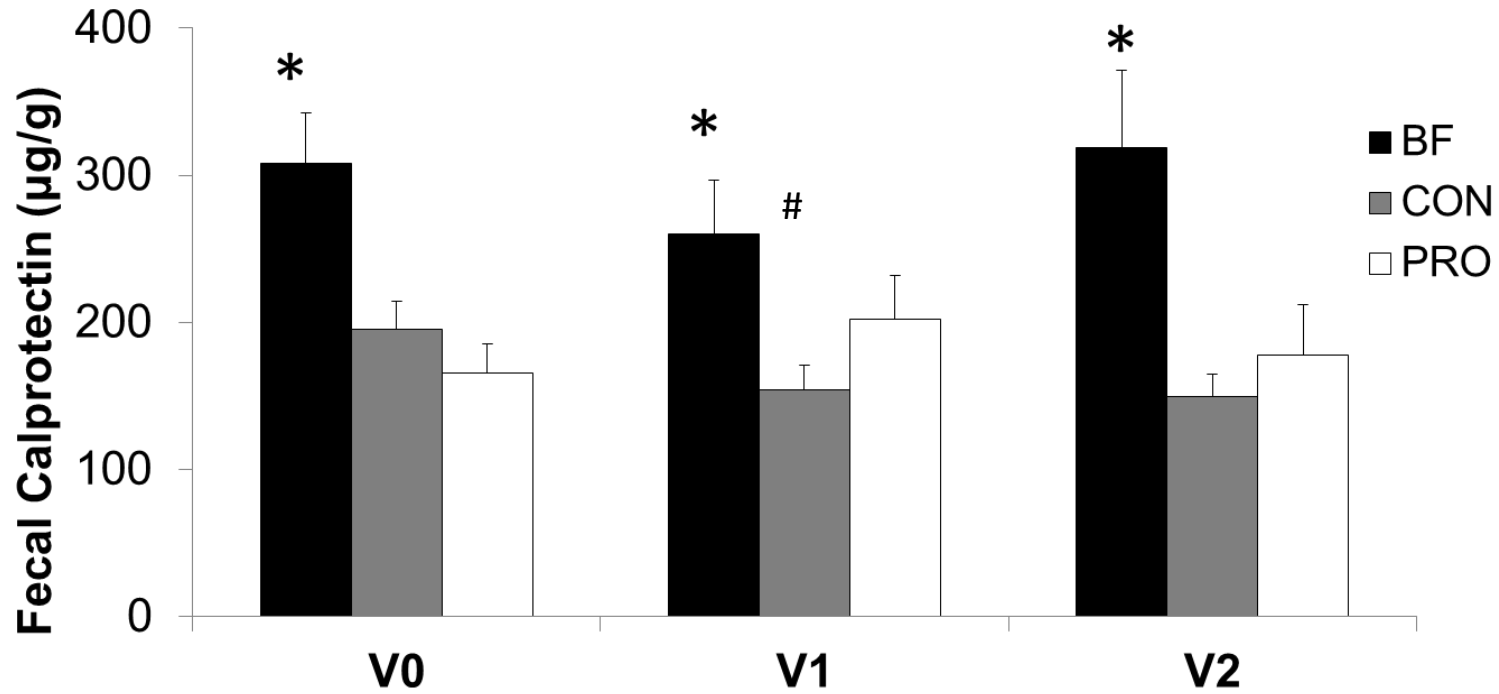


Figure 3.7. Effect of feeding method on fecal calprotectin concentration (in microgram per gram feces) in infants at baseline (V0), after 2 weeks (V1) and after 6 weeks (V2). BF infants did have significantly higher calprotectin levels than CON and PRO infants at V0 ($p=0.0002$), V1 ($p=0.028$), and V2 ($p=0.0024$). Fecal calprotectin concentration also increased from V0 to V1 in PRO ($P=0.043$) vs. CON denoted with the #. Data are expressed as means + SEM. CON: control infant formula group; PRO: probiotic infant formula group.

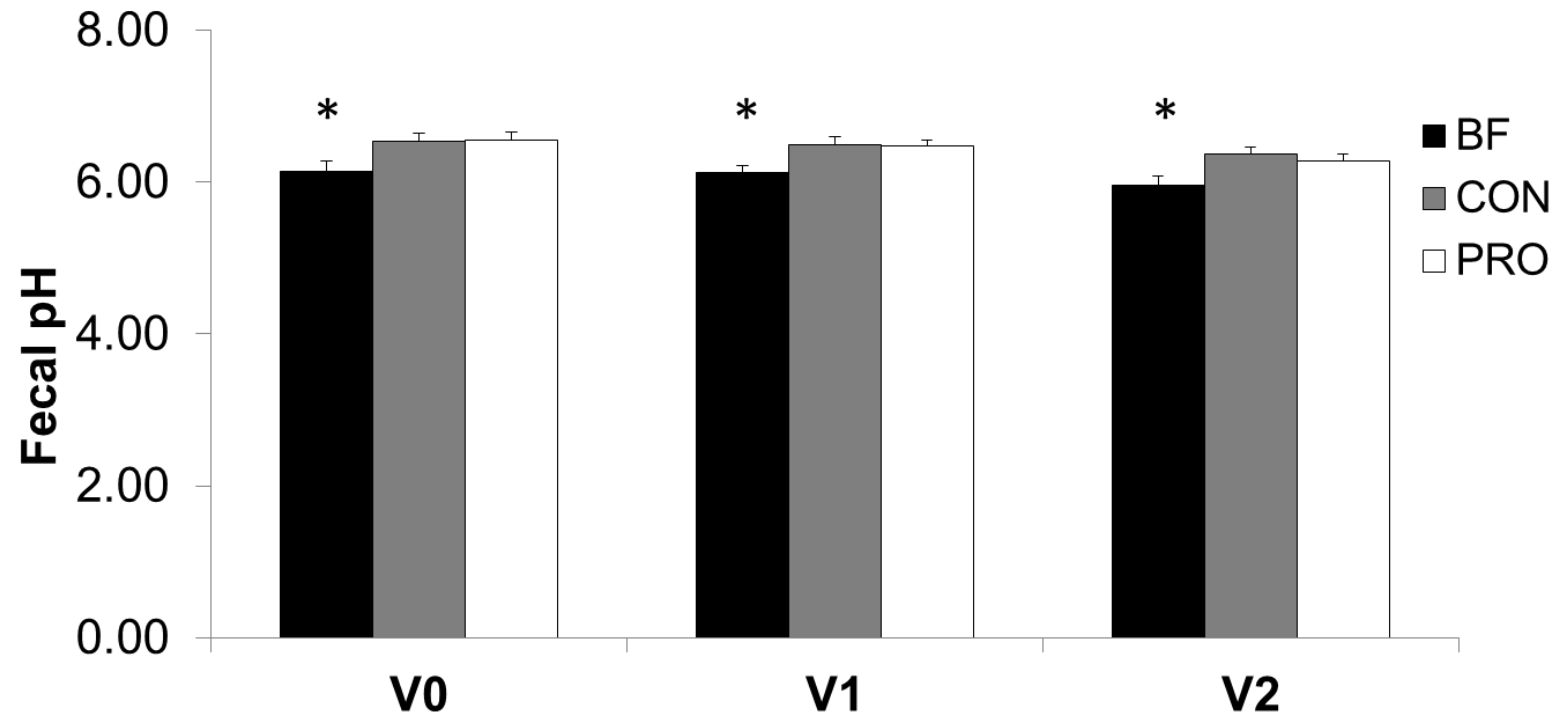


Figure 3.8. Breast fed infants had significantly lower fecal pH at baseline (V0), after 2 weeks (V1) and after 6 weeks (V2) as compared to PRO and CON. Data are expressed as means + SEM. BF: breastfed CON: control infant formula group; PRO: probiotic infant formula group. * $p < 0.05$.

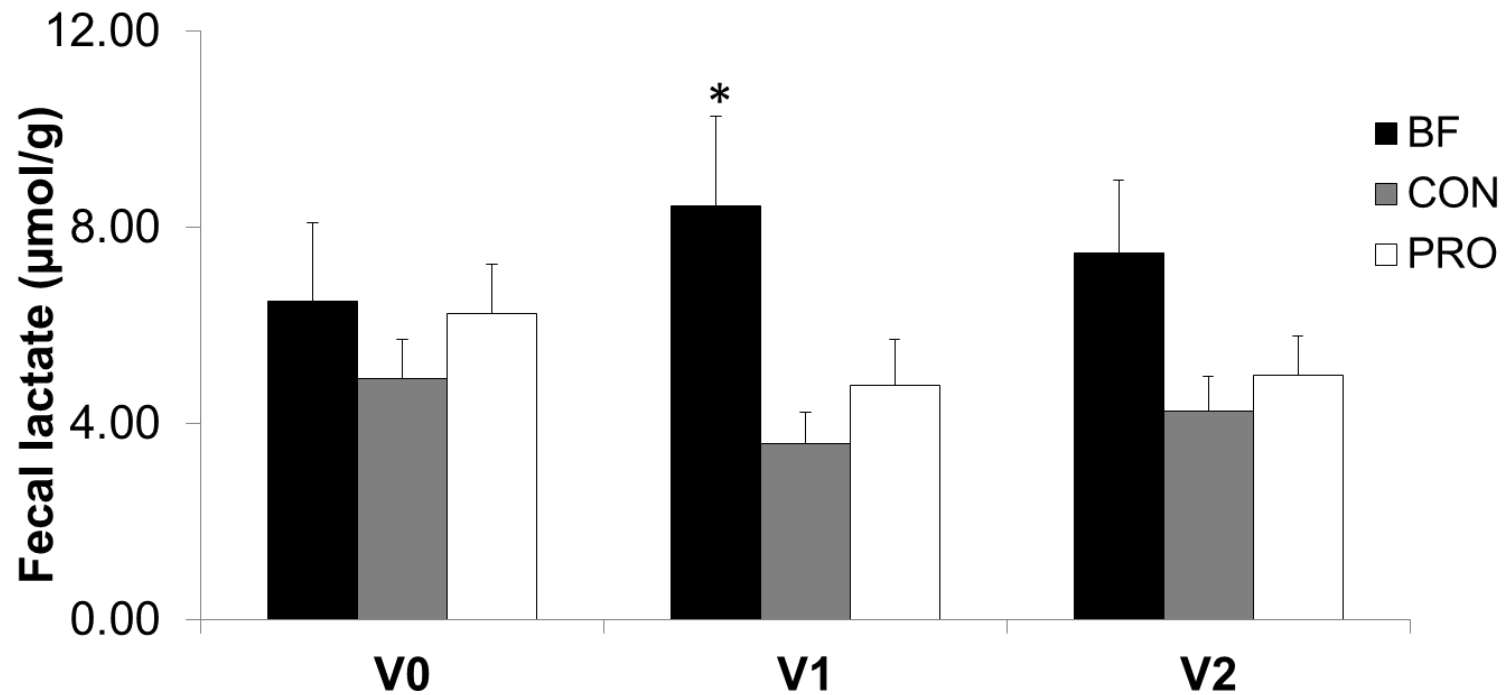


Figure 3.9. Breast fed infants had significantly higher fecal lactate concentration at after 2 weeks (V1) compared to PRO and CON. Data are expressed as means + SEM. BF: breastfed CON: control infant formula group; PRO: probiotic infant formula group. * $p < 0.05$.

CHAPTER 4

EFFECTS OF PREBIOTIC-CONTAINING INFANT FORMULA ON GASTROINTESTINAL TOLERANCE AND FECAL MICROBIOTA IN A RANDOMIZED CONTROLLED TRIAL²

ABSTRACT

Background: Infant formula containing prebiotics may beneficially impact gastrointestinal tolerance and composition of the commensal microbiota. *Objective:* Assess gastrointestinal tolerance and fecal microbiota, pH, and short-chain fatty acid (SCFA) concentrations of infants consuming formula with or without prebiotics. *Design:* Healthy, full-term formula-fed infants were enrolled in a randomized, double-blind, controlled trial with 2 groups studied in parallel to a breastfed comparison group (BF). Formula-fed infants (FF) were randomized to consume a partially hydrolyzed whey formula with (PRE) or without (CON) 4g/L of galacto-oligosaccharides and fructo-oligosaccharides (9:1) for 6 weeks. Fecal bacteria, pH and SCFA were assessed at baseline, 3, and 6 weeks. Caregivers of subjects recorded stool characteristics and behavior for 2 days before the 3- and 6-week visits. *Results:* Feces from infants fed PRE had a higher absolute number ($p=0.0083$) and proportion ($p=0.0219$) of bifidobacteria than feces of CON-fed infants and did not differ from feces of BF. BF had a higher proportion of bifidobacteria than CON ($p=0.0219$) and a lower number of *Clostridium difficile* than FF ($p=0.0087$). Feces from formula-fed infants had higher concentrations of acetate ($p<0.001$),

² The final, definitive version of this paper has been published in the Journal of Parenteral and Enteral Nutrition, 36, Supplement 1, January 2012 by SAGE Publications, Inc., All rights reserved. © 2012 A.S.P.E.N. Holscher, H.D., Faust, K.L., Czerkies, L.A., Litov, R., Ziegler, E.E., Lessin, H., Hatch, T., Sun, S. & Tappenden, K.A. 2012b, "Effects of Prebiotic-Containing Infant Formula on Gastrointestinal Tolerance and Fecal Microbiota in a Randomized Controlled Trial", *Journal of Parenteral and Enteral Nutrition*, vol. 36, no. 1 suppl, pp. 95S-105S. DOI: 10.1177/0148607111430087

butyrate ($p<0.001$), propionate ($p<0.001$) and total SCFAs ($p=0.0230$) than BF; however, fecal pH was lower ($p=0.0161$) in PRE and BF than CON. Prebiotic supplementation did not alter stool patterns, tolerance, or growth. Compared to FF, BF had more frequent stools that were yellow ($p<0.0001$) and more often liquid ($p<0.0001$). *Conclusions:* Infant formula containing the studied oligosaccharides is well-tolerated, increased abundance and proportion of bifidobacteria, and reduced fecal pH in healthy infants.

CLINICAL RELEVANCY STATEMENT

Breast milk, the optimal source for infant nutrition, provides a source of prebiotics in the form of human milk oligosaccharides which in turn partially contribute to the distinct bifidobacteria-dominant microbiota of a breastfed infant as compared to the formula-fed infant. Although the addition of oligosaccharides from human milk is not clinically feasible at this time, the addition of prebiotics in the form of galacto-oligosaccharides and fructo-oligosaccharides to infant formula has been reported to facilitate development of a more desirable microbiota pattern in the formula-fed infant. This study demonstrates that supplementation of infant formula with galacto-oligosaccharides and fructo-oligosaccharides is well-tolerated and modestly increased fecal bifidobacteria and lowered fecal pH.

INTRODUCTION

Breastfeeding promotes the establishment of intestinal microbiota typically characterized by predominance of bifidobacteria. This effect is not seen with infant formulas in routine use. One strategy to improve infant formula is to modify it in ways that can influence the composition and activity of the intestinal commensal microbiota of the formula-fed infant to better reflect that of the breastfed infant. Altering the microbiota of the formula fed infant is thought to provide

benefits related to enhanced mucosal immunity. Studies by Bakker-Zierikzee et al. and Scholtens et al. have demonstrated that supplementation of infant formulas with GOS/FOS is associated with enhanced fecal secretory IgA levels (Bakker-Zierikzee et al., 2005; Scholtens et al., 2008). Secretory IgA is found within breast milk and provides an important benefit of decreased pathogen adherence within the gastrointestinal tract. Additionally, other studies in infants have found that providing GOS/FOS can enhance immunity with outcomes such as decreased incidence of atopic dermatitis and infection (Moro et al., 2006; Arslanoglu et al., 2007; Gruber et al., 2010).

Colonization of the infant's gastrointestinal tract primarily begins at birth, with the mode of delivery impacting the types and numbers of bacteria present. Vaginal delivery results in inoculation of bacteria from the mother's cervix, vagina and feces while cesarean delivery, under more sterile conditions, provides the infant with bacterial exposure related to the hospital environment, tools, hospital personnel and other infants (Bettelheim et al., 1974; Lennox-King et al., 1976a; Lennox-King et al., 1976b; Brook et al., 1979). However, after delivery the infant's diet soon begins to impact the microbiota. Breast milk provides both microbes (up to 108microbes/L) and prebiotics for bacterial fermentation (human milk oligosaccharides in ranges of 7-12g/L) (Collins and Gibson, 1999; Heikkila and Saris, 2003; Martín et al., 2003; Boehm et al., 2004).

Prebiotics are carbohydrates that are resistant to digestion by human enzymes and selectively stimulate the growth and activity of beneficial bacteria in the gastrointestinal tract (Gibson and Roberfroid, 1995). Oligosaccharides have been shown to increase the number and concentration of bifidobacteria in the feces in a dose-dependent manner (Moro et al., 2002). Therefore, it has been postulated that the dominance of bifidobacteria in breastfed infants'

microbiota may be responsible for some of the health benefits the breastfed infant possess in comparison to the formula-fed infant.

A number of studies have shown that infant feeding with the prebiotics galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) is safe for healthy term infants and promotes satisfactory growth (Moro et al., 2002; Schmelzle et al., 2003; Haarman and Knol, 2005; Knol et al., 2005a; Knol et al., 2005b; Waligora-Dupriet et al., 2007; Costalos et al., 2008; Braegger et al., 2011). Additionally, a large number of clinical trials in term infants have shown that supplementation of infant formula with GOS and FOS at varying concentrations and blends increases bifidobacteria in feces as compared to control (Moro et al., 2002; Moro et al., 2003; Schmelzle et al., 2003; Ben et al., 2004; Boehm et al., 2004; Bakker-Zierikzee et al., 2005; Haarman and Knol, 2005; Knol et al., 2005a; Knol et al., 2005b).

The majority of studies in the literature of prebiotic-containing infant formulas have been conducted outside of the United States and in the context of several different formula matrices. The intent of the study described herein is to investigate the effects of a prebiotic-containing partially hydrolyzed whey infant formula on tolerance, stool characteristics, and commensal microbial populations in infants from the United States. It is hypothesized that consumption of the prebiotic-containing formula would be well-tolerated and increase fecal bifidobacteria proportions as compared with the control formula.

METHODS

This randomized, controlled, double-blind prospective clinical trial of two formula-fed groups with an exclusively breastfed reference group was conducted from November 2005 to May 2006 in the United States at 4 study sites (Pediaresearch, Owensboro KY; University of

Iowa, Iowa City IA; Children's Medical Group, Poughkeepsie NY; Carle Clinic, Urbana IL).

The randomized trial included healthy, full-term (37-42 weeks gestation) infants who were 2-8 weeks old and whose mothers had elected not to continue breastfeeding and who had not received solid foods. All study infants were required to live within 45 minutes of a study center and have a working freezer in their home. Infants with any of the following conditions were excluded from the study: known or suspected cow-milk allergy; major deformities and/or illness including cardiovascular, gastrointestinal, renal, neurological and/or metabolic; admission to intensive care or required readmission to hospital in first 14-28 days of life; diarrhea requiring treatment in the 7 days prior to enrollment; receiving therapeutic iron supplementation or medication other than multivitamin supplement. In addition, infants receiving antibiotic therapy, or breastfed subjects whose mothers had received antibiotic therapy, within 30 days prior to enrollment were not eligible for the study. Parents of all participating infants provided written informed consent at the time of enrollment. The study was approved by the relevant Institutional Review Board (IRB) of each study site and the University of Illinois IRB, where laboratory analyses occurred. The procedures followed were in accordance with the ethical standards of the responsible institutional or regional committee on human experimentation.

The primary objective of the study was to compare the proportions of fecal bifidobacteria in infants fed formula with or without prebiotics for 6 weeks. Secondary outcomes were absolute and relative fecal lactobacilli, bacteroides, and *C. difficile*; fecal pH and short chain fatty acid (SCFA) concentrations; gastrointestinal (GI) tolerance; and body weight.

Formula-fed (FF) infants were randomized to receive either the control formula (CON) or the prebiotic containing formula (PRE). The control formula used was a commercially available partially hydrolyzed whey infant formula (Nestlé Good Start Supreme DHA/ARA, Florham

Park, NJ). The test formula was nutritionally identical to the control formula with the exception of the addition of 4 g/L of GOS (Vivinal® GOS; Domo, the Netherlands) and short-chain FOS (Beneo™ P95 ; Orafiti N.V., Belgium) (9:1) (**Table 4.1**). A dose of 4 g/L was chosen to conservatively mimic the neutral oligosaccharide content of human breast milk (Thurl et al., 1993). As the fermentability of GOS/FOS is higher than that of human milk oligosaccharides, provision of a lower concentration of prebiotic than that of breast milk was hypothesized to provide adequate substrate for enhancing commensal bacteria such as bifidobacteria (Chaturvedi et al., 2001a; Coppa et al., 2001; Smiricky-Tjardes et al., 2003). Randomization for FF subjects was stratified according to route of delivery (vaginal versus cesarean-section). Subjects received their assigned formula as exclusive feeding for a period of 6 weeks. The self-selected breastfed reference group was exclusively breastfed and followed for a period of 6 weeks. Weight of each subject was taken at baseline and after 3 and 6 weeks of feeding at study visits.

The caregiver of each subject was responsible for keeping records for 2 days before the 3- and 6-week visits to assess nutritional intake, stool assessment, and GI tolerance indicators. The amount of formula consumed at each feeding was recorded for formula fed infants whereas length of each feeding at the breast, or the amount of expressed breast milk given at each feeding, was documented for breastfed infants. Stool color (green, yellow, brown, black), consistency (hard, formed, soft, liquid), odor (normal or unusual) and number of stools passed within each 24-hour period were recorded. In addition, the frequency of gas, spitting up, vomiting, crying bouts lasting more than 10 minutes, fussing bouts lasting more than 30 minutes, “colic” as perceived by the caregivers as number of episodes of intense, unsoothable crying with painful facial expressions and pulling up of legs were documented.

Within 72 hours of each study visit, stool samples were collected within 30 minutes of passing at home in 30 ml polypropylene fecal collection tubes (Globe Scientific, Paramus, NJ). Caregivers then brought the fresh sample in a cooler with ice packs to the study site within 30 minutes of collection, or the sample was placed in a home freezer for 2-72 hours. In the latter case, samples were then transported to a study site in a cooler with ice packs with a maximum transit time of 45 minutes. At the study site, samples were stored at -20°C for a maximum of 2 weeks prior to storage at -80°C until analysis.

Enumeration of Fecal Microbiota by Fluorescent In Situ Hybridization (FISH)

To study the gastrointestinal microbiota, enumeration was conducted using FISH to quantify bacteria present in fecal samples. Specifically, *Bifidobacteria* spp., *Bacteroides/Prevotella*, *C. difficile*, and *Lactobacillus* spp. were quantified (Ribo Technologies Microbial Diagnostics, Groningen, The Netherlands) (Matteuzzi et al., 2004). Briefly, fecal samples were homogenized vigorously with glass beads and centrifuged at 80 x g to remove debris. One hundred microliters supernatant, which contained the bacteria, was removed and added to a clean microcentrifuge tube and incubated with 10 µl fixative at 4°C for 30 minutes. The bacterial cells were then dehydrated with 70% ethanol and hybridized with the species-specific rRNA probes at 50°C in the dark for 16 to 24 hours to quantify the number of each respective fluorescein isothiocyanate (FITC)-labeled bacterial target strain. The bacterial cells were counted visually using 100 x magnification on an Axioskop 40 Carl Zeiss epifluorescence microscope (Goettingen, Germany) fitted with a 50 watt arc lamp and double filter for FITC (excitation 495 nm and emission 520 nm) and Cy3 (excitation 550 nm and emission 570 nm). The quantitative value was then normalized to total bacterial counts using Eub 338, specific for the domain Bacteria. Each fecal microbiota population was analyzed in

two ways: first, quantitatively, $\log^{7-10}$ cells per gram feces, as well as normalized to total bacteria as a percentage at each time point.

Short Chain Fatty Acid Quantification

Concentrations of SCFAs were determined using gas chromatography (GC)/mass spectrometry (MS) based on the method of Knol and colleagues (Knol et al., 2005b). Three hundred and fifty milligrams of feces were vortexed with 350 μ L 1.2 g/L 2-ethylbutyric acid, 350 μ L distilled sterile water, and 100 μ L of formic acid for 2 minutes. Samples were then centrifuged for 5 minutes at 16,000 x g to remove large particles and the supernatant was stored at -20°C in GC vials until analysis. Acetate, butyrate, propionate, isobutanate and valerate were quantified using a GC/MS (Agilent 6890N Santa Clara, CA) equipped with an electron impact ionization and chemical ionization, flame ionization detector, thermal conductivity detector, 7683B autosampler, and 5973N mass selective detector. Five microliters of sample were injected at 80°C into the column (Zebron-wax Plus, 30 m x 250 μ m, film thickness 0.25 μ m, Phenomenex, Torrance, California) using helium as a gas carrier (11.61 psi). After injection of the sample into a splitless inlet, the oven was heated to 230°C where it was transferred to the MS at the same temperature with a scan range of 25 to 800. Commercial Wiley and National Institute of Standards and Technology (NIST) libraries were used to interpret the mass spectra. The levels of SCFAs were determined using 2-ethylbutyric acid as an internal standard in combination with an external standard curve. SCFAs were expressed as absolute quantity (μ mol per gram feces) and on a relative basis (normalized to total concentration of SCFAs) at each time point.

Fecal pH

Fecal pH was used as a surrogate marker for the acidity of the colonic contents, altered primarily by the degree of fermentation occurring in the colon. Fecal pH value was obtained by

submerging the pH probe (Futura Refillable Probe, Beckman Instrument, Inc., Fullerton, CA) into the sample immediately upon thawing and waiting until the pH meter (UP-5, Denver Instrument, Denver, CO) reading stabilized.

Dry Matter Determination

Dry matter concentrations of fecal samples were used to normalize bacterial and SCFA measurements. Dry matter concentrations of fecal samples were determined by AOAC methods by obtaining the dry weight of crucibles by baking them overnight in a 105°C oven, then allowing the crucibles to reach room temperature within desiccators. Once the dry weight of the crucible was obtained, an aliquot of 0.025-1.0 g feces was treated in the same manner so that the dry matter proportion of the fecal sample could be determined (Association of Official Analytical Chemists, 1984).

Statistical Methods

Statistical analyses were carried out using an analysis of variance (ANOVA) for a 2 x 3 factorial design for fecal bacterial populations, pH and SCFAs. The random and fixed effects were visit (visit = 3) and treatment (treatment = 3). Sources of variation were the visit, treatment, and visit by treatment interaction. The random effect for route of delivery and study site were included in the model. If a significant visit by treatment interaction existed, the interaction was broken down into its component parts. In addition, a longitudinal analysis for repeated measurements of fecal bacterial and SCFA data at baseline, visits 1, and visit 2 using a mixed effects model was also done. Normality was tested using the Shapiro-Wilk statistic. Log transformations were utilized as needed. To compare the differences in tolerance and body weight data between the two formula groups and the breastfed group, a general linear model analysis was performed followed by a Tukey-Kramer procedure to control overall type 1 error

rate (Tukey, 1954; Kramer, 1956). Computations were conducted using SAS (Version 9.1; SAS Institute, Cary, NC). Statistical significance was defined as $p \leq 0.05$. All values are shown as mean $\pm$ SEM.

RESULTS

Of 146 recruited infants, 139 gave a baseline stool sample and continued in the study (**Figure 4.1**). At the baseline visit, 50 breastfed infants were enrolled, and 89 formula-fed infants were randomized to PRE (43 subjects) or CON (46 subjects). 123 infants completed all visits (attrition rate of 12%). Three subjects in PRE, 10 subjects in CON, and 3 BF subjects did not complete the study. While CON group had a higher attrition rate, there were no differences in tolerance or occurrence of adverse events during the study. Birth weight, length, gestational age, or route of delivery did not differ between feeding groups. Average formula intake was 798 ± 30 milliliters per day for both formula groups. Breastfed infants were fed an average of 7.0 ± 0.1 times per day.

Stool samples from 102 infants were analyzed. Reasons for excluding samples in stool analyses include false inclusion (3), incorrect timing of stool collection (1), non-compliance (4), and antibiotic use (13). Tolerance outcomes and growth are reported for all recruited subjects on an intention-to-treat basis. Data on fecal samples are reported on per protocol subjects only. Both statistical approaches employed in analyzing fecal bacteria and SCFA gave similar results whether looking at data for dry or wet fecal matter. Unless otherwise indicated, for these measures, the ANOVA model results are reported for wet weight of stools.

Absolute and relative bacterial abundance in fecal samples at all time points are reported in **Table 4.2**. Dependent variables impacted by visit and/or treatment are outlined on the figure

specified in the following text. Stool of subjects consuming PRE had a higher absolute number ($p=0.0083$) and proportion ($p=0.0219$) of bifidobacteria than CON for all visits combined (**Table 4.2 and Figure 4.2**). Neither the absolute number, nor proportion of fecal bifidobacteria differed between PRE and BF. BF had a higher proportion of bifidobacteria than CON ($p=0.0218$), though the absolute number of bifidobacteria did not differ between BF and CON.

BF had a lower number of *C. difficile* in feces than FF ($p=0.0087$; **Table 4.2, Figure 4.3**). Formula type did not alter fecal total bacteria abundance, nor the absolute and relative abundance of *Bacteroides/Prevotella*, *C. difficile*, or *Lactobacillus* spp (**Table 4.2**). On a dry matter basis, number of *Bacteroides/Prevotella* was higher in PRE than BF though there was no difference between PRE and CON (**Table 4.2**).

Fecal pH was significantly lower ($p=0.0161$) in PRE (6.59 ± 0.11) and BF (6.41 ± 0.11) than CON (6.69 ± 0.11) for all visits combined. Fecal SCFA concentrations are reported at all time points in **Table 4.3**. FF infants (PRE and CON combined) had significantly higher concentrations of acetate, butyrate, propionate ($p<0.0001$), and total SCFAs ($p=0.0230$) than BF infants. Comparing PRE and CON to each other, PRE had lower concentrations of propionate ($p<0.0001$) and butyrate ($p<0.0001$) than CON. PRE infants also had a lower proportion of propionate ($p=0.0026$) and a higher proportion of acetate ($p=0.0007$) compared to CON. Fecal valerate or isobutanate were not altered by visit or treatment.

Formula differences did not alter fecal attributes (frequency, consistency, color, or odor). BF had significantly more frequent stools ($p<0.0001$) compared to FF (3.0/day versus 1.4/day). FF subjects had soft stools 83% of the time. BF had a higher ($p<0.0001$) proportion of liquid and lower ($p<0.0001$) proportion of soft stools than FF. Confirming this subjective information recorded by caregivers, the objective measurements taken by weighing actual fecal matter

showed FF had a significantly higher ($p<0.001$) proportion of dry matter in their stools compared to BF. In formula-fed subjects, stool color was green 70% of the time. Parents of BF infants reported yellow stools 80% of the time. These differences in color were statistically significant ($p<0.0001$).

Formula type did not impact care-giver perceived incidence of crying, fussing, parent-perceived “colic”/cramps, spitting up, vomiting, and flatulence frequency. Nor did formula type alter body weight. Mean weight percentiles generated from the World Health Organization growth charts did not differ among PRE, CON, and BF. A trend was seen for BF girls to be heavier than girls fed either formula ($p=0.067$).

DISCUSSION

A combination of GOS and FOS has demonstrated potential health promoting characteristics in numerous human studies by preferentially targeting specific bacteria, such as bifidobacteria, which have been associated with enhanced mucosal immunity. Although the prebiotic source utilized in this study is different from others that have been studied in previous literature, results of this study add to this body of evidence as indicated by positive alterations in fecal microbial populations.

Infants recruited into this study were between 2 and 8 weeks of age and therefore received either human milk or infant formula prior to enrollment. The demographics of the pre-study feeding method regarding type of formula and any amount of breast milk formula-fed infants received prior to study enrollment were similar between PRE and CON. The age of infants at enrollment into the study was also similar within each treatment group. Although both of these factors likely play a role in the initial composition of the microbiota, it is interesting that

they did not prevent the detection of a statistically significant increase in fecal bifidobacteria numbers. However, this variation may have attributed to the lack of detection in differences in *C. difficile* numbers between formula groups. It is clinically noteworthy that consumption of GOS/FOS resulted in a positive shift within the commensal microbiota population even among a group of diverse infants.

Prebiotic consumption increased the number of *Bifidobacterium* spp. when combining data for all visits. The enhanced colonization of bifidobacteria in the PRE group was accompanied by suppressed growth of the pathogenic bacteria *C. difficile* in comparison to CON. However this was not statistically significant, possibly due to the significant variability of *C. difficile* at baseline among treatment groups. Despite this variation at baseline, formula type impacted the *C. difficile* response over time wherein PRE decreased fecal *C. difficile* concentration 39%, whereas CON increased it 80%. This observation is similar to the results from *in vivo* and *in vitro* studies that showed stimulation of bifidobacteria and suppression of potential pathogen, such as clostridia associated with prebiotic consumption and inoculation, respectively (Wang and Gibson, 1993; Gibson and Roberfroid, 1995; Penders et al., 2006). In healthy, term infants (0-14 days), Costalos and colleagues reported that consumption of a prebiotic formula for 6-weeks reduced fecal clostridia and increased fecal bifidobacteria (Costalos et al., 2008). The carbohydrate portion of oligosaccharides is largely indigestible and may be acting as a receptor analog to preclude pathogen colonization by prevention of epithelial adherence (Schroten et al., 1992; Schwertmann et al., 1999; Kunz et al., 2000).

Overall, while statistically significant, the differences in fecal microbiota studied herein were relatively small when considering the magnitude of the resident microbiota of the human infant. Increases in bifidobacteria were observed, however the changes that occurred were no

greater than 1 log of CFU. In comparison, Moro and colleagues reported bifidobacteria increases > 1 log of CFU in infants fed for the first 28 days of life with an intact whey and casein-containing formula with 4g/L of a prebiotic mixture similar to that used in this study (Moro et al., 2002). Several aspects of the current study should be considered in light of this. First, the studied population was healthy, full-term infants at the ages of 2-8 weeks with a more diverse microbiota established, as discussed above. In this context, a dose > 4g/L of a prebiotic mixture may be more effective. Second, the GOS:FOS combination used in the bulk of other prebiotic-containing infant formula literature is referred to as long-chain FOS and short-chain GOS; whereas, the combination utilized in this study had short-chain FOS with degree of polymerization of 2-7 and GOS containing typically between 1-7 galactose units in comparison (Moro et al., 2002; Moro et al., 2003; Schmelzle et al., 2003; Boehm et al., 2004). Therefore, differences in fermentability of these combinations could affect various outcomes and explain inconsistencies seen between the current study and previous literature. Third, the formula matrix studied herein was a whey-based, lower phosphate, lactose-containing formula, with and without prebiotics. These attributes may already beneficially support a healthy microbiota as compared to a casein-dominant, higher phosphate, lactose-free formula (Balmer et al., 1989; Petschow and Talbott, 1990; Langhendries et al., 1995). Finally, the low protein content of human milk is an attribute postulated to enhance its bifidogenicity within the infant intestine (Willis et al., 1973; Bullen et al., 1977). It is possible that the use of an infant formula with hydrolyzed whey may reduce the amount of undigested protein reaching the colon and thus available for fermentation, as compared to an infant formula with intact protein. Addition of this prebiotic combination of GOS/FOS to a healthy microbiota-supportive formula based on hydrolyzed whey may have

limited the magnitude of effect of the prebiotic studied in the current trial albeit statistically significant and presumably clinically-relevant changes were indeed observed.

Microbial composition and its associated metabolic activity in response to diet impacts the abundance of SCFA produced. Hence, a change in the composition of the intestinal microbiota may result in changes in levels of SCFA present in feces (Midtvedt and Midtvedt, 1992; Knol et al., 2005b). In the present study, propionate and butyrate concentrations were lower in feces of infants consuming breast milk. These data agree with Flickinger and co-workers who reported that breastfed infants produced a lower proportion of propionate and butyrate compared with proportions produced by formula-fed infants (Flickinger et al., 2002). Acetate has been reported as the major acid in feces of breastfed infants, however, we found a higher concentration of acetate in our formula-fed infants in comparison to the breastfed infants (Flickinger et al., 2002; Bakker-Zierikzee et al., 2005). Lack of consistent data in fecal SCFA in the published literature reflects the difficulties of obtaining this information in a healthy population of infants as effects of oxidation in fecal matter are great with these highly volatile materials. Consideration of the relevance of such data is also under debate as concentrations of SCFAs in fecal matter may not accurately reflect those present at the epithelial interface. Similarly, measurement of intestinal fermentation using pH is difficult because it is an indirect measure and numerous other factors can also impact fecal pH such as mucosal bicarbonate secretion.

The current study adds to the mounting body of evidence that prebiotic-containing infant formulae are well-tolerated and affect the microbiota of the formula-fed infant. Future studies could concentrate on how these alterations in the microbiota may lead to clinical effects and optimal dosing. Recent studies have looked at potential effects of prebiotics on immune-related

outcomes such as primary prevention of allergy and atopic dermatitis and infection rates (Arslanoglu et al., 2007; Arslanoglu et al., 2008; Bruzzese et al., 2009; Gruber et al., 2010). In addition, greater effects of prebiotics could be seen in a potentially less bifidogenic formula matrix within a population who may already have a suboptimal intestinal microbiota due to underlying etiologies. The use of prebiotics in such cases could render greater realization of their benefits in at-risk populations. Such knowledge would help change the composition of infant formula to simulate the health advantages currently achieved in the breastfed infant population.

In conclusion, feeding healthy full-term infants a partially hydrolyzed whey formula with 4g/L of GOS/FOS results in a modification of the established microbiota that more closely resembles the breastfed infant as assessed by an increase proportion of bifidobacteria in the stool. Additionally, this remodeled microbiota was associated with lower fecal pH and changes in the fermentation profile. This randomized controlled trial contributes further to the growing literature supporting the safety and tolerance of infant formula containing prebiotics. More study is needed to clarify the clinical relevance of these fecal alterations and if such shifts persist after cessation of prebiotic use.

ACKNOWLEDGEMENTS

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TABLES AND FIGURES

	<i>Breast milk¹</i> <i>(BF)</i>	<i>Control Formula</i> <i>(CON)</i>	<i>Prebiotic Formula</i> <i>(PRE)</i>
Energy (kcal/L)	640-670	670	670
Protein (g/L)	11 ²	14.7 ³	14.7 ³
Carbohydrate (g/L)	75 ⁴	75 ⁵	75 ⁵
Prebiotic (g/L)	7-12 ⁶	----	4 ⁷
Fat (g/L)	42 ⁸	34.2 ⁹	34.2 ⁹

Table 4.1. Composition of Infant Diets

¹Typical composition of mature breast milk (Jensen, 1995).

²Casein and whey protein.

³Enzymatically hydrolyzed whey protein concentrate.

⁴Lactose.

⁵Lactose and corn maltodextrin.

⁶Oligosaccharide content is predominantly lactose-N-tetraose and its monofucosylated derivatives, representing about 10% of total milk carbohydrate (Kunz et al., 2000).

⁷Galactooligosaccharides:short-chain fructooligosaccharides (9:1); Vivinal[®] GOS (Domo, the Netherlands); FOS (Beneo[™] P95 ; Orafiti N.V., Belgium).

⁸Triglycerides and polyunsaturated fatty acids.

⁹Vegetable oils (Palm Olein, Soy, Coconut, and High-Oleic Sunflower), DHA and ARA represents about 3.2% and 0.64% of total fat content, respectively.

	BF			CON			PRE		
	W0	W3	W6	W0	W3	W6	W0	W3	W6
Absolute Abundance CFU/g wet weight stool									
<i>Bifidobacterium</i> spp ³	2.04x10 ⁹ ± 7.04x10 ⁸	2.85x10 ⁹ ± 8.24x10 ⁸	3.02x10 ⁹ ± 8.47x10 ⁸	1.91x10 ⁹ ± 7.08x10 ⁸	1.90x10 ⁹ ± 6.81x10 ⁸	2.05x10 ⁹ ± 7.00x10 ⁸	2.61x10 ⁹ ± 8.04x10 ⁸	4.60x10 ⁹ ± 1.02x10 ⁹	3.72x10 ⁹ ± 9.27x10 ⁸
<i>Bacteroides/Prevotella</i>	6.08x10 ⁷ ± 2.41x10 ⁷	7.19x10 ⁷ ± 2.74x10 ⁷	8.91x10 ⁷ ± 3.23x10 ⁷	7.06x10 ⁷ ± 2.63x10 ⁷	7.18x10 ⁷ ± 2.54x10 ⁷	8.50x10 ⁷ ± 3.18x10 ⁷	6.29x10 ⁷ ± 2.14x10 ⁷	1.24x10 ⁸ ± 4.27x10 ⁷	1.02x10 ⁸ ± 3.79x10 ⁷
<i>Clostridium difficile</i> ⁴	6.55x10 ⁶ ± 2.58x10 ⁶	1.85x10 ⁷ ± 6.30x10 ⁶	1.93x10 ⁷ ± 5.88x10 ⁶	2.56x10 ⁷ ± 6.84x10 ⁶	2.69x10 ⁷ ± 5.40x10 ⁶	2.51x10 ⁷ ± 5.40x10 ⁶	3.32x10 ⁷ ± 7.13x10 ⁶	2.49x10 ⁷ ± 5.10x10 ⁶	1.79x10 ⁷ ± 4.06x10 ⁶
<i>Lactobacillus</i> spp ⁵	4.80x10 ⁷ ± 1.62x10 ⁷	8.21x10 ⁷ ± 2.68x10 ⁷	1.36x10 ⁸ ± 4.35x10 ⁷	1.19x10 ⁸ ± 3.28x10 ⁷	2.07x10 ⁸ ± 5.79x10 ⁷	1.39x10 ⁸ ± 3.98x10 ⁷	1.36x10 ⁸ ± 3.68x10 ⁷	1.34x10 ⁸ ± 3.55x10 ⁷	1.94x10 ⁸ ± 5.66x10 ⁷
<i>Total bacteria</i> ⁶	4.40x10 ⁹ ± 7.14x10 ⁸	5.30x10 ⁹ ± 8.59x10 ⁸	5.65x10 ⁹ ± 9.01x10 ⁸	5.65x10 ⁹ ± 9.02x10 ⁸	7.35x10 ⁹ ± 1.17x10 ⁹	7.04x10 ⁹ ± 1.14x10 ⁹	6.64x10 ⁹ ± 1.69x10 ⁹	1.06x10 ¹⁰ ± 1.62x10 ⁹	8.12x10 ⁹ ± 1.28x10 ⁹
Relative Abundance (% of total)									
<i>Bifidobacterium</i> spp ⁷	36.38 ± 6.73	48.77 ± 7.33	43.48 ± 6.77	36.70 ± 6.68	30.85 ± 5.87	32.62 ± 8.09	45.59 ± 7.35	49.46 ± 7.12	44.10 ± 6.74
<i>Bacteroides/Prevotella</i>	1.54 ± 0.62	1.29 ± 0.49	1.29 ± 0.46	1.26 ± 0.47	0.90 ± 0.32	1.27 ± 0.48	0.86 ± 0.28	1.08 ± 0.36	1.19 ± 0.44
<i>Clostridium difficile</i>	0.16 ± 0.08	0.32 ± 0.13	0.34 ± 0.12	0.45 ± 0.14	0.34 ± 0.08	0.37 ± 0.07	0.42 ± 0.10	0.21 ± 0.05	0.18 ± 0.05
<i>Lactobacillus</i> spp	0.98 ± 0.30	1.68 ± 0.51	2.20 ± 0.66	2.10 ± 0.54	2.76 ± 0.72	2.16 ± 0.57	2.20 ± 0.55	1.36 ± 0.34	2.82 ± 0.84

Table 4.2. Absolute and Relative Bacterial Abundance in Fecal Samples^{1,2}

¹Data are expressed as means ± SEM.

²BF: breastfed; CON: control infant formula group; PRE: prebiotic infant formula group; W0: week 0; W3: week 3; W6: week 6; CFU: colony forming units; spp: species.

³For means pooled for diet, PRE>CON; PRE=BF; CON=BF (p=0.0083). Refer to Figure 4.2a.

⁴A significant interaction between diet and time was seen (p=0.0414). Refer to Figure 4.3.

⁵For means pooled for diet, PRE=CON>BF (p=0.0104).

⁶For means pooled for diet, PRE=CON>BF (p=0.0007).

⁷For means pooled for diet, PRE=BF; PRE>CON; BF>CON (p=0.0218). Refer to Figure 4.2b.

	BF			CON			PRE		
	W0	W3	W6	W0	W3	W6	W0	W3	W6
Absolute Abundance Units(μmol/g wet feces)									
Acetate ³	17.87 $\pm$ 2.80	17.10 $\pm$ 2.68	19.91 $\pm$ 3.12	33.22 $\pm$ 5.22	31.04 $\pm$ 4.88	33.92 $\pm$ 5.38	26.33 $\pm$ 4.07	34.76 $\pm$ 5.38	35.21 $\pm$ 5.38
Propionate ⁴	3.58 $\pm$ 0.93	3.63 $\pm$ 1.02	4.83 $\pm$ 1.26	12.07 $\pm$ 2.35	9.39 $\pm$ 1.76	9.96 $\pm$ 1.88	7.58 $\pm$ 1.28	7.03 $\pm$ 1.28	7.42 $\pm$ 1.38
Butyrate ⁵	1.66 $\pm$ 0.52	1.85 $\pm$ 0.64	1.40 $\pm$ 0.44	5.26 $\pm$ 1.30	3.43 $\pm$ 0.81	4.45 $\pm$ 1.04	2.96 $\pm$ 0.68	3.25 $\pm$ 0.77	2.87 $\pm$ 0.68
Valerate	2.06 $\pm$ 1.74	1.41 $\pm$ 1.37	0.97 $\pm$ 0.87	2.38 $\pm$ 1.91	1.46 $\pm$ 1.13	1.23 $\pm$ 0.98	1.71 $\pm$ 1.36	1.18 $\pm$ 0.94	1.09 $\pm$ 0.90
Isobutanoic	5.59 $\pm$ 3.76	5.30 $\pm$ 8.59	4.10 $\pm$ 4.40	1.92 $\pm$ 0.39	1.38 $\pm$ 0.46	1.52 $\pm$ 0.49	1.12 $\pm$ 0.39	1.47 $\pm$ 0.64	1.36 $\pm$ 0.63
Total ⁶	35.29 $\pm$ 8.48	30.22 $\pm$ 8.37	37.20 $\pm$ 9.01	57.92 $\pm$ 9.03	45.99 $\pm$ 6.63	50.49 $\pm$ 7.15	40.95 $\pm$ 5.77	51.36 $\pm$ 7.40	45.73 $\pm$ 6.62
Relative Abundance (% of total)									
Acetate ⁷	67.76 $\pm$ 3.53	76.67 $\pm$ 3.99	77.82 $\pm$ 3.51	65.45 $\pm$ 2.31	67.89 $\pm$ 2.14	70.08 $\pm$ 2.12	68.73 $\pm$ 2.03	74.61 $\pm$ 2.08	75.62 $\pm$ 2.13
Propionate ⁸	20.30 $\pm$ 3.96	12.14 $\pm$ 2.71	11.53 $\pm$ 2.24	21.43 $\pm$ 2.62	20.83 $\pm$ 2.33	20.01 $\pm$ 2.19	19.60 $\pm$ 2.08	15.34 $\pm$ 1.67	15.68 $\pm$ 1.73
Butyrate	9.16 $\pm$ 2.09	7.09 $\pm$ 1.95	6.46 $\pm$ 1.66	10.59 $\pm$ 1.43	8.27 $\pm$ 1.16	9.57 $\pm$ 1.22	8.62 $\pm$ 1.15	7.49 $\pm$ 1.10	7.27 $\pm$ 1.10

Table 4.3. Absolute and Relative Short Chain-Fatty Acid Abundance in Fecal Samples^{1,2}

¹Data are expressed as means $\pm$ SEM.

²BF: breastfed; CON: control infant formula group; PRE: prebiotic infant formula group; W0: week 0; W3: week 3; W6: week 6; CFU: colony forming units; spp: species.

³For means pooled for diet, CON=PRE>BF (p<0.0001).

⁴For means pooled for diet, BF<PRE<CON (p<0.0001).

⁵For means pooled for diet, BF<PRE<CON (p<0.0001).

⁶For means pooled for diet, CON=PRE>BF (p=0.0230).

⁷For means pooled for diet, PRE=BF; PRE>CON; BF>CON (p=0.0007).

⁸For means pooled for diet, PRE=BF; CON >PRE; CON>BF (p=0.0026).

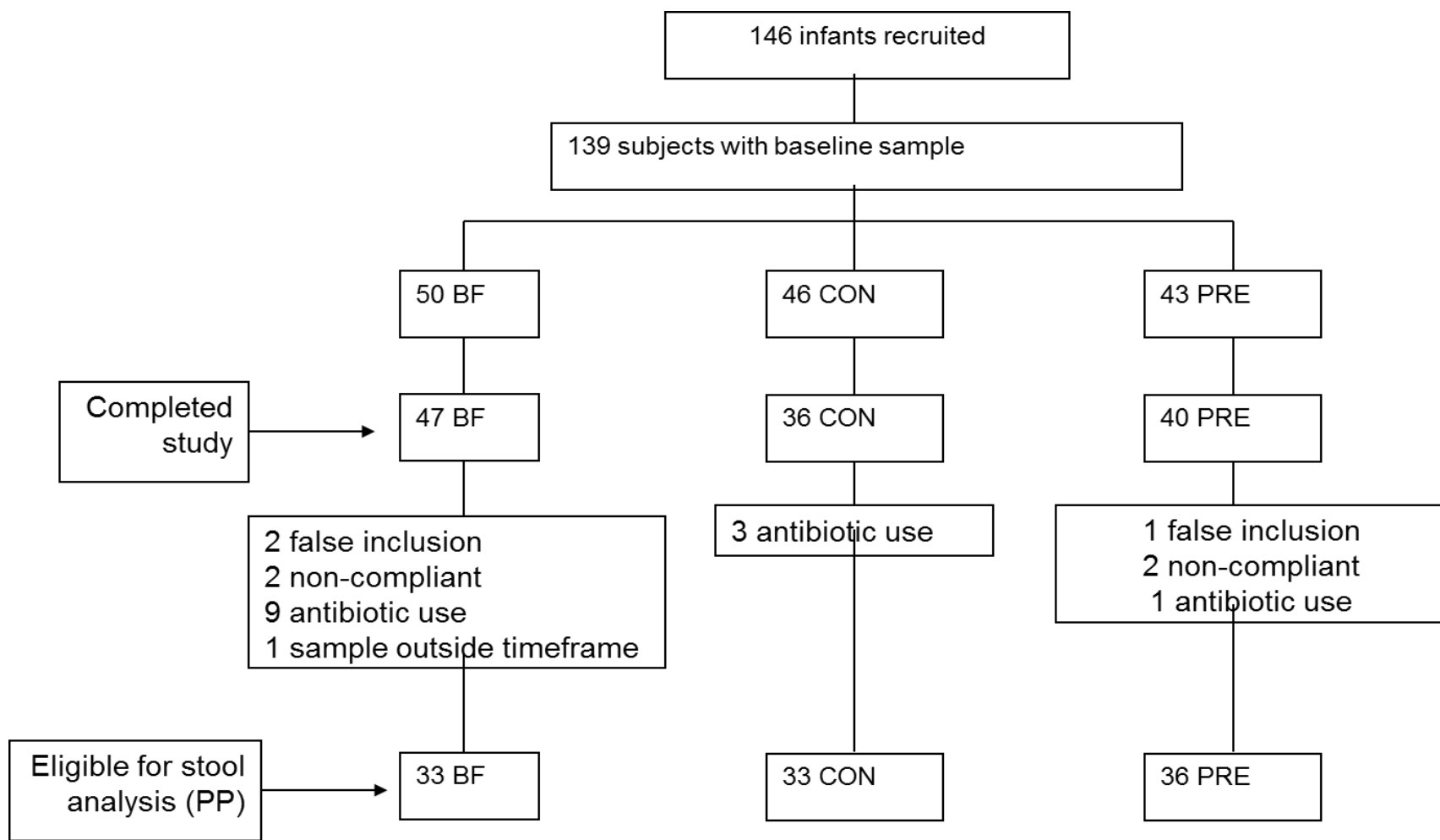


Figure 4.1. Experimental Design and Subject Completion

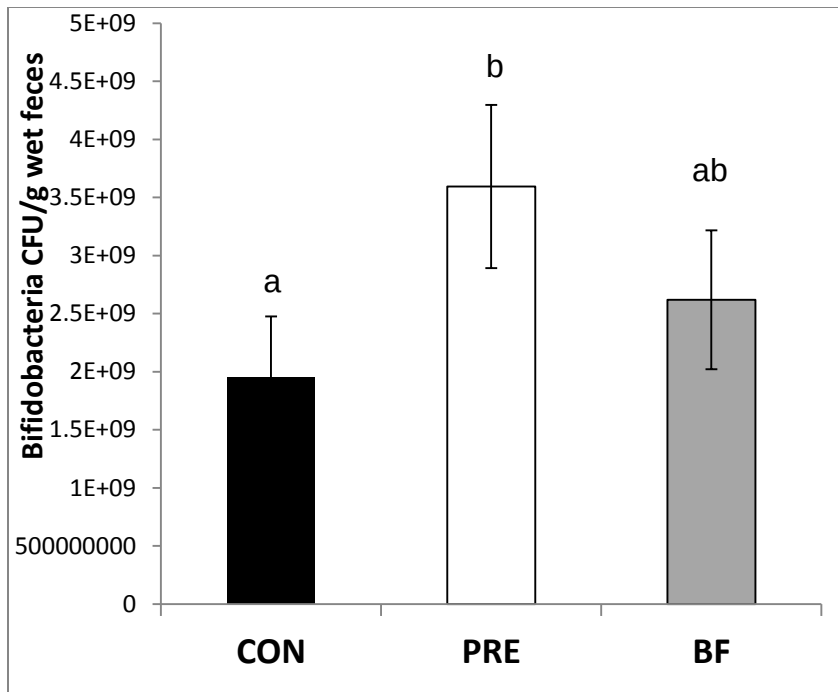


Figure 4.2a

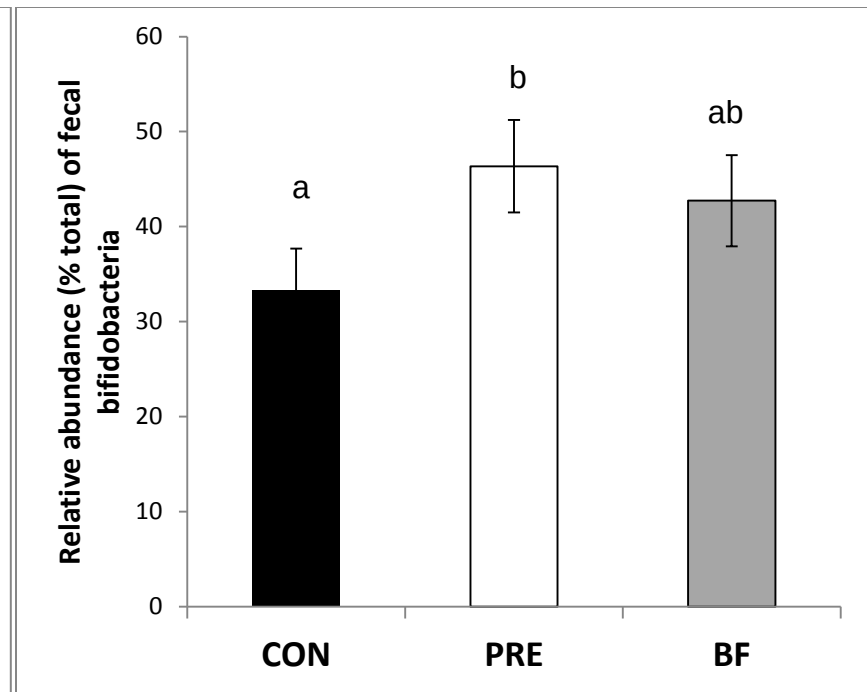


Figure 4.2b

Figure 4.2. Subjects consuming prebiotic formula had more fecal bifidobacteria than those consuming the control formula. Bars with different superscripts indicate a statistically significant difference. Data are expressed as means (pooled for time) $\pm$ SEM. Table 4.2 contains data for each group at each time point. BF: breastfed; CON: control infant formula group; PRE: prebiotic infant formula group; CFU: colony forming units. Figure 4.2a. Absolute abundance (CFU/g) of fecal bifidobacteria was significantly higher ($p=0.0083$) in subjects consuming prebiotic formula as compared to the control formula. Figure 4.2b. Relative abundance (percentage of total) of fecal bifidobacteria was significantly higher ($p=0.0219$) in subjects consuming prebiotic formula as compared to the control formula.

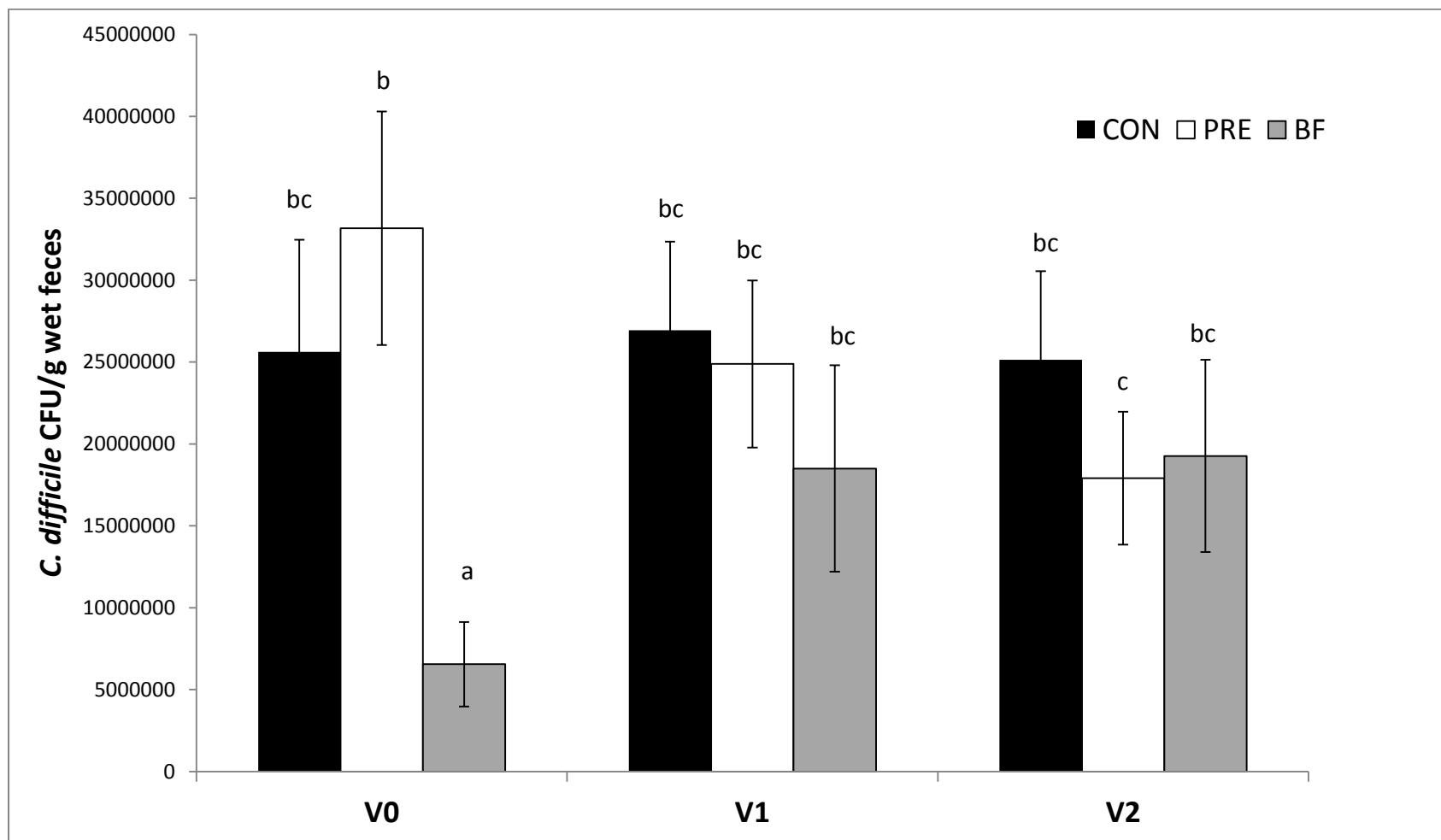


Figure 4.3. Subjects consuming prebiotic formula had significantly less *C. difficile* abundance over time. Bars with different superscripts indicate a statistically significant difference. Data are expressed as means $\pm$ SEM. Table 4.2 contains data for each group at each time point. BF: breastfed; CON: control infant formula group; PRE: prebiotic infant formula group.

CHAPTER 5

HUMAN MILK OLIGOSACCHARIDES INFLUENCE INTESTINAL MATURATION *IN VITRO*

ABSTRACT

Human milk is considered the gold standard of infant nutrition and recommended exclusively until 6 months of age; however, by one month of age nearly 40% of infants are receiving infant formula as their sole source of nutrition (Li et al., 2005; Eidelman et al., 2012). Formula-fed infants are at increased risk of morbidity and mortality compared to their breastfed counterparts (Grulee et al., 1934; Lopez-Alarcon et al., 1997; Sisk et al., 2007). As such, research is needed to identify infant formula ingredients that impart some of the benefits of breast feeding onto formula-fed infants. Human milk oligosaccharides (HMOs) are abundant in breast milk (≥ 15 g/L) and associated with decreased disease risk and enhanced intestinal maturation *in vitro* (Newburg et al., 2004; Kuntz et al., 2008; Kuntz et al., 2009). Therefore, we aimed: 1) to determine the time course of impact of a model HMO; and 2) to assess the induction of epithelial differentiation by three individual HMOs using an *in vitro* epithelial model of the crypt-villus axis. In study 1, pre-confluent HT-29, pre-confluent Caco-2Bbe, confluent Caco-2Bbe, and post-confluent Caco-2Bbe cells were cultured individually and randomized to 1 of 5 treatments: lacto-N-neotetrose (LNnT) at 0, 100, 200 or 400 mg/L or energy control (0 mg/L plus lactose + N-acetylglucosamine) for 24, 48 or 72 hours of incubation to assess effects on proliferation and differentiation. In study 2, pre-confluent HT-29, pre-confluent Caco-2Bbe, and post-confluent Caco-2Bbe cells were randomized to treatments with one of three HMOs at the following levels for 72 hours: a) LNnT at 0, 20, 200 or 2000 mg/L or energy control (0 mg /L plus lactose + N-acetylglucosamine); b) 2'-fucosyllactose (2'FL) at 0, 20, 200 or 2000 mg/L or

energy control (0 mg/L plus lactose + fucose); c) 6'-sialyllactose (6'SL) at 0, 40, 400, 4000 mg/L or energy control (0 mg/L plus lactose + sialic acid). Dependent variables included proliferation, differentiation, cell cycle analysis, apoptosis, disaccharidase activity, and absorption and barrier function via modified Ussing chambers. Treatment effects of individual HMO treatments were assessed by one-way ANOVA and paired-comparisons. Statistical significance was set at $p \leq 0.05$. In study 1, compared to 0 mg/L control, LNnT decreased cell proliferation in pre-confluent HT-29 cells at 48 hours ($p < 0.05$) and 72 hours ($p < 0.01$) and pre-confluent CaCo-2Bbe at 72 hours ($p < 0.01$). AP activity increased over time and was highest in post-confluent CaCo-2Bbe cells ($p < 0.001$) but was not affected by treatment. In study 2, pre-confluent HT-29 cell proliferation was reduced ($p < 0.05$) with 200 and 2000 mg/L LNnT, 2000 mg/L 2'FL, and 400 and 4000 mg/L 6'SL. Pre-confluent CaCo-2Bbe cell proliferation was reduced ($p < 0.05$) with 2000 mg/L LNnT, 4000 mg/L 6'SL, and 2000 mg/L 2'FL. Reduced proliferation was associated with cell cycle arrest in the G2/M phase in pre-confluent HT-29 cells treated with 200 ($p = 0.027$) and 2000 mg/L ($p = 0.003$) of LNnT or 2000 mg/L 2'FL ($p = 0.021$), and pre-confluent CaCo-2Bbe cells treated with 4000 mg/L 6'SL. Interestingly, in pre-confluent HT-29 cells, treatment with 4000 mg/L 6'SL resulted in S phase arrest ($p < 0.001$), whereas there was a trend ($p = 0.058$) for cell cycle arrest in the G2/M phase with 400 mg/L 6'SL. AP activity increased 31% ($p = 0.030$) in pre-confluent HT-29 cells and tended ($p = 0.071$) to increase by 33% in post-confluent CaCo-2Bbe treated with 2000 2'FL. Further, post-confluent CaCo-2Bbe cell sucrase activity increased 54% ($p = 0.036$) compared to control when treated with 2000 mg/L 2'FL. Transepithelial resistance increased 21% ($p = 0.002$) in post-confluent CaCo-2Bbe cells with 200 mg/L LNnT, and tended ($p = 0.059$) to increase by 12% with 2000 mg/L LNnT, compared to control. In summary, study 1 identified 72 hours as the most sensitive incubation period to measure treatment effects. In study

2, individual HMOs were found to inhibit proliferation in transformed small intestinal cell lines with associated cell cycle arrest, primarily within the G2/M phase. More importantly, the inhibition of proliferation was associated with enhanced differentiation. Well differentiated enterocytes, modeled by post-confluent CaCo-2Bbe cells, expressed greater digestive capabilities and barrier function, following treatment with physiologically relevant doses of 2'FL and LNnT, respectively. Further study is needed to determine if these effects translate *in vivo*, however, these results suggest addition of HMO may promote intestinal maturation *in vitro*.

INTRODUCTION

Human milk is regarded as the gold standard of infant nutrition because of the magnitude of functional nutritive and non-nutritive components it contains that aid in the maturation and protection of the neonatal gastrointestinal (GI) tract (Carver and Barness, 1996). The American Academy of Pediatrics recommends that infants be provided human milk exclusively for about six months followed by continued breastfeeding during the first year or longer as complementary foods are introduced (Eidelman et al., 2012). Nevertheless, by one month of age over one-third of infants are receiving formula as their sole source of nutrition (Li et al., 2005). As a large proportion of infants are lacking exposure to human milk during infancy, research is needed to identify formula ingredients that may be able impart some of the benefits of human milk to formula-fed infants.

Human milk contains nearly 200 unique oligosaccharides, a concentration $\geq$ 15 grams per liter which varies between women and also over time; moreover, human milk oligosaccharides (HMOs) are the third most abundant solid component following lactose (70 g/L) and lipids (40 g/L) and narrowly surpassing protein (8g/L) (Coppa et al., 1999; Chaturvedi et al., 2001a; Ninonuevo et al., 2006; German et al., 2008b; Thurl et al., 2010; Wu et al., 2010).

Human milk is unique in its oligosaccharide composition and complexity, as milk from animals is nearly completely lacking in free complex oligosaccharides (Kunz et al., 2000). Infant formula is completely lacking in HMOs, only plant derived or synthetic fructo- and galacto-oligosaccharides are currently supplemented (Tungland and Meyer, 2002; Kunz and Rudloff, 2006).

Interestingly, recent studies have reported that HMOs may have an effect on intestinal maturation. Kuntz and colleagues showed that neutral and acidic HMO fractions in pre-confluent HT-29 and CaCo-2 transformed intestinal cell lines induced growth inhibition by two different mechanism 1) suppression of cell cycle progression through induction of differentiation and/or 2) induction of apoptosis (Kuntz et al., 2008). To expand on these research findings we aimed: 1) to determine the time course of impact of a model HMO on proliferation and differentiation in transformed intestinal cell lines (HT-29 and CaCo-2Bbe cells); and 2) to assess the induction of epithelial differentiation by three individual HMO, 2'-fucosyllactose (2'FL), lacto-N-neotetraose (LNnT), and 6'-sialyllactose (6'SL), using an *in vitro* epithelial model of the crypt-villus axis consisting of pre-confluent HT-29 cells, pre-confluent CaCo-2Bbe cells, and post-confluent CaCo-2Bbe cells.

MATERIALS AND METHODS

Study Design

Our model consisted of pre-confluent HT-29 cells, pre-confluent CaCo-2Bbe cells, confluent CaCo-2Bbe, and post-confluent CaCo-2Bbe cells cultured individually to provide an *in vitro* model of the small intestinal crypt-villus axis and simulate the varying levels of differentiation within the constantly renewing villus *in vivo* (**Figure 5.1**). HT-29 cells are a low

differentiated phenotype reflective of crypt colonocytes—these cells are not able to differentiate even in post-confluent states under normal culture conditions (Lesuffleur et al., 1990). HT-29 cells have properties of chloride secreting cells related to a chloride channel that can also be found within human intestinal epithelial cells (Kreusel et al., 1991). CaCo-2Bbe cells, a well characterized model of absorptive intestinal epithelium, exhibit moderate differentiation in a pre-confluent state and in a post-confluent state exhibit a process of differentiation similar to that seen in a villus enterocyte (Peterson and Mooseker, 1992). CaCo-2Bbe cells will grow as a monolayer, continuing to differentiate and form cylindrical polarized morphology with tight junctions between cells (Peterson and Mooseker, 1992). Additionally, CaCo-2Bbe cells have apical microvilli with associated brush-border enzymes (sucrase and lactase) in post-confluent states which are similar to human fetal enterocytes (Peterson and Mooseker, 1992). Furthermore, CaCo-2 cells grown as a monolayer are also a well-established model of intestinal absorptive function and provide an avenue by which transepithelial resistance and nutrient transport can be assessed (Delie and Rubas, 1997).

Overall, our model allowed us to assess relevant outcomes along the length of the crypt-villus axis *in vitro* as pre-confluent HT-29 cells model secretory crypt cells, pre-confluent CaCo-2Bbe cells model proliferating absorptive enterocytes as they migrate up the villus, and post-confluent CaCo-2Bbe cells model well differentiated functional absorptive villus cells. The *in vitro* model as a whole simulates the neonatal infant's maturing gastrointestinal epithelial. Furthermore, incubation of the cells with HMO provides a simulation of the suckling infant's nearly continuous enteral feedings of human milk. As such, our model proved an avenue for novel insight into the effects of HMO administration on small intestinal cellular dynamics and function *in vitro*.

Cell Culture

To model the crypt-villus axis and investigate the effects of incubation with HMO, pre-confluent HT-29, pre-confluent CaCo-2Bbe, confluent CaCo-2Bbe and post-confluent CaCo-2Bbe cells were utilized. The CaCo-2Bbe cell line was obtained from American Type Culture Collection (CRL-2102, ATCC, Manassas, VA) clone of CaCo-2 (ATCC: ATB-37). They were used between passages 55 and 65. The transformed human intestinal cells, HT-29 cells, were obtained from American Type Culture Collection (ATCC, Manassas, VA). They were used between passages 5 and 15. Both cell lines were separately maintained in 75 cm² tissue culture flasks (TP90075, Midwest Scientific, St. Louis, MO) and maintained in a humidified atmosphere of 37°C and 5% CO₂. HT-29 cells were maintained in McCoy's 5A media (pH 7.4) supplemented with 10% fetal bovine serum (Atlanta Biological, Lawrenceville, GA). CaCo-2Bbe cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (pH 7.4) supplemented with 10% fetal bovine serum (Atlanta Biological, Lawrenceville, GA) and 0.01 mg/ml human apotransferrin (Sigma, St. Louis, MO). Growth medium was replaced as needed, but no fewer than twice per week. Cell lines were subcultured weekly at pre-confluent densities with 0.25% trypsin-EDTA (25200, Invitrogen, Carlsbad, CA).

HMO Treatments

Study 1 and 2

Prior to treatment, each of the HMOs (provided by Abbott Nutrition, Columbus, OH) and energy controls were added to either DMEM or McCoy's 5A cell culture media at a concentration of 20 mg/mL, passed through a Detoxi-Gel endotoxin removing column (Pierce), and stored at -80°C until use. The columns utilize immobilized polymyxin B to remove pyrogens by binding their lipid A domains.

Study 1

Treatments included LNnT at 0, 100, 200 or 400 mg/L or energy control (0 mg/L plus lactose + N-acetylglucosamine) for 24, 48 or 72 hours of incubation. The LNnT treatment doses centered on the physiologically relevant level of 200 mg/L in human milk.

Study 2

Pre-confluent HT-29, pre-confluent Caco-2Bbe, and post-confluent Caco-2Bbe cells were randomized to treatments with one of three HMOs at the following levels for 72 hours of incubation: a) LNnT at 0, 20, 200 or 2000 mg/L or energy control (0 mg /L plus lactose + N-acetylglucosamine); b) 2'FL at 0, 20, 200 or 2000 mg/L or energy control (0 mg/L plus lactose + fucose); c) 6'SL at 0, 40, 400, 4000 mg/L or energy control (0 mg/L plus lactose + sialic acid) (**Table 5.1**). Final treatment solutions maintained a pH of 7.4. LNnT and 6'SL treatments centered on their physiologically relevant doses of 200 and 400 mg/L, respectively. The highest treatment dose of 2'FL at 2000 mg/L is the physiologically relevant level found in human milk.

Cell Proliferation

In study 1, cell proliferation was measured using the BrdU & Ki-67 Assay (HCS213, Millipore) according to manufacture directions. Prior to assay, 96 well plates were seeded with HT-29 or CaCo-2Bbe cells at a density of 1500 cells/well and allowed to adhere for 48 hours in the case of pre-confluent cells, 7 days for confluent cell types, and 21 days for post-confluent cells. Thereafter, the medium was replaced with treatment medium containing LNnT at variable concentrations for 24, 48, or 72 hours. At the end of the incubation period, 20 μ M BrdU was added to cell culture wells for 1 hour at 37°C prior to a one hour fixation period at room temperature. After removing the fixation solution, wells were washed two times with wash buffer. Next, the primary antibody solution containing mouse anti-BrdU and rabbit Anti-Ki-76

was allowed to incubate for 1 hour at room temperature. Following a second wash step, the secondary antibodies (FITC-Donkey Anti-Mouse, Cy3-Donkey Anti-Rabbit) and Hoechst nuclear stain were added and incubated at room temperature for one hour protected from light. After a third wash step, the immunofluorescence buffer was added and plates were read at the following excitation/emission wavelengths 360/460, 480/535, 585/600. Results were normalized to number of cells and expressed as a percentage of 0 mg/L control.

In study 2, the CellTiter 96 AQueous non-radioactive cell proliferation assay (G5421, Promega, Madison, WI) was utilized to assess cell proliferation. The assay utilizes the soluble compound 3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-3-(4-sulfophenyl)-2H-tetrazolium (MTS) with an electron coupling reagent, phenazine methosulfate (PMS). MTS is bio-reduced by actively proliferating cells into a formazan product which is measured at 490nm absorbance and is directly proportional to the number of living cells in culture. Proliferation was measured by the MTS assay as described by the manufacturer. Briefly, 96 well plates were seeded with HT-29 or CaCo-2Bbe cells at a density of 1500 cells/well and allowed to adhere for 24 hours followed by incubation with HMO treatments for 72 hours. During the last hour of the incubation period, MTS/PMS was added and plates continued to incubate at 37°C at 5% CO₂. Absorbance was then measured at 490 nm. Results are expressed as a percent of control (0 mg/L).

Cellular differentiation

HT-29 cells and CaCo-2Bbe cells were seeded separately in 75 cm² tissue culture flasks at a density of 10⁴ cells/cm² and maintained for either 48 hours (pre-confluent HT-29 and pre-confluent CaCo-2Bbe cells) or 19 days (post-confluent CaCo-2Bbe cells). Following the adherence period, media were changed and HMO treatments applied and allowed to incubate for

72 hours in the incubator. After the 72 hour incubation period, cells were trypsinized, pelleted by centrifugation at 500 x g for 5 minutes and washed two times with PBS. Cell pellets were flash frozen in liquid nitrogen and stored at -80°C until analysis. Cellular differentiation was measured by alkaline phosphatase (AP) activity. AP activity was measured by commercially available fluorometric and colorimetric assay kits (K422 and K412, BioVision, Mountain View, CA). AP activity in HT-29 cells was determined fluorometrically following homogenization of cells in Assay Buffer then centrifuging for 3 minutes at 13,000 x g to remove insoluble material. Cell supernatants will then be added to black 96 wells plate and incubated with 4-methylumbelliferyl phosphate disodium salt (MUP) substrate for 3 hours at room temperature protected from light. The reaction was stopped by the addition of Stop Solution and read at excitation=360nm and emission=440nm. Background controls were used for all samples by adding Stop Solution to the wells prior to addition of MUP. Final AP activity was determined by subtracting background control values and quantifying activity based on standard curve methodology. AP activity was normalized to protein content as measured by the Lowry assay. AP activity in CaCo2-Bbe cells was quantified in a similar manner with the following exceptions: p-nitrophenyl phosphate was used as a phosphatase substrate and measured colorimetrically in clear 96 wells plates at 405 nm in a plate reader after 30 minutes incubation at room temperature. Results are expressed as AP activity units per milligram protein.

Apoptosis

Caspase-3 activity was used as a marker of cellular apoptosis and measured by enzymatic assay. The caspase-3 colorimetric assay utilizes the hydrolysis of the peptide substrate acetyl-Asp-Glu-ValAsp p-nitroanilide (Ac-DEVD-pNA) by caspase-3, resulting in the release of the p-nitroaniline (pNA) moiety which can be measured at an absorbance of 405 nm and calculated

using standard curve methodology. The caspase-3 assay kit (CASP-3-C, Sigma) was used according to manufacture directions. Briefly, CaCo-2Bbe cells were seeded at a density of 10^4 cells/cm² in 75 cm² tissue culture flasks, maintained for 19 days, then media containing HMO treatments were applied. After the 72 hour incubation period, cells were washed three times with ice-cold PBS then removed by scraping with a plastic cell scraper into 3.5 ml of PBS. Following centrifugation at 500 x g for 5 minutes, PBS was removed and cell pellets flash frozen in liquid nitrogen and stored at -80°C until analysis. Caspase-3 activity was quantified following cells lysis and centrifugation at 20,000 x g for 15 minutes at 4°C. Cell lysate was added in triplicate to a 96 well plate. Assay buffer and caspase-3 substrate were added to each well and incubated at 37°C for 90 minutes. Absorbance was read at 405 nm and results calculated based on a p-nitroaniline calibration curve. Positive and negative controls were included on each plate. Caspase-3 activity was normalized to protein content measure by BCA assay. Results are expressed as caspase-3 activity units per milligram protein.

Cell cycle analysis

HT-29 cells and CaCo-2Bbe cells were seeded separately in 75 cm² tissue culture flasks at a density of 10^4 cells/cm² and allowed to adhere for 48 hours. Thereafter, media were changed, HMO treatments applied, and cells were then allowed to grow for an additional 72 hours. At the end of the treatment period, pre-confluent HT-29 and pre-confluent CaCo-2Bbe cells were trypsinized, pelleted by centrifugation at 500 x g for 5 minutes, and washed two times with PBS. Cells were fixed by adding 1×10^6 cells to 9 ml of ice cold 90% ethanol and stored at -20°C until analysis. For cell cycle analysis, cells fixed in ethanol were pelleted by centrifugation at 200 x g for 15 minutes at 4°C then washed two times with cold PBS. Cells were resuspended in propidium iodide (PI)/Triton X-100 staining solution containing DNase-

free RNase and strained through nylon filter capped Falcon tubes. Following incubation at room temperature for 30 minutes, tubes were transfer to ice and protected from light until data acquisition by a Epics XL benchtop flow cytometer. Data are expressed as the distribution of cell populations in different cell cycle stages.

Sucrase and Lactase Activity

CaCo-2Bbe cells were seeded in 75 cm² tissue culture flasks at a density of 10⁴ cells/cm² and maintained for 19 days. Following the adherence period, media was changed and treatments applied. After the 72 hour incubation period, cells were washed three times with ice-cold PBS then removed by scraping with a plastic cell scraper into 3.5 ml of PBS. Following centrifugation at 500 x g for 5 minutes, PBS was removed and cell pellets flash frozen in liquid nitrogen and stored at -80°C until analysis. For measurement of disaccharidase activity, cell pellets were suspended in 1 mL of homogenization buffer (0.9% saline solution containing 1 uM phenylmethylsulfonyl fluoride and 223 uM of iodoacetic acid) and homogenized for 30 seconds on setting 5 on a homogenizer. Homogenates were incubated with either sucrose or lactose buffers for 1 hour in a 37°C water bath. Enzyme activity was stopped by the addition of Ba(OH)₂ and ZnSO₄. Background control tubes were immediately stopped with Ba(OH)₂ and ZnSO₄ prior to incubation to quantify non-specific disaccharidase activity. Samples were centrifuged for 20 minutes at 3000 rpm at 4°C. Supernatants were added in triplicate to a clear 96 well plates, glucose oxidase reagent (Sigma) was added and allowed to incubate at room temperature for 10 minutes in order to detect the amount of glucose released from the hydrolysis of the disaccharides. The plates were read at 500 nm in a spectrophotometer and quantified using standard curve technique. Disaccharidase activity was normalized to cellular protein

content of the same homogenate as measured by the Lowry assay and expressed as the amount of glucose release per hour per milligram protein.

Transepithelial barrier function and electrogenic nutrient transport

CaCo-2Bbe cells were seeded at a density of 1×10^5 cells/cm² into polyester, 0.4 micron pore size, 1.12 cm² Transwell Snapwell inserts (Sigma) and maintained in an incubator for 18 days with twice weekly medium renewal. On day 18, media were replaced and cells were exposed to media containing HMO treatments. On day 21, Transwell Snapwell inserts were mounted in modified Ussing chambers connected to dual channel voltage/current clamps interfaced with a computer system allowing for real-time data acquisition and analysis (Physiologic Instruments, Inc, San Diego, CA). Chambers containing 4 milliliters of modified Kreb's solution on both the apical chamber and basolateral chamber were maintained at 37°C by a circulating water bath (Fischer Scientific, Ithasca, IL). Basal transepithelial resistance, short-circuit current, and potential differences were measured after a 10 to 15 minute equilibration period. Sodium-dependent nutrient transport was quantified by measuring changes in the short circuit current induced following the addition of 10 mmol/L glucose, glutamine, arginine or glysar.

Statistical Analysis

Statistical differences between treatments were determined using a one-way analysis of variance (ANOVA) with paired comparisons versus the 0 mg/L control. Normality was tested using the Shapiro-Wilk statistic. Logarithmic transformations were utilized as needed. Computations were conducted using a mixed model in SAS (Version 9.1; SAS Institute, Cary, NC). Statistical significance was defined as $p \leq 0.05$, and trends were reported at $p \leq 0.01$. Data are reported as mean $\pm$ SEM.

RESULTS

Study 1

The first study determined the time course of impact of a model HMO, LNnT. We found that LNnT significantly reduced proliferation in pre-confluent HT-29 cells following incubation for 24 ($p=0.021$), 48 ($p=0.012$), and 72 hours ($p=0.0039$) (**Figure 5.2**); however, a consistent dose effect was not seen until 72 hours of incubation. After 72 hours of incubation with 100, 200 and 400 mg/L LNnT, cellular proliferation was reduced to 67.5%, 68.3% and 70.6% (SEM 10.4%), respectively, as compared to the 0 mg/L control. Pre-confluent CaCo-2Bbe cell proliferation was significantly reduced after 24 hours ($p<0.001$) and 72 hours ($p=0.005$); however, no significant effect was seen during the 48 hour incubation period ($p=0.12$) likely related to higher variation at this time point (**Figure 5.3**). LNnT did not affect markers of proliferation in the slowly proliferating confluent and post-confluent cells at any time point (data not shown).

Cellular differentiation, measured by AP activity, was not significantly affected by incubation with LNnT in the pre-confluent, confluent, or post-confluent CaCo-2Bbe cells at any time point. The ability to detect significant differences was limited by the high variability in AP activities. Although treatment did not affect differentiation, time and cell type (pre-confluent, confluent and post-confluent) did have significant effects (**Figure 5.4**).

The conclusion of study 1 was that incubation with LNnT for 72 hours most efficaciously reduced proliferation in both CaCo-2Bbe and HT-29 cells and was therefore used as the incubation period for the second trial.

Study 2

In the second study, dependent variables were assessed at one time point, 72 hours of incubation, with 3 different HMOs at 3 different levels each. The effects of treatment with each of the three individual HMO on proliferation were assessed by MTS assay in the second preliminary study (**Table 5.2**). Treatment with LNnT reduced proliferation ($p=0.041$) in pre-confluent HT-29 cells with significant treatment effects seen at 200 and 2000 mg/L as compared to 0 mg/L control. Treatments of 200 and 2000 mg/L LNnT resulted in proliferation rates of 94.4% and 92.1%, respectively, as compared to the 0 mg/L control. Incubation with 2000 mg/L 2'FL decreased proliferation ($p<0.001$) in pre-confluent HT-29 cells to 75.3% as compared to 0mg/L control and 6'SL decreased proliferation ($p=0.024$) at 400 mg/L and 4000 mg/L to 92.3% and 88.3%, respectively, as compared to the 0 mg/L control.

Pre-confluent CaCo-2Bbe cells were less sensitive to treatment with HMOs. Proliferation was significantly reduced in the highest doses of LNnT, 2'FL or 6'SL (**Table 5.2**). Energy controls within each HMO treatment group in both cell types were not significantly different from 0 mg/L controls suggesting that the reductions in proliferation were related to the presence of the HMOs and not the terminal lactose plus n-acetyllactosamine, fucose, or sialic acid.

Cell cycle analysis by flow cytometry was conducted in pre-confluent HT-29 (**Figures 5.5, 5.6, and 5.7**) and CaCo-2Bbe cells (**Figure 5.8**). Treatment with 200 mg/L LNnT in pre-confluent HT-29 cells induced changes in all three cell cycle phases as compared to 0 mg/L with cells exhibiting G0/G1 ($p=0.026$) and G2/M arrest ($p=0.027$) in coordination with a significant decrease ($p<0.001$) in the percentage of gated cells in the S phase (**Figure 5.5**). Additionally, treatment with 2000 mg/L of LNnT also resulted in G2/M arrest ($p=0.003$) and tended to

decrease ($p=0.085$) the proportion of cells in G0/G1 as compared to 0 mg/L controls (**Figure 5.5**). Similar to the effects of the neutral LNnT treatment, 2'FL treatment at 2000 mg/L induced cell cycle arrest ($p=0.021$) in the G2/M phase which was balanced by a decrease ($p=0.034$) in the proportion of cells in the G0/G1 phase (**Figure 5.6**). Treatment with the acidic HMO fraction 6'SL revealed arrest ($p<0.001$) in the S phase at the highest dose of 4000 mg/L with a corresponding decrease ($p=0.003$) in the proportion of cells in the G2/M phase (**Figure 5.7**). Interestingly, a significant decrease ($p=0.001$) in the proportion of cells in the S phase was observed in the 400 mg/L 6'SL treatment group with cell arrest tending ($p=0.058$) to occur in the G2/M phase (**Figure 5.7**).

A significant treatment effect was also observed at the 4000 mg/L 6'SL in the CaCo-2Bbe cells with cell cycle arrest ($p=0.016$) in the G2/M phase and decreased ($p=0.0013$) proportion of gated cells in the G0/G1 phase (**Figure 5.8**). Conversely, the effect of 400 mg/L 6'SL on pre-confluent CaCo-2Bbe cells resulted in accumulation ($p=0.013$) of cells in the G0/G1 phase. This was balanced with a decrease ($p=0.035$) in the percentage of gated cells in the G2/M phase. Neither LNnT nor 2'FL affected the cell cycles in the pre-confluent CaCo-2Bbe cells (data not shown). As with proliferation, cell cycle kinetics were not affected by the energy controls within any HMO treatment group in either cell type suggesting that progression through the cell cycle is related to the presence of the respective HMOs and not the terminal lactose plus n-acetyllactosamine, fucose, or sialic acid.

Cellular differentiation was measured via AP activity in post-confluent CaCo-2Bbe cells and pre-confluent HT-29 cells. Of the three HMOs, only 2'FL had a significant effect or tended to significantly affect differentiation. Pre-confluent HT-29 cells (**Figure 5.9**) exhibited a 30.9% (SEM 6.8%) increase ($p=0.030$) in AP activity as compared to 0 mg/L control while post-

confluent CaCo-2Bbe cells (**Figure 5.10**) tended to exhibit a 33.1% (SEM 13.7%) increase ($p=0.071$) in AP activity at 2000 mg/L 2'FL.

Cellular apoptosis, measured by caspase-3 activity, was determined in post-confluent CaCo-2Bbe cells. In cells treated with LNNt (**Figure 5.11**) and 6'SL (**Figure 5.12**), a significant increase in apoptosis was exhibited in the positive energy control groups (lactose + n-acetyllactosamine and lactose + sialic acid) and the highest treatment doses (2000 mg/L LNNt and 4000 mg/L 6'SL). In the cells treated with LNNt, caspase-3 activity increased ($p=0.036$) by 79.6% (SEM 13.6%) in the 2000 mg/L treatment group and increased ($p=0.010$) by 101.0 % (SEM 12.1%) in the energy control group (lactose + n-acetyllactosamine). Treatment with 6'SL resulted in an increase ($p=0.033$) of 71.4% (SEM 14.1%) in the 4000 mg/L treatment group while the energy control group (sialic acid + lactose) increased ($p=0.034$) by 76.3% (SEM 16.7%) as compared to the 0 mg/L control. 2'FL did not affect caspase-3 activity in post-confluent CaCo-2Bbe cells.

Functional indices were also assessed in post-confluent CaCo-2Bbe cells including sucrase and lactase activity, transepithelial resistance, and nutrient transport. Cells treated with 2000 mg/L of 2'FL (**Figure 5.13**) exhibited a 53.5% (SEM 6.6%) increase ($p=0.036$) in sucrase activity as compared to 0 mg/L control. There were no other significant treatment effects with regard to HMO treatments as compared to 0 mg/L controls for either sucrase or lactase activity. Interestingly, there was a significant treatment effect of the positive energy control (**Figure 5.14**), lactose + sialic acid, in which sucrase activity was enhanced ($p=0.042$) by 48.5% (SEM 15.4%) as compared to the 0 mg/L control. Of the outcomes measured in modified Ussing chambers, transepithelial resistance (**Figure 5.15**) significantly increased ($p=0.002$) by 21.1% (SEM 3.3%) in post-confluent CaCo-2Bbe cells treated with 200 mg/L LNNt and tended to

increase ($p=0.059$) by 12% (SEM 3.6%) in the 2000 mg/L treatment group as compared to 0 mg/L control. Treatment with LNnT, 2'FL or 6'SL did not alter basal short-circuit current, potential difference or electrogenic nutrient transport.

Table 5.3 provides a summary of all epithelial cytokinetic and epithelial function results for each cell type.

DISCUSSION

The GI tract undergoes numerous structural and functional changes as it adapts to extra-uterine life (Carver and Barness, 1996). Only recently have HMOs been identified as dietary components which may aid in maturation of the developing infant's GI tract. Studies by Kuntz and colleagues (2008) demonstrated that neutral and acidic HMO fractions and individual oligosaccharides affect proliferation, differentiation, and apoptosis *in vitro* in non-transformed fetal intestinal crypt cell line, HIEC, and in transformed human intestinal cell lines, HT-29 and CaCo-2 (Kuntz et al., 2008). The present study investigated the effects of three individual HMOs (2'FL, LNnT, and 6'SL) on epithelial cell differentiation utilizing a more comprehensive model of the crypt-villus axis consisting of pre-confluent HT-29 cells and CaCo-2Bbe cells at pre-confluent, confluent, and post-confluent states. A cell culture model was the most appropriate model for assessing the effects of HMOs on specific regions along the crypt-villus axis. Utilizing a well-controlled, reproducible *in vitro* model allowed for characterization of cellular kinetics following treatment with specific HMOs. Further, it allowed us to overcome the obstacle of limited availability of the HMOs of interest.

With regard to selection of HMOs studied, although nearly 200 oligosaccharides are present in human milk, the neutral and acidic oligosaccharide fractions of human milk are

primarily made up of fourteen neutral and six acidic oligosaccharides (Thurl et al., 2010). The three HMOs investigated in this trial were selected based on their predominance in human milk, with 2'FL and LNnT being dominant oligosaccharide within the neutral fraction and 6'SL representing the largest proportion of the acidic fraction.

We demonstrated that each of the three individual HMOs dose-dependently inhibited proliferation in pre-confluent HT-29 cells with an associated cell cycle arrest primarily within the G2/M phase, with 2'FL also increasing differentiation. AP activity, a marker of cell differentiation was enhanced with treatment of the physiologically relevant dose of 2000 mg/L 2'FL. Our cell proliferation and cell cycle results were similar to those of Kuntz and colleagues. We differed though in that we found a single neutral oligosaccharide to enhance differentiation where they found the total acidic HMO fraction as well as 6'SL to have the greatest stimulatory effect on AP activity. A potential explanation for our differing results in HT-29 cell differentiation may relate to the medium. While we cultured our HT-29 cells in the ATCC recommended McCoy's 5A, Kuntz et al. used RPMI 1640. As culture conditions such as limiting glucose and supplementing galactose have been shown to enhance HT-29 cell differentiation it is possible that their use of RPMI 1640 was more conducive to enhancing differentiation (Lindley et al., 2012). Further, while we utilized HT-29 cells between passages 5 and 15, they utilized later passages (40 to 50) which have been shown in some case to have enhanced differentiation when compared to early passages (Chantret et al., 1994).

We found pre-confluent Caco-2Bbe cells to be less sensitive to inhibition of proliferation with only the highest doses of the individual HMO having a significant effect and only 6'SL effecting progression through the G2/M phase. Differentiation was assessed in CaCo-2BBe cells at 21 days post-confluence and similar to above, 2'FL tended to have a stimulatory effect on AP

activity. We also quantified disaccharidase activities in the post-confluent CaCo-2Bbe cells and found sucrase activity to be a more sensitive and specific measure of differentiation within these cells, demonstrating a statistically significant 50% increase in activity with 2000 mg/L 2'FL. Our proliferation results were again similar to Kuntz and colleagues (Kuntz et al., 2008). As they reported cell cycle differences in fractions at 15 g/L after 24 hours, it is likely that the timing of our treatments allowed for changes to be detected in cell cycle at such low doses as 6'SL at 4000 mg/L for 72 hours was found to arrest pre-confluent Caco-2Bbe cells within the G2/M phase. To the best of our knowledge, this is the first time that an individual human milk oligosaccharide has been shown to enhance sucrase activity *in vitro*.

Cellular caspase-3 activity, a measure of apoptosis, increased in post-confluent Caco-2Bbe cells treated with 2000 mg/L LNnT. Interestingly, LNnT also enhanced transepithelial resistance in post-confluent CaCo-2Bbe cells. The increase in apoptosis paralleled enhancement of transepithelial resistance leading us to conclude that LNnT may have stimulated maturation of these cells to enhance an especially important intestinal characteristic in developing neonates. This is further evidenced by the increase in apoptosis in post-confluent CaCo-2Bbe cells treated with lactose + sialic acid and their subsequent enhancement in sucrase activity. These enhancements in intestinal maturation *in vitro* are important findings when one considers the protective effects of human milk in premature infants, a population with an especially immature gut. It is well established that human milk decreases the incidence of necrotizing enterocolitis (NEC) in infants when compared to infant formula (Sisk et al., 2007). Recently Jantscher-Krenn and co-workers demonstrated that a single human milk oligosaccharide, disialyllactos-N-tetraose (DSLNT), increased survival in a rat pup model of NEC (Jantscher-Krenn et al., 2011). While the etiology of NEC remains undefined, impaired intestinal epithelial barrier function is often

implicated in the pathogenesis. Taken together, the enhancement of transepithelial resistance following treatment with LNT in our trial and the increased NEC survival following treatment with DSLNT demonstrated by Jantscher-Krenn et al suggest that HMOs may specifically have a role in the overall protective effects of human milk against NEC. More *in vivo* research is needed to determine if our *in vitro* effects of LNT will translate into pre-clinical models.

In summary, we observed that 2'FL, LNT, and 6'SL individually inhibited proliferation in pre-confluent transformed small intestinal cell lines HT-29 and Caco-2Bbe with associated G2/M cell cycle arrest. More importantly, within our model of the crypt-villus axis, the inhibition of proliferation was associated with increased epithelial differentiation within HT-29 cells treated with a physiologically relevant dose of 2'FL. Post-confluent CaCo-2Bbe cells used to model the well differentiated villus enterocytes demonstrated greater digestive and barrier function following treatment with doses of 2'FL and LNT that reflect concentrations found in human milk. Further study is needed to assess whether synergist effects occur following concomitant administration of HMOs as these results indicate that HMOs may promote maturation of the small intestine. *In vivo* research is needed to determine if these *in vitro* effects on epithelial cytokinetics and epithelial function continue within the complex milieu of the GI tract.

FIGURES AND TABLES

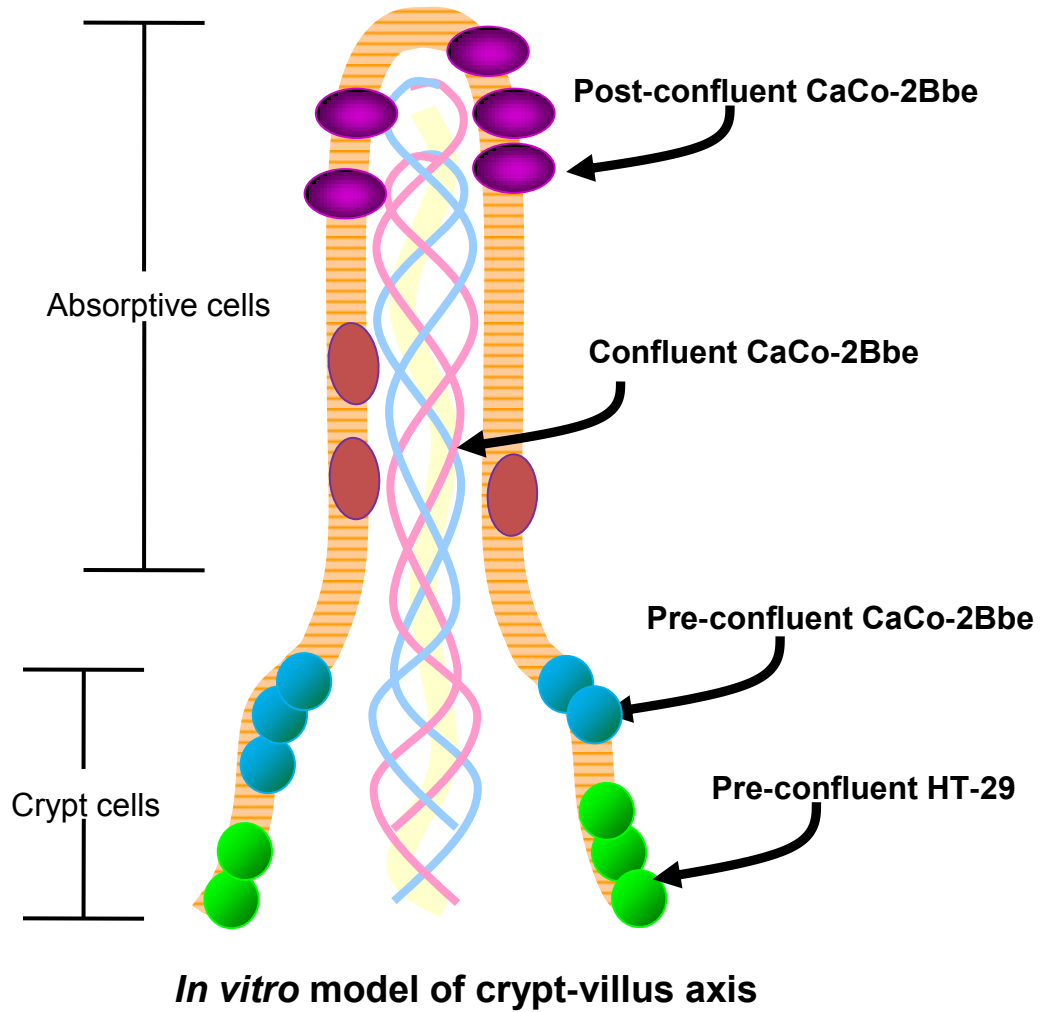


Figure 5.1. *In vitro* model of the crypt-villus axis consisting of individually cultured pre-confluent HT-29 cell, pre-confluent CaCo-2Bbe, confluent CaCo-2Bbe, and post-confluent CaCo-2Bbe cells to represent the increasing levels of differentiation progressing from crypt to villus tip.

Treatment Groups	Concentration (mg/L)
LNnT Control	0
LNnT Energy Control ¹ (lactose + n-acetyllactosamine)	154
LNnT – low	20
LNnT – physiological	200
LNnT – high	2000
2'FL Control	0
2'FL Energy Control (lactose + fucose)	154
2'FL – low	20
2'FL – low	200
2'FL – physiological	2000
6'SL Control	0
6'SL Energy Control (lactose + sialic acid)	154
6'SL – low	40
6'SL – physiological	400
6'SL – high	4000

Table 5.1. Study 2 treatment groups. 2'FL, 2'-fucosyllactose; LNnT, lacto-N-neotetraose; 6'SL, 6'-sialyllactose. ¹60 mg/L lactose plus 94 mg/L of other sugar.

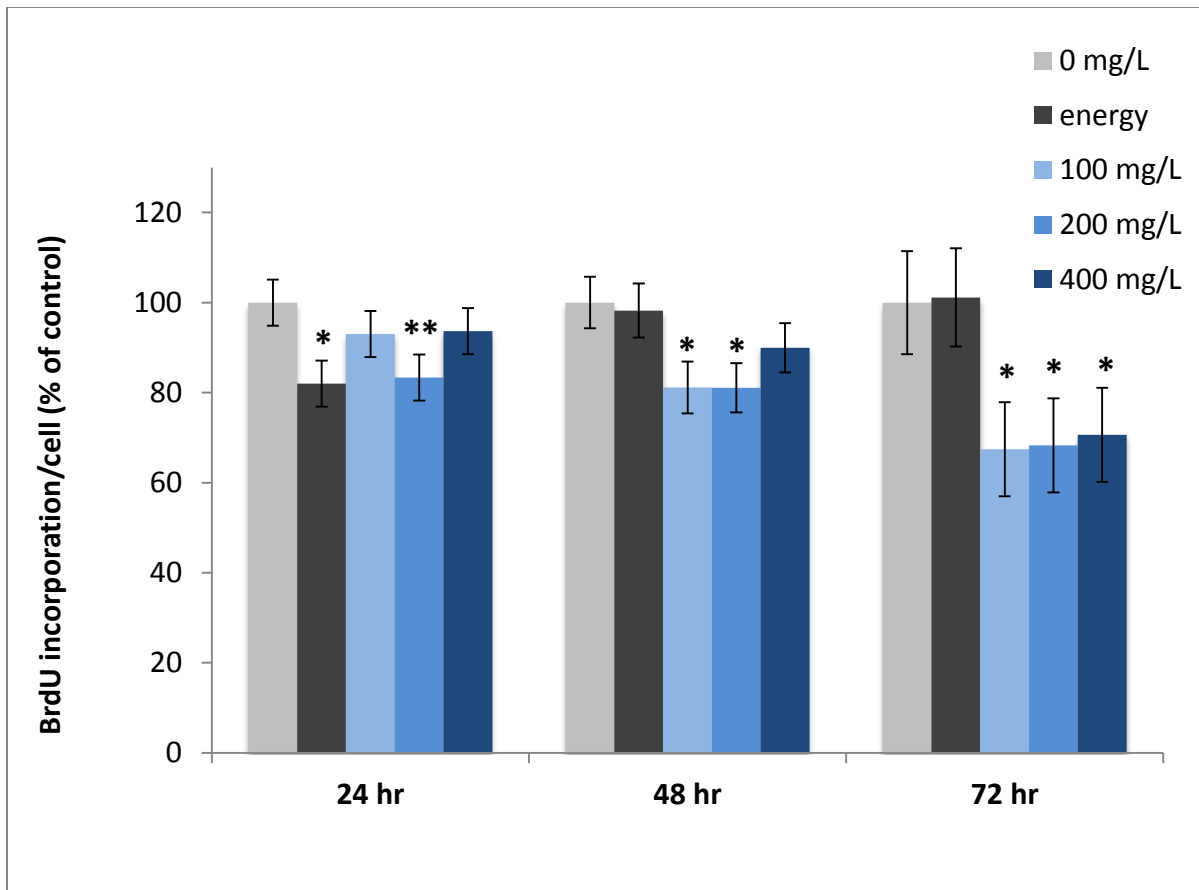


Figure 5.2. Effect of incubation with lacto-N-neotetraose (LNnT) for 24, 48 or 72 hours on proliferation in pre-confluent HT-29 cells. Proliferation significantly decreased following incubation with LNnT for 24 ($p=0.021$), 48 ($p=0.012$) and 72 ($p=0.0039$) hours, with the most consistent effect at 72 hours. Energy references to lactose + n- acetyllactosamine. Values are mean $\pm$ SEM. Asterisks denote statistically significant difference in treatment means as compared to 0 mg/L control within each time period by one-way ANOVA and paired-comparisons (* $p<0.05$, ** $p<0.01$).

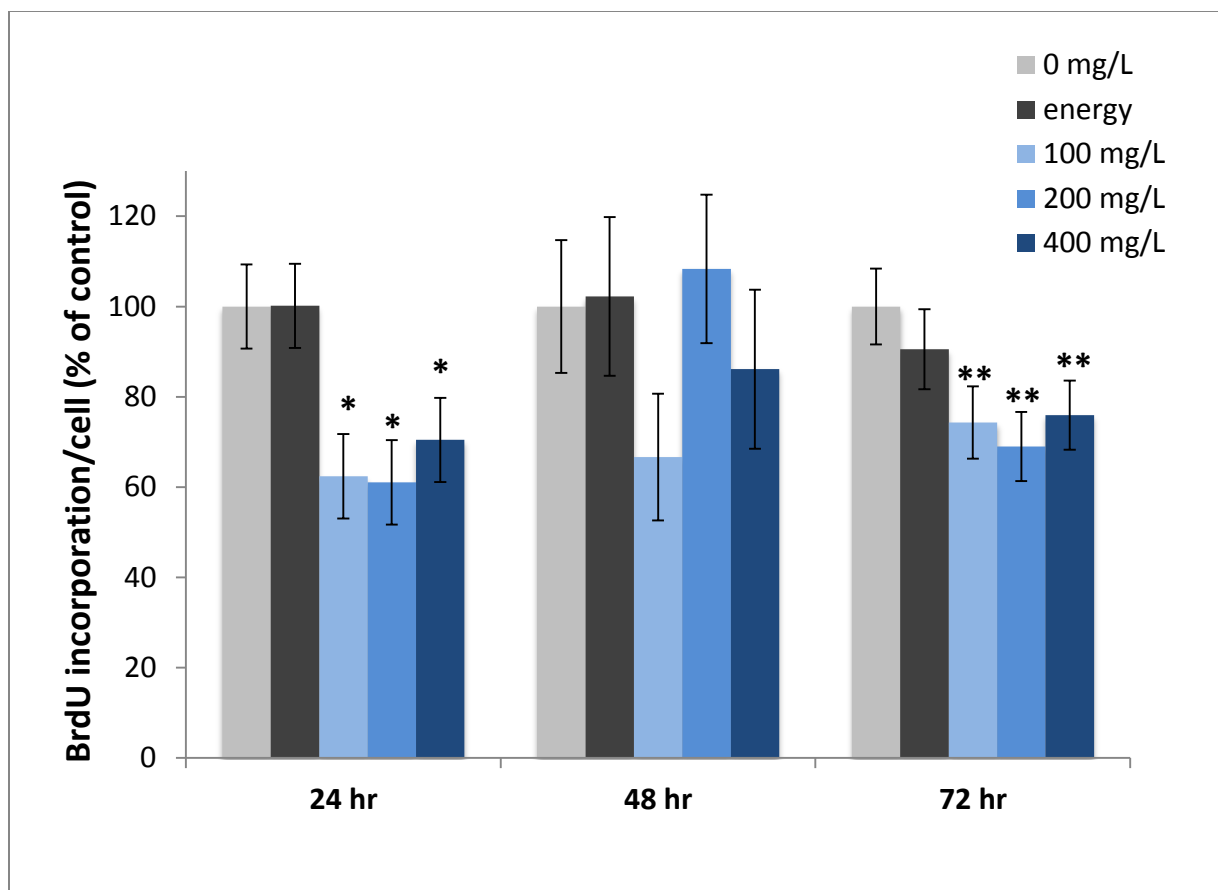


Figure 5.3. Effect of incubation with lacto-N-neotetraose (LNnT) for 24, 48 or 72 hours on proliferation in pre-confluent CaCo-2Bbe cells. Proliferation significantly decreased following incubation with LNnT for 24 ($p<0.001$) and 72 ($p=0.0054$) hours, with the most consistent effect at 72 hours. No treatment effect was seen at 48 hours ($p=0.12$). Energy references to lactose + n- acetyllactosamine. Values are mean $\pm$ SEM. Asterisks denote statistically significant difference in treatment means as compared to 0 mg/L control within each time period by one-way ANOVA and paired-comparisons (* $p<0.05$, ** $p<0.01$).

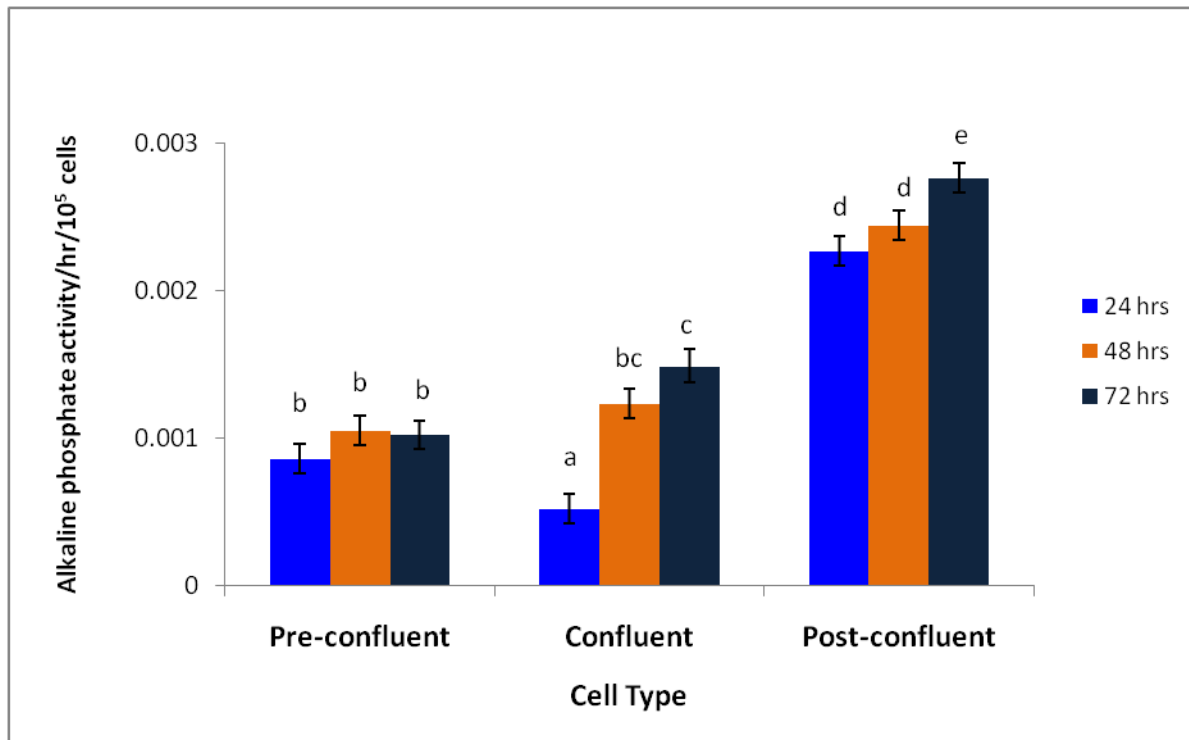


Figure 5.4. Effect of incubation with lacto-N-neotetraose (LNnT) for 24, 48 or 72 hours on differentiation in pre-confluent, confluent, and post-confluent CaCo-2Bbe cells.

Differentiation was not altered by treatment ($p=0.47$); however, alkaline phosphatase (AP) activity significantly increased with time ($p<0.001$), cell type ($p<0.001$) and in the interaction between cell type and time ($p=0.001$). Values are pooled means of all LNnT treatments (0 mg/L, 100 mg/L, 200 mg/L, 400 mg/L, and lactose +n-acetylglucosamine) for the respective time point and cell type. Values are means $\pm$ SEM. Means that do not share a common letter are significantly different as determined by a three-way ANOVA and paired-comparisons.

		HT-29			CaCo-2	
Treatment Groups	Concentration (mg/L)	Mean %	SEM %		Mean %	SEM %
LNnT Control	0	100.0%	2.0		100.0%	2.3
LNnT Energy (lactose + n-acetyllactosamine)	154	96.0	2.0		93.8	2.3
LNnT	20	94.8	2.0		102.4	2.3
LNnT	200	94.4*	2.0		101.3	2.3
LNnT	2000	92.1**	2.0		87.13**	2.3
2'FL Control	0	100%	2.3		100%	2.1
2'FL Energy (lactose + fucose)	154	101.1	2.3		95.6	2.1
2'FL	20	99.5	2.3		105.2	2.1
2'FL	200	97.1	2.3		101.8	2.1
2'FL	2000	75.3***	2.3		89.7*	2.1
6'SL Control	0	100%	2.9		100%	2.2
6'SL Energy (lactose + sialic acid)	154	95.1	3.0		103.2	2.6
6'SL	40	96.7	2.9		107.1*	2.2
6'SL	400	92.3*	2.9		105.4	2.2
6'SL	4000	88.3**	3.0		83.0***	2.2

Table 5.2. Effects of HMO treatments on proliferation in pre-confluent HT-29 and pre-confluent CaCo-2Bbe cells. Values represent the percent proliferation as compared to respective 0 mg/L controls (set at 100.0%). HT-29 cells were more sensitive to reduction in proliferation with significant reductions seen at 200 and 2000 mg/L LNnT, 2000 mg/L 2'FL and 400 and 4000 mg/L 6'SL. Significant reductions were seen at 2000 mg/L LNnT, 2000 mg/L 2'FL, and 4000 mg/L 6'SL in the CaCo-2Bbe cells. Values are mean $\pm$ SEM. Asterisks denote statistically significant difference in treatment means as compared to 0 mg/L control within each treatment group by one-way ANOVA and paired-comparisons (*p<0.05, **p<0.01, ***p<0.001). 2'FL, 2'-fucosyllactose; LNnT, lacto-N-neotetraose; 6'SL, 6'-sialyllactose.

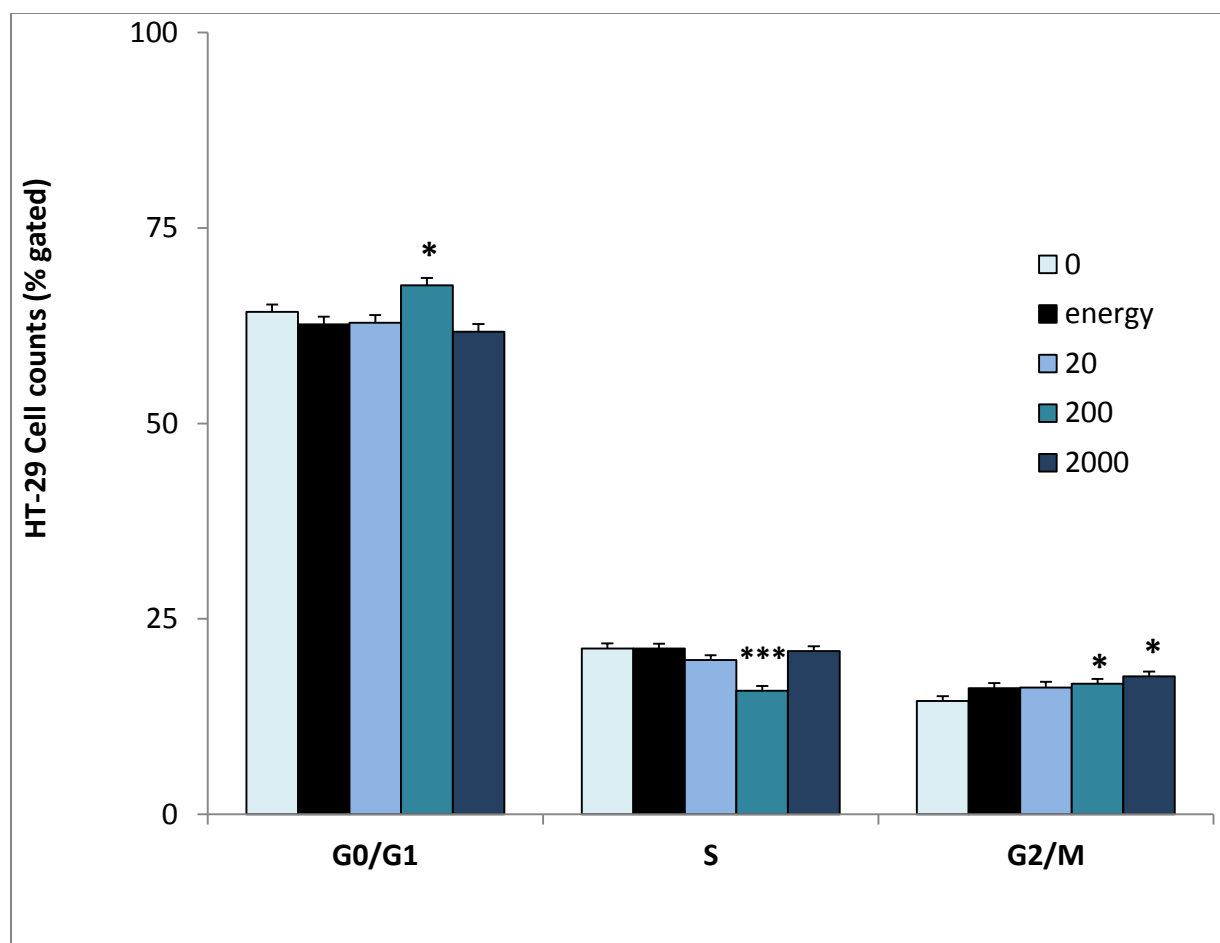


Figure 5.5. Effect of incubation with lacto-N-neotetraose (LNnT) for on cell cycle events in pre-confluent HT-29 cells. Treatment with 200 mg/L of LNnT resulted in both G0/G1 ($p=0.026$) and G2/M arrest ($p=0.027$) in coordination with a decreased ($p<0.001$) proportion of cells in the S phase. Treatment with 2000 mg/L of LNnT resulted in G2/M arrest ($p=0.0033$) and tended to decrease ($p=0.085$) the proportion of cells in G0/G1 as compared to 0 mg/L controls. Energy refers to lactose + n-acetyllactosamine. Values are mean $\pm$ SEM. Asterisks denote statistically significant difference in treatment means as compared to 0 mg/L control within each cell cycle event by one-way ANOVA and paired-comparisons (* $p<0.05$, *** $p<0.001$).

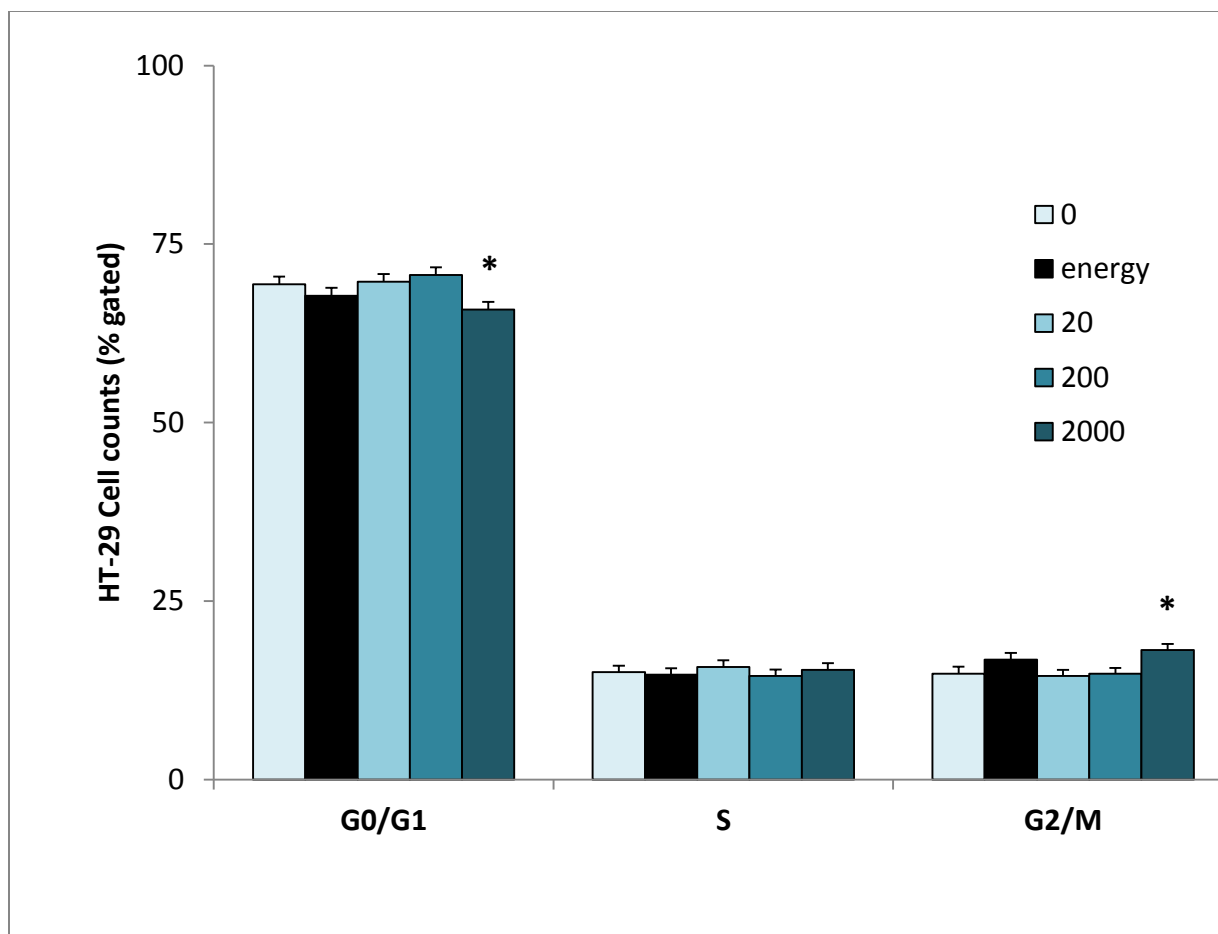


Figure 5.6. Effect of incubation with 2'-fucosyllactose (2'FL) on cell cycle events in pre-confluent HT-29 cells. Treatment of 2000 mg/L 2'FL resulted in cell cycle arrest ($p=0.021$) in the G2/M phase and a decreased ($p=0.034$) proportion of cells in the G0/G1 phase. Energy refers to lactose + fucose. Values are mean $\pm$ SEM. Asterisks denote statistically significant difference in treatment means as compared to 0 mg/L control within each cell cycle event by one-way ANOVA and paired-comparisons ($*p<0.05$).

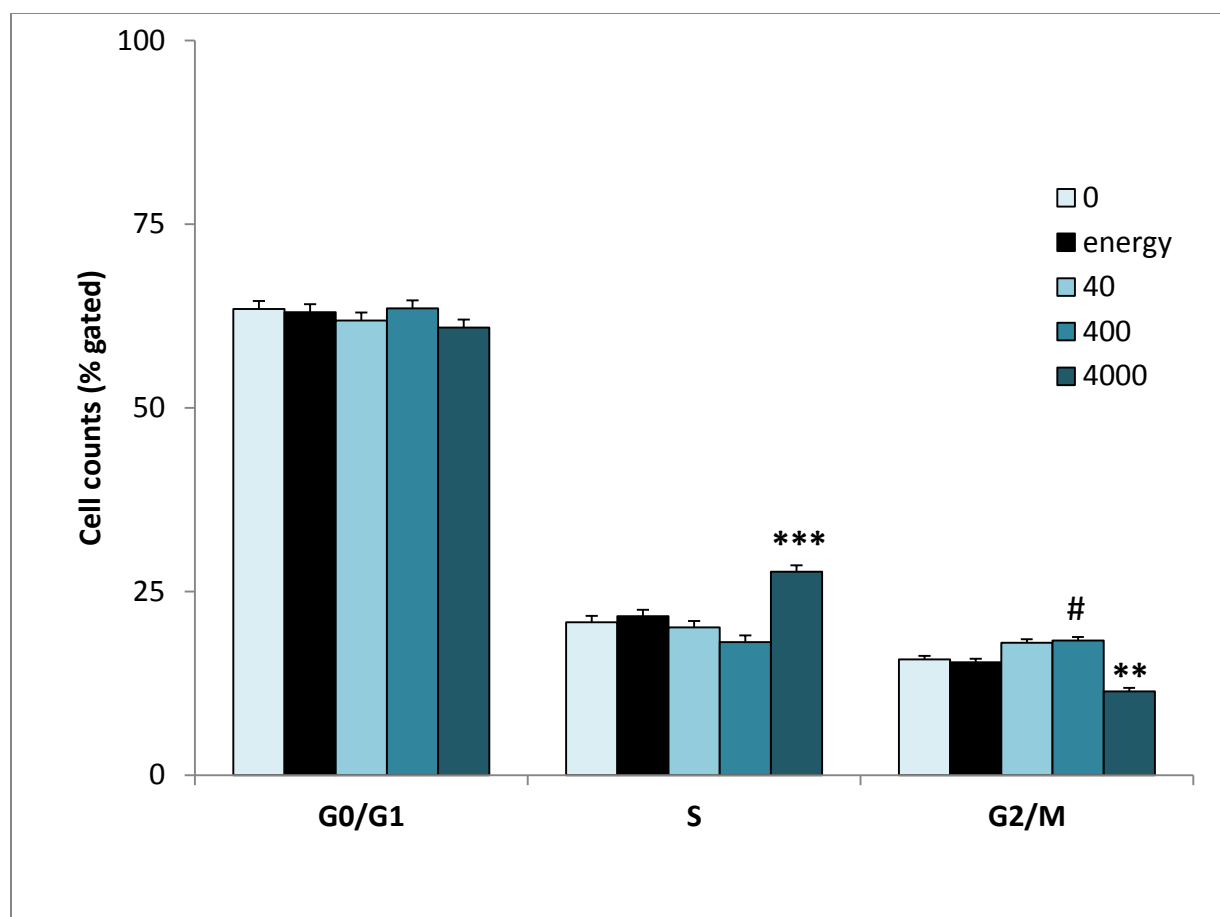


Figure 5.7. Effect of incubation with 6'-sialyllactose (6'SL) on cell cycle events in pre-confluent HT-29 cells. Treatment with 4000 mg/L 6'SL resulted in cell cycle arrest ($p < 0.001$) in the S phase and a decreased ($p = 0.0035$) proportion of cells in the G2/M phase. Treatment with 400 mg/L 6'SL resulted in a significant decrease ($p = 0.0013$) in the proportion of cells in the S phase with cell arrest tending ($p = 0.058$) to occur in the G2/M phase. Energy refers to lactose + sialic acid. Values are mean $\pm$ SEM. Asterisks denote statistically significant difference in treatment means as compared to 0 mg/L control within each cell cycle event by one-way ANOVA and paired-comparisons (# $p < 0.1$; ** $p < 0.01$, *** $p < 0.0001$).

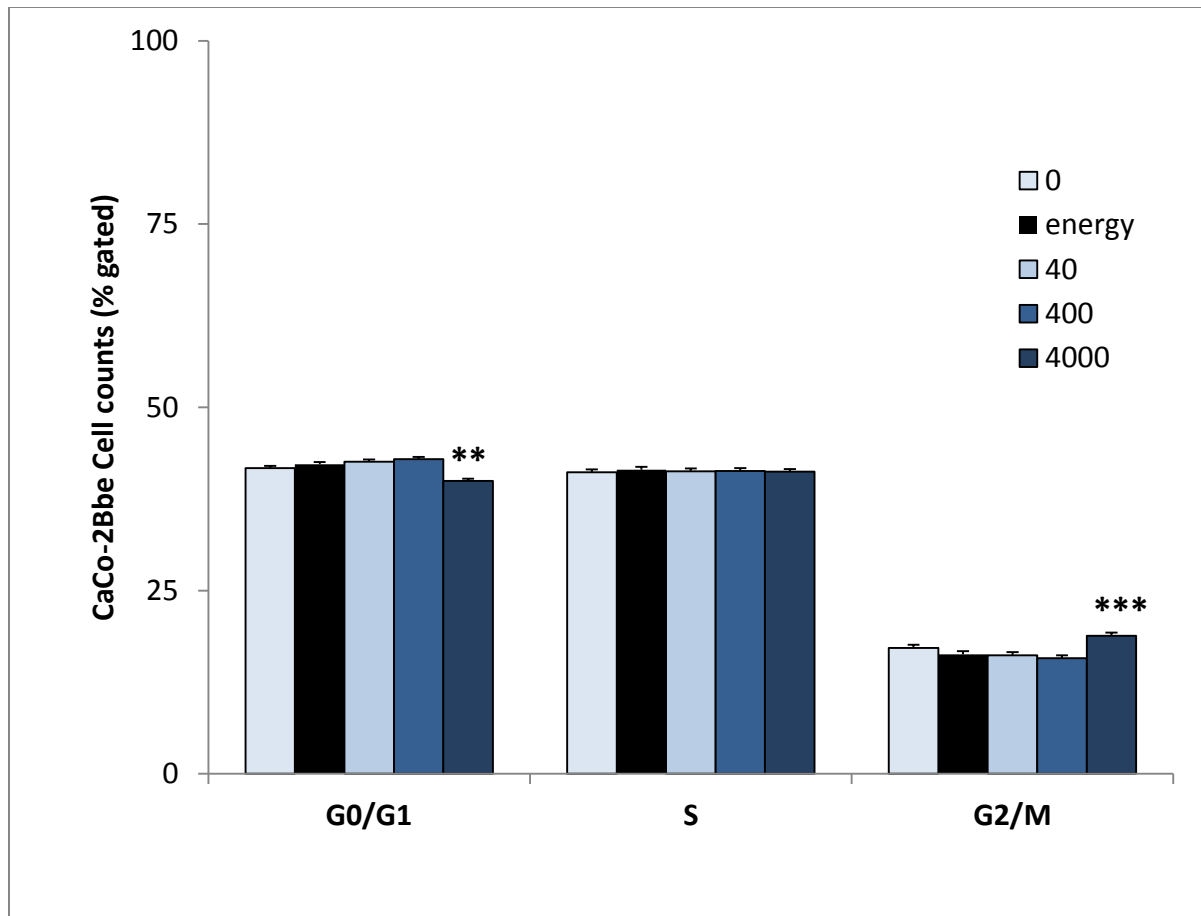


Figure 5.8. Effect of incubation with 6'-sialyllactose (6'SL) on cell cycle events in pre-confluent CaCo-2Bbe cells. Treatment with 4000 mg/L 6'SL resulted in cell cycle arrest ($p=0.016$) in the G2/M phase and a decreased ($p=0.0013$) proportion of cells in the G0/G1 phase. Treatment with 400 mg/L 6'SL resulted in arrest ($p=0.013$) in the G0/G1 phase with a corresponding decrease ($p=0.035$) in the percentage of cells in the G2/M phase. Energy refers to lactose + sialic acid. Values are mean $\pm$ SEM. Asterisks denote statistically significant difference in treatment means as compared to 0 mg/L control within each cell cycle event by one-way ANOVA and paired-comparisons (** $p<0.01$, *** $p<0.0001$).

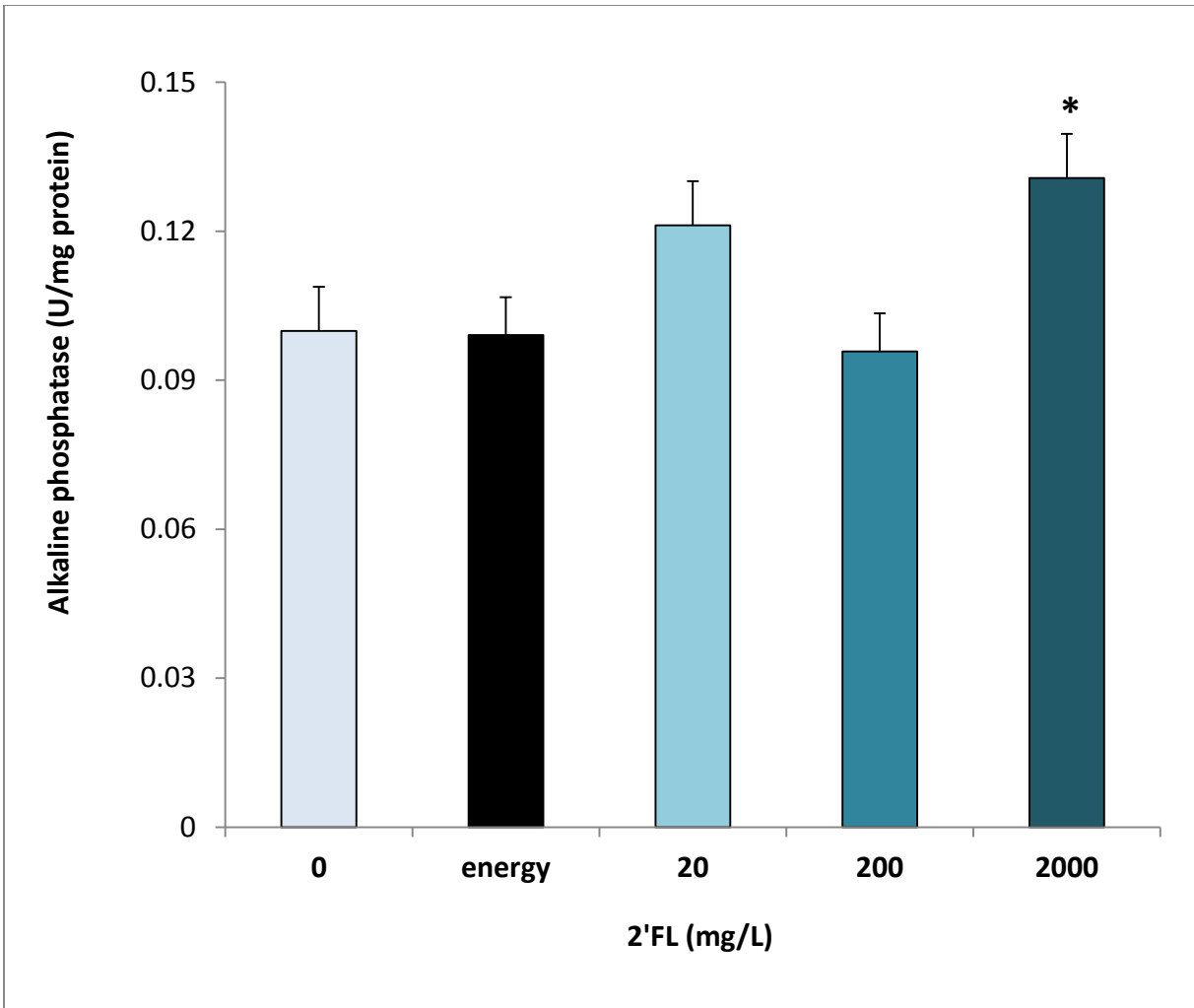


Figure 5.9. Effect of incubation with 2'-fucosyllactose (2'FL) on differentiation in pre-confluent HT-29 cells. A 30.9% (SEM 6.8%) increase ($p=0.030$) in alkaline phosphatase (AP) activity occurred in cells treated with 2000 mg/L 2'FL as compared to 0 mg/L control. Energy refers to lactose + fucose. Values are mean $\pm$ SEM. Asterisks denote statistically significant difference in treatment means as compared to 0 mg/L control by one-way ANOVA and paired-comparisons ($*p<0.05$).

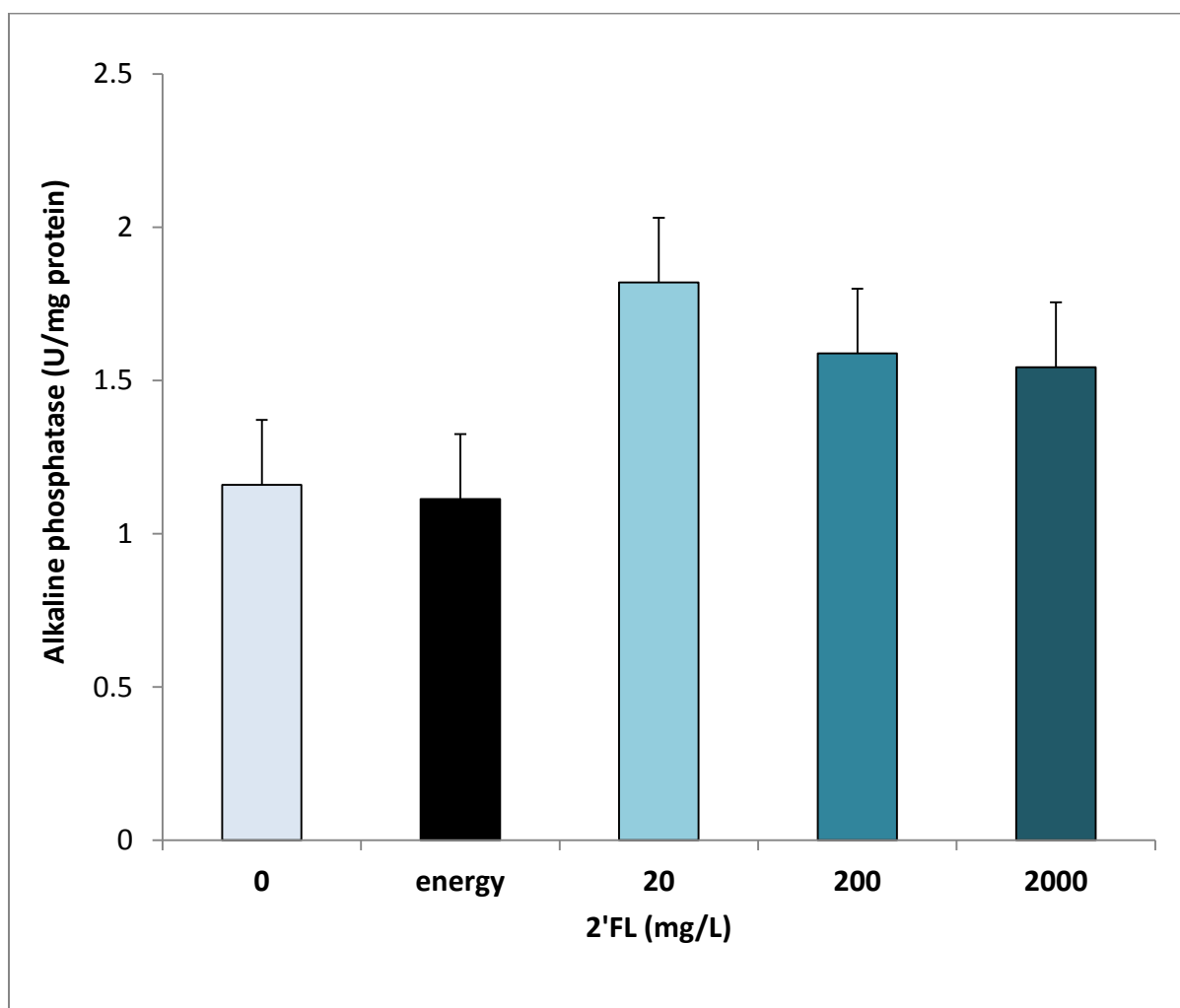


Figure 5.10. Effect of incubation with 2'-fucosyllactose (2'FL) on differentiation in post-confluent CaCo-2Bbe cells. Treatment with 2000 mg/L 2'FL tended to increase ($p=0.071$) alkaline phosphatase (AP) activity by 33.1% (SEM 13.7%) as compared to 0 mg/L control. Energy refers to lactose + fucose. Values are mean $\pm$ SEM. There was no statistically significant difference in treatment means as compared to 0 mg/L control by one-way ANOVA and paired-comparisons.

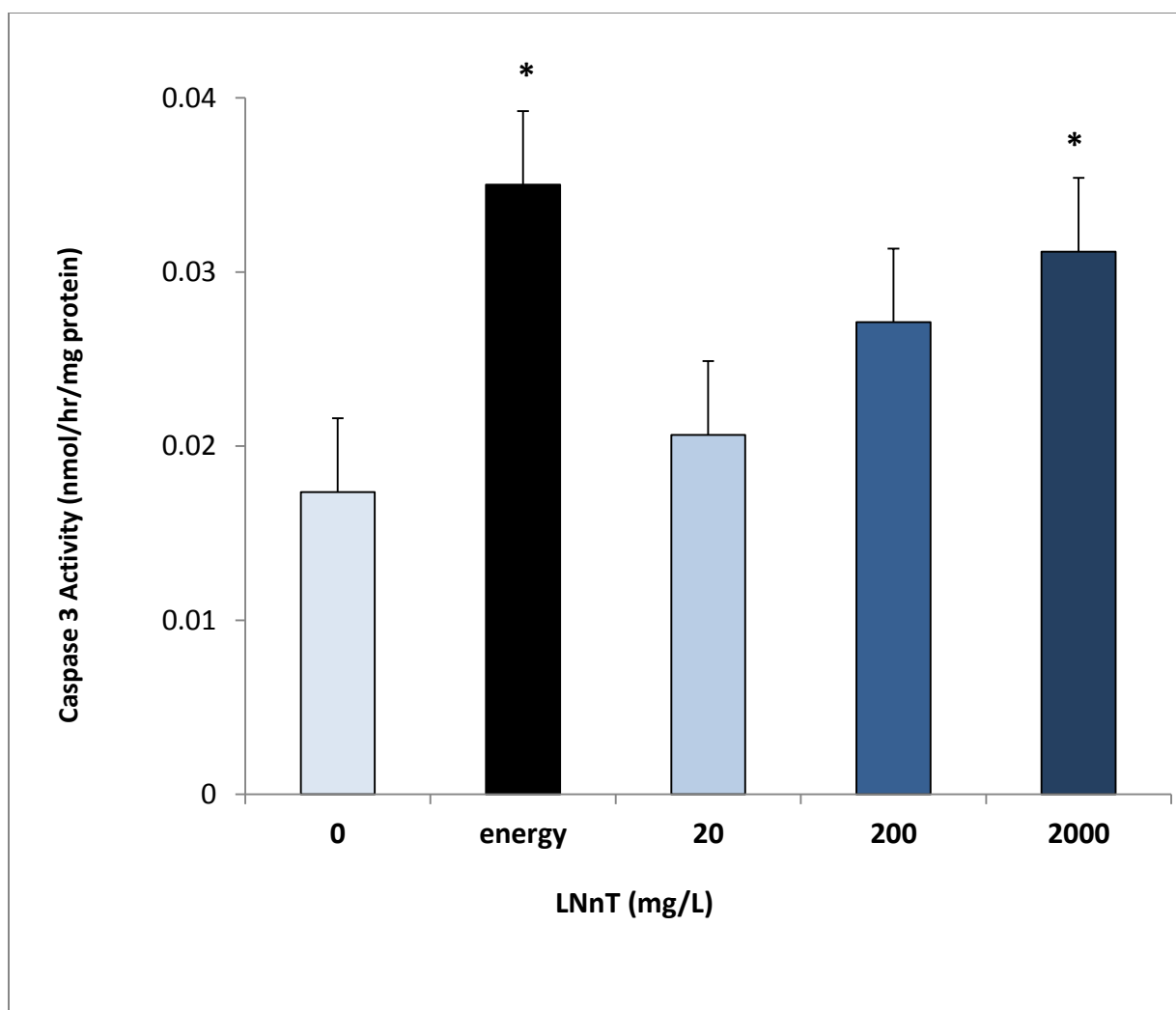


Figure 5.11. Effect of incubation with lacto-N-neotetraose (LNNt) on apoptosis in post-confluent CaCo-2Bbe cells. Treatment with 2000 mg/L LNNt increased ($p=0.036$) caspase-3 activity 79.6% (SEM 13.6%). Lactose plus sialic acid (energy) increased ($p=0.010$) caspase-3 activity 101.0 % (SEM 12.1%) as compared to the 0 mg/L control. Values are mean $\pm$ SEM. Asterisks denote statistically significant difference in treatment means as compared to 0 mg/L control by one-way ANOVA and paired-comparisons (* $p<0.05$).

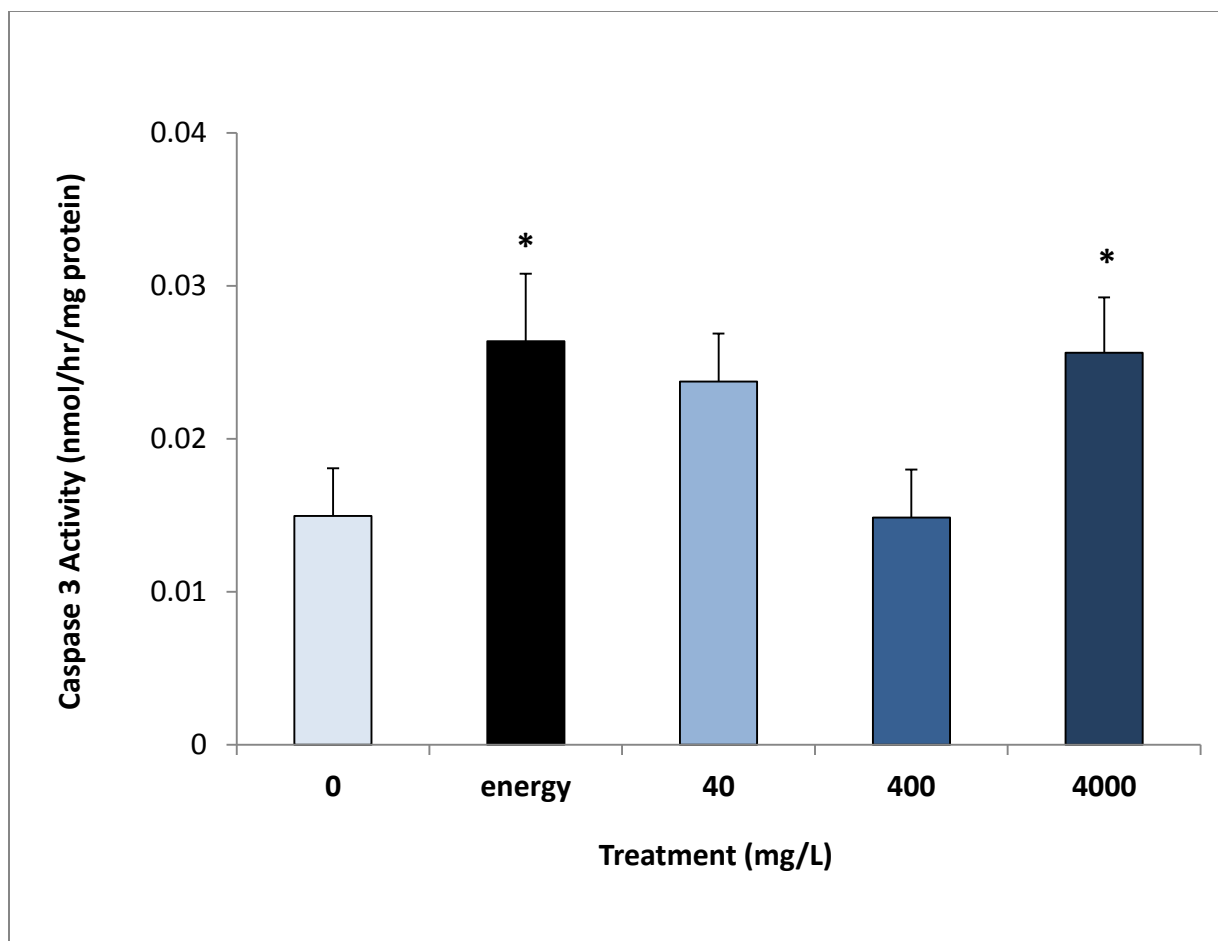


Figure 5.12. Effect of incubation with 6'-sialyllactose (6'SL) on apoptosis in post-confluent CaCo-2Bbe cells. Treatment with 6'SL increased ($p=0.033$) caspase-3 activity 71.4% (SEM 14.1%) in the 4000 mg/L treatment while the energy control group (sialic acid plus lactose) increased ($p=0.034$) by 76.3% (SEM 16.7%) as compared to the 0 mg/L control. Values are mean $\pm$ SEM. Asterisks denote statistically significant difference in treatment means as compared to 0 mg/L control by one-way ANOVA and paired-comparisons ($*p<0.05$).

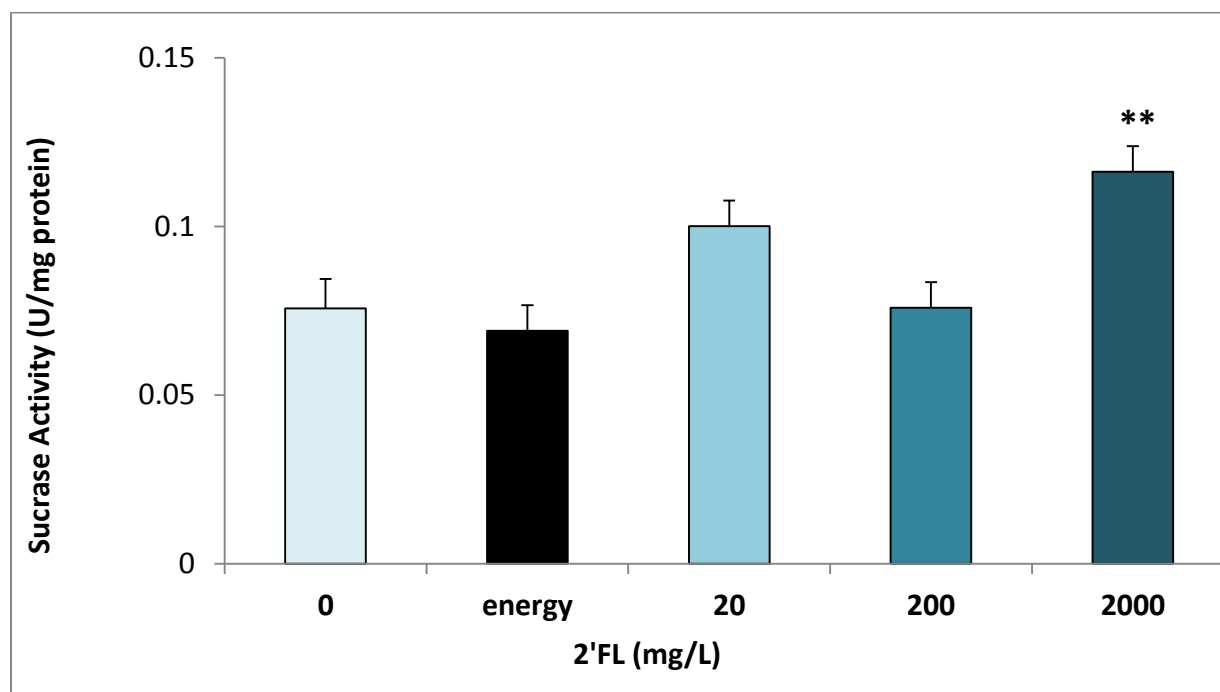


Figure 5.13. Effect of incubation with 2'fucosyllactose (2'FL) on sucrase activity in post-confluent CaCo-2Bbe cells. Cells treated with 2000 mg/L of 2'FL had a 53.5% (SEM 6.6%) increase ($p=0.036$) in sucrase activity as compared to 0 mg/L controls. Energy refers to lactose + fucose. Values are mean $\pm$ SEM. Asterisks denote statistically significant difference in treatment means as compared to 0 mg/L control by one-way ANOVA and paired-comparisons (* $p<0.05$).

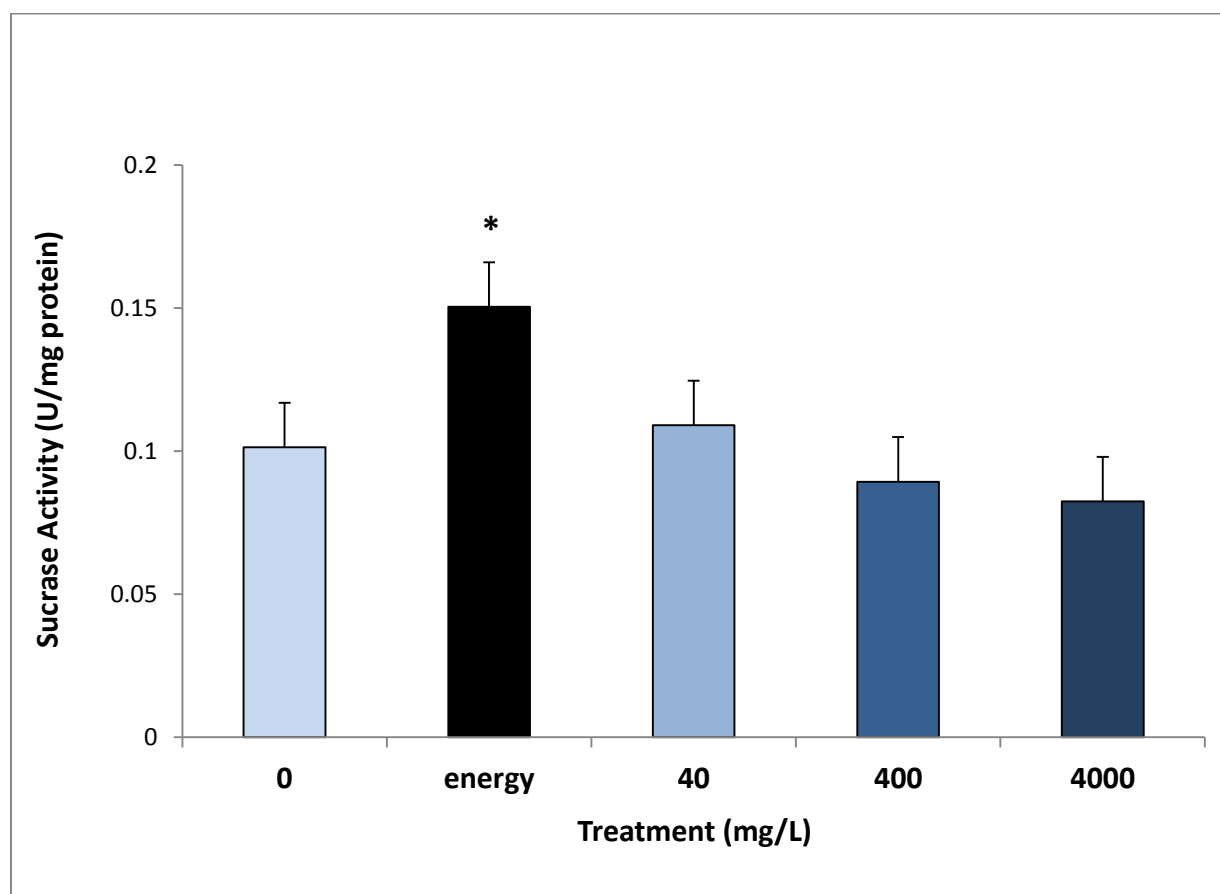


Figure 5.14. Effect of incubation with 6'sialyllactose (6'SL) on sucrase activity in post-confluent CaCo-2Bbe cells. Energy (lactose + sialic acid) increased ($p=0.042$) sucrase activity by 48.5% (SEM 15.4%) as compared to the 0 mg/L control. Values are mean $\pm$ SEM. Asterisks denote statistically significant difference in treatment means as compared to 0 mg/L control by one-way ANOVA and paired-comparisons (* $p<0.05$).

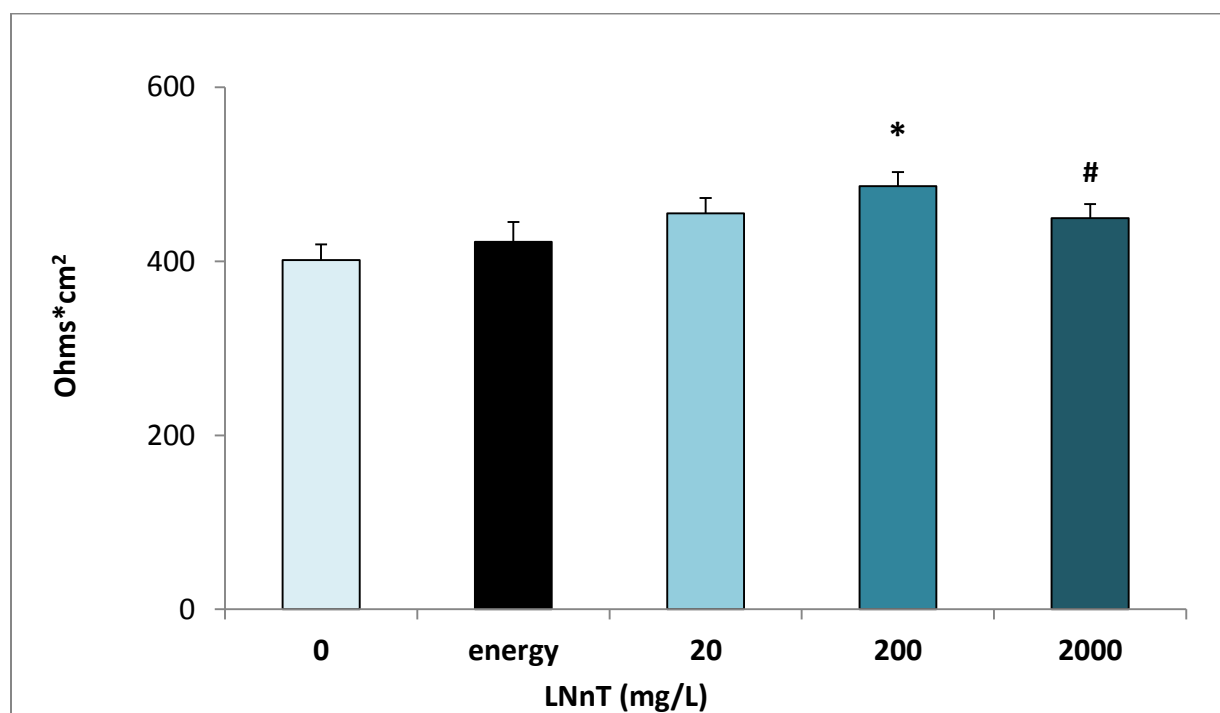


Figure 5.15. Effect of incubation with lacto-N-neotetraose (LNnT) on transepithelial resistance in post-confluent CaCo-2Bbe cells. Transepithelial resistance increased ($p=0.002$) by 21.1% (SEM 3.34%) in cells treated with 200 mg/L LNnT and tended to increase ($p=0.059$) by 12% (SEM 3.61%) in the 2000 mg/L treatment group as compared to 0 mg/L control. Energy refers to lactose + n-acetyllactosamine. Values are mean $\pm$ SEM. Asterisks denote statistically significant difference in treatment means as compared to 0 mg/L control by one-way ANOVA and paired-comparisons ($\#p<0.1$; $*p<0.05$).

	LNnT	6'SL	2'FL
Pre-confluent HT-29			
Proliferation	Decreased	Decreased	Decreased
Cell Cycle	G2/M arrest	G2/M & S arrest	G2/M arrest
Differentiation			Increased
Pre-confluent CaCo-2Bbe			
Proliferation	Decreased	Decreased	Decreased
Cell Cycle		G2/M arrest	
Differentiation			Increased
Post-confluent CaCo-2Bbe			
Differentiation			Increased
Apoptosis	Increased		
Barrier Function	Increased		
Sucrase			Increased
Lactase			

Table 5.3. Summary of HMO effects in each cell type. 2'FL, 2'-fucosyllactose; LNnT, lacto-N-neotetraose; 6'SL, 6'-sialyllactose.

CHAPTER 6

CONCOMITANT ADMINISTRATION OF HUMAN MILK OLIGOSACCHARIDES INFLUENCES INTESTINAL MATURATION *IN VITRO*

ABSTRACT

Human milk oligosaccharides (HMOs) are abundant in human milk (> 15 g/L) and are associated with enhanced intestinal maturation *in vitro* (Kuntz et al., 2008; Kuntz et al., 2009). Therefore, we aimed to assess the induction of epithelial differentiation by individual and concomitant administration of three HMOs, 2'-fucosyllactose (2'FL), 3'-sialyllactose (3'SL), and 6'-sialyllactose (6'SL), using an *in vitro* epithelial model of the crypt-villus axis made up of pre-confluent HT-29, pre-confluent CaCo-2Bbe and post-confluent CaCo-2Bbe cells. Cells were randomized to 1 of 18 treatments for 72 hours of incubation: individual HMO treatments at low and high doses (3'SL at 0.2 and 1.0 g/L, 6'SL at 0.4 and 1.0 g/L, 2'FL at 0.2 and 2.0 g/L), concomitant treatments of the previous HMOs at both low and high doses, and controls (0 g/L, media, 4 g/L total HMO, and lipopolysaccharide (LPS)). Pre-confluent HT-29 cells had reduced proliferation ($p<0.05$) with individual high dose treatments of 6'SL and 2'FL. Additionally, concomitant administration of the high doses of HMOs ($p<0.05$) significantly reduced proliferation as did the total pooled HMO ($p<0.001$). Pre-confluent CaCo-2Bbe cells were less sensitive to reduction in proliferation where only high dose concomitant treatments containing 2'FL ($p<0.01$) significantly reduced proliferation in addition to the total HMO ($p<0.001$). Interestingly, 2'FL did not enhance differentiation in HT-29 cells as we had previously shown; however, it did increase ($p<0.05$) differentiation in pre-confluent CaCo-2Bbe cells. HMO treatments did not enhance alkaline phosphatase (AP) activity, a general marker of differentiation, nor did it increase disaccharidase activity in post-confluent CaCo-2Bbe cells.

Disaccharidase activity was only affected by time ($p<0.05$) in that lactase activity was higher at 12 days as compared to 21 days and the opposite was found for sucrase activity. Apoptosis and necrosis were both significantly decreased ($p<0.001$) in post-confluent CaCo-2Bbe cells treated with 4.0 g/L total pooled HMO as compared to 0 g/L control. In summary, HMO treatments consistently inhibit proliferation with some associated enhancement of epithelial differentiation. Although total pooled HMO reduced apoptosis and necrosis, it is less clear if this is associated with functional outcomes. Further study is needed to determine if these effects translate *in vivo*, however, results of our *in vitro* model of the crypt-villus axis suggest addition of HMO to infant formula may promote intestinal maturation.

INTRODUCTION

Human milk, a complex physiological fluid, is nutritionally adequate to meet the needs of the developing neonate and promotes maturation of the neonatal gastrointestinal (GI) tract (Carver and Barness, 1996). Human milk oligosaccharides (HMOs) are the third most abundant solid component in human milk making up nearly 15 grams per liter in mature milk (Coppa et al., 1999; Chaturvedi et al., 2001a; Ninonuevo et al., 2006; German et al., 2008b; Thurl et al., 2010; Wu et al., 2010). Human milk is unique in its oligosaccharide complexity and composition, containing up to 200 unique oligosaccharides (Coppa et al., 2001). In contrast, bovine milk is nearly completely lacking in free complex oligosaccharides (Kunz et al., 2000). Infant formula also essentially lacks oligosaccharides except in cases where plant derived or synthetic prebiotic oligosaccharides are supplemented (Tungland and Meyer, 2002; Kunz and Rudloff, 2006).

As a large portion of U.S. infants are solely fed formula during the key period of neonatal development, more research is needed to determine potential ingredients which can aid in narrowing the compositional gap between formula and human milk in order to impart some of the benefits of human milk on to formula-fed infants. Recent studies have sparked interest in HMOs as potential ingredients for supplementation to infant formula. Kuntz and colleagues showed that neutral and acidic HMO fractions in effect cell dynamics and promote maturation of the small intestine *in vitro* (Kuntz et al., 2008)

To our knowledge, this is the first study to investigate *in vitro* small intestinal effects as related to concomitant administration of specific HMOs which are of interest for use as infant formula ingredients. As such, the results of this study will help fill the gap in knowledge surrounding synergistic effects of some of the most prevalent HMOs within the immature small intestine *in vitro*.

In this study, we aimed to assess the induction of epithelial differentiation by individual and concomitant administration three HMOs, 2'-fucosyllactose (2'FL), 3'-sialyllactose (3'SL), and 6'-sialyllactose (6'SL), using an *in vitro* epithelial model of the crypt-villus axis consisting of pre-confluent HT-29 cells, pre-confluent CaCo-2Bbe cells, and post-confluent CaCo-2Bbe cells.

MATERIALS AND METHODS

Study Design

Our model consisted of pre-confluent HT-29 cells, pre-confluent CaCo-2Bbe cells and post-confluent CaCo-2Bbe cells cultured individually to provide an *in vitro* model of the small intestinal crypt-villus axis and simulate the varying levels of differentiation within the constantly renewing villus *in vivo* (**Figure 5.1**). HT-29 cells are a low differentiated phenotype reflective of

crypt colonocytes—these cells are not able to differentiate even in post-confluent states under normal culture conditions (Lesuffleur et al., 1990). HT-29 cells have properties of chloride secreting cells related to a chloride channel that can also be found within human intestinal epithelial cells (Kreusel et al., 1991). CaCo-2Bbe cells, a well characterized model of intestinal absorptive epithelium, exhibit moderate differentiation in a pre-confluent state and in a post-confluent state exhibit a process of differentiation similar to that seen in a villus enterocyte (Peterson and Mooseker, 1992). CaCo-2Bbe cells will grow as a monolayer, continuing to differentiate and form cylindrical polarized morphology with tight junctions between cells (Peterson and Mooseker, 1992). Additionally, CaCo-2Bbe cells have apical microvilli with associated brush-border enzymes (sucrase and lactase) in post-confluent states which are similar to human fetal enterocytes (Peterson and Mooseker, 1992). Furthermore, CaCo-2 cells grown as a monolayer are also a well-established model of intestinal absorptive function and provide an avenue by which transepithelial resistance and nutrient transport can be assessed (Delie and Rubas, 1997).

Overall, our model allowed us to assess relevant outcomes along the length of the crypt-villus axis *in vitro* as pre-confluent HT-29 cells model secretory crypt cells, pre-confluent CaCo-2Bbe cells model proliferating absorptive enterocytes as they migrate up the villus, and post-confluent CaCo-2Bbe cells model well differentiated functional absorptive villus cells. The *in vitro* model as a whole simulates the neonatal infant's maturing gastrointestinal epithelium (Kuntz et al., 2008). Furthermore, incubation of the cells with HMOs provides a simulation of the suckling infant's nearly continuous enteral feedings of human milk. As such, our model will proved an avenue for novel insight into the synergistic effects of concomitant HMO administration on small intestinal cellular dynamics and function *in vitro*.

Treatments

In order to decipher synergism effect of concomitant administration of HMOs, each treatment replicate included the individual HMO at the levels that will be tested in the concomitant groups. HMOs were provided by Abbott Nutrition. Thus, the treatment group included 3'SL, 6'SL and 2'FL individually at their physiologically relevant levels, 0.2 g/L, 0.4 g/L and 2.0 g/L, respectively. Additionally, each HMO was provided at a second dose of 1.0 g/L 3'SL, 1.0 g/L 6'SL, and 0.2 g/L 2'FL. Concomitant treatment groups included pairs of the HMOs at two levels each and all three HMOs at two levels. Positive and negative controls were also included in each experiment. Total HMO (donated by Lars Bode, University of San Diego) at 4g/L acted as the positive control. The 4g/L level was selected to match the highest concomitant treatment group tested (1.0 g/L 3'SL + 1.0 g/L 6'SL + 2.0 g/L 2'FL = 4.0 g/L).

Lipopolysaccharide (LPS) control groups were utilized to match the remaining endotoxin units measured in the respective total HMO fraction following one passage through the endotoxin removal column. The media control group was utilized to determine the potential effects of the endotoxin column on the 0 g/L. The 0 g/L control contained the matched volume of medium passed through the endotoxin removal columns needed to make the 4 g/L concomitant treatment and pooled HMO treatment.

Prior to treatment, each of the HMOs as well as the energy controls were added to either Dulbecco's Modified Eagle Medium (DMEM) or McCoy's 5A cell culture media at a concentration of 20 mg/mL, passed through a Detoxi-Gel endotoxin removing column (Pierce), and stored at -80°C until use. The columns utilize immobilized polymyxin B to remove pyrogens by binding their lipid A domains. Solutions were tested prior to use to ensure endotoxin levels were below a detectable level using the Limulus Amebocyte Lysate (LAL) Endotoxin test kit

(Roche) according to manufacture guidelines. 3'SL, 6'SL, and 2'FL solutions did not contain detectable levels of endotoxin after passage through the endotoxin removal columns. The total pooled HMO sample in DMEM contained 2050 endotoxin units and the total pooled HMO sample in McCoy's 5A contained 2600 endotoxin units following one passage through the columns. Thus, LPS controls were created by adding the respective amounts of endotoxin units to complete media prior to treatment.

Cell Culture

To model the crypt-villus axis and investigate the effects of incubation with HMOs, pre-confluent HT-29, pre-confluent CaCo-2Bbe, confluent CaCo-2Bbe and post-confluent CaCo-2Bbe cells were utilized. The CaCo-2Bbe cell line was obtained from American Type Culture Collection (CRL-2102, ATCC, Manassas, VA) clone of CaCo-2 (ATCC: ATB-37). They were used between passages 55 and 65. The transformed human intestinal cells, HT-29 cells, were obtained from American Type Culture Collection (ATCC, Manassas, VA). They were used between passages 5 and 15. Both cell lines were separately maintained in 75 cm² tissue culture flasks (TP90075, Midwest Scientific, St. Louis, MO) and maintained in a humidified atmosphere of 37°C and 5% CO₂. HT-29 cells were maintained in McCoy's 5A media formulation supplemented with 10% fetal bovine serum (Atlanta Biological, Lawrenceville, GA). CaCo-2Bbe cells were maintained in DMEM supplemented with 10% fetal bovine serum (Atlanta Biological) and 0.01 mg/ml human apotransferrin (Sigma). Growth medium was replaced as needed, but no fewer than twice per week. Cell lines were subcultured weekly at pre-confluent densities with 0.25% trypsin-EDTA (25200, Invitrogen, Carlsbad, CA).

Cell Proliferation

The CellTiter 96 AQueous non-radioactive cell proliferation assay (G5421, Promega, Madison, WI) was utilized to assess cell proliferation. The assay utilizes the soluble compound 3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-3-(4-sulfophenyl)-2H-tetrazolium (MTS) with an electron coupling reagent, phenazine methosulfate (PMS). MTS is bio-reduced by actively proliferating cells into a formazan product which is measured at 490nm absorbance and is directly proportional to the number of living cells in culture. Proliferation was measured by the MTS assay as described by the manufacturer. Briefly, 96 well plates were seeded with HT-29 or CaCo-2Bbe cells at a density of 1500 cells/well and allowed to adhere for 24 hours followed by incubation with HMO treatments for 72 hours. During the last hour of the incubation period, MTS/PMS was added and plates continued to incubate at 37°C at 5% CO₂. Absorbance was then measured at 490 nm. Results are expressed as a percent of control (0 mg/L).

Cellular differentiation

HT-29 cells and CaCo-2Bbe cells were seeded separately in 75 cm² tissue culture flasks at a density of 10⁴ cells/cm² and allowed to adhere for 48 hours in the case of the pre-confluent HT-29 and pre-confluent CaCo-2Bbe cells and 18 days for the post-confluent CaCo-2Bbe cells. Following the adherence period, media were changed and HMO treatments applied. After the 72 hour incubation period, cells were trypsinized, pelleted by centrifugation at 500 x g for 5 minutes and washed two times with PBS. Cell pellets were flash frozen in liquid nitrogen and stored at -80°C until analysis. Cellular differentiation was measured by alkaline phosphatase (AP) activity. AP activity was measured by commercially available fluorometric and colorimetric assay kits (K422 and K412, BioVision, Mountain View, CA). AP activity in HT-29 cells was determined

fluorometrically following homogenization of cells in Assay Buffer then centrifuging for 3 minutes at 13,000 x g to remove insoluble material. Cell supernatants were added to black 96 wells plate and incubated with 4-methylumbelliferyl phosphate disodium salt (MUP) substrate for 3 hours at room temperature protected from light. The reaction was stopped by the addition of Stop Solution and read at excitation=360nm and emission=440nm. Background controls were used for all samples by adding Stop Solution to the wells prior to addition of MUP. Final AP activity was determined by subtracting background control values and quantifying activity based on standard curve methodology. AP activity was normalized to protein content as measured by the BCA protein assay. AP activity in CaCo2-Bbe cells was quantified in a similar manner with the following exceptions: p-nitrophenyl phosphate was used as a phosphatase substrate and measured colorimetrically in clear 96 wells plates at 405 nm in a plate reader after 30 minutes incubation at room temperature. Results are expressed as AP activity units per milligram protein.

Apoptosis and Necrosis

Apoptosis and necrosis were quantified by the Cell Death Detection ELISA^{PLUS} (Roche), a photometric enzyme-immunoassay for *in vitro* determination of cytoplasmic histone-associated-DNA-fragments according to manufacture directions. Briefly, for quantification in pre-confluent cells, 96 well plates were seeded with HT-29 or CaCo-2Bbe cells at a density of 1500 cells/well and allowed to adhere for 24 hours followed by incubation with HMO treatments for 72 hours. For assessment in post-confluent CaCo-2Bbe cells, cells were seeded at a density of 1×10^4 cells/cm² into the 96 well plates and maintained in an incubator for 18 days with twice weekly medium renewal. On day 18, media were removed and media containing HMO treatments applied. At the end of the 72 hour treatment period, plates were centrifuged for 10

minutes at 200 x g and supernatant was collected and stored at 4°C until assay to quantify necrotic cells. Lysis buffer was added to each well and incubated at room temperature for 30 minutes followed by centrifugation for 10 minutes at 200 x g. Supernatant was collected to measure cellular apoptosis. Next, 20 ul of the cytoplasmic fractions collected following the two centrifugation phases were transferred to the streptavidin coated microplate and 80 ul of the immunoreagent, containing anti-histone-biotin and anti-DNA-peroxidase, was added. During the incubation period, the anti-histone antibody binds the nucleosomes histone-component while the biotin simultaneously complexes with the streptavidin coating the microplate and the anti-DNA-peroxidase antibody reacts with the nucleosomal DNA-component. At the end of the 2 hour incubation period, the solutions were removed and the plate washed 3 times with incubation buffer. The ABTS substrate was then added and allowed to incubate for 10 minutes prior to stopping the reaction. The photometric color development was measured at 490nm. Results are expressed as a percent of control (0 mg/L).

Cell cycle analysis

HT-29 cells and CaCo-2Bbe cells were seeded separately in 75 cm² tissue culture flasks at a density of 10⁴ cells/cm² and were grown in media for 48 hours. Following the adherence period, media were changed, HMO treatments applied, and cells were then allowed to grow for an additional 72 hours. At the end of the treatment period, cells were trypsinized, pelleted by centrifugation at 500 x g for 5 minutes, and washed two times with PBS. Cells were then fixed by adding 1 x 10⁶ cells to 9 ml of ice cold 90% ethanol and stored at -20°C until analysis. For cell cycle analysis, cells fixed in ethanol were pelleted by centrifugation at 200 x g for 15 minutes at 4°C then washed two times with cold PBS. Cells were resuspended in propidium iodide (PI)/Triton X-100 staining solution containing DNase-free RNase and strained through

nylon filter capped Falcon tubes. Following incubation at room temperature for 30 minutes, tubes were transfer to ice and protected from light until data acquisition by a Epics XL benchtop flow cytometer. Data are expressed as the distribution of cell populations in different cell cycle stages.

Sucrase and Lactase Activity

CaCo-2Bbe cells were seeded in 75 cm² tissue culture flasks at a density of 10⁴ cells/cm² and maintained for 9 or 18 days. Following the adherence period, media was changed and treatment applied and allowed to incubate for 72 hours in the incubator. After the incubation period, cells were washed three times with ice-cold PBS then removed by scraping with a plastic cell scraper into 3.5 ml of PBS. Following centrifugation at 500 x g for 5 minutes, PBS was removed and cell pellets flash frozen in liquid nitrogen and stored at -80°C until analysis. For measurement of disaccharidase activity, cell pellets were suspended in 1 mL of homogenization buffer (0.9% saline solution containing 1 uM phenylmethylsulfonyl fluoride and 223 uM of iodoacetic acid) and homogenized for 30 seconds on setting 5 on a homogenizer. Homogenates were then incubated with either sucrose or lactose buffers for 1 hour in a 37°C water bath. Enzyme activity was stopped by the addition of Ba(OH)₂ and ZnSO₄. Background control tubes were immediately stopped with Ba(OH)₂ and ZnSO₄ prior to incubation to quantify non-specific disaccharidase activity. Samples were then be centrifuged for 20 minutes at 3000 rpm at 4°C. Supernatants were added in triplicate to a clear 96 well plates, glucose oxidase reagent (Sigma) was then added and allowed to incubate at room temperature for 10 minutes in order to detect the amount of glucose released from the hydrolysis of the disaccharides. The plates were read at 500 nm in a spectrophotometer and quantified using standard curve technique. Disaccharidase

activity was normalized to cellular protein content of the same homogenate as measured by the BCA assay and expressed as the amount of glucose release per hour per milligram protein.

Statistical Analysis

Statistical differences between treatments were determined using a one-way analysis of variance (ANOVA) with Dunnett's adjustment for multiple comparisons. Source of variation were treatment (n=18) and replicate (n=3). Contrast statements were also utilized: 1) to compare the pooled means of each of the individual HMO treatments verses the 0 g/L control; and, 2) to compare the pooled treatment means of each of the individual HMOs versus its respective concomitant HMO treatment group. Normality was tested using the Shapiro-Wilk statistic. Logarithmic and reciprocal transformations were utilized as needed. Computations were conducted using a mixed model in SAS (Version 9.1; SAS Institute, Cary, North Carolina). Statistical significance was defined as $p \leq 0.05$, and trends were reported at $p \leq 0.01$. Data are reported as mean $\pm$ SEM.

RESULTS

In this trial, each dependent variable was assessed following a 72 hour incubation period with the HMO treatment groups discussed above. The effect of each treatment on HT-29 cell proliferation is listed in **Table 6.1**. Neither the media nor the LPS control groups were different from the 0 g/L control group. Unless otherwise noted, all comparisons in proliferation rates were made to the 0 g/L control group. Treatment with two of the individual HMOs, 6'SL and 2'FL, reduced proliferation in pre-confluent HT-29 cells. Both 1.0 g/L 6'SL and 2.0 g/L 2'FL significantly reduced proliferation ($p < 0.05$) by 8.7% and 9%, respectively as compared to 0 mg/L control. Further, pooled treatment means of the high and low doses of the individual

HMOs reveals that both 3'SL and 2'FL reduced ($p<0.05$) proliferation as compared to 0 g/L control (**Figure 6.1**). Thus, all three individual HMOs reduced proliferation in pre-confluent HT-29 cells.

Concomitant treatments had a slightly additive effect on the reduction of proliferation in pre-confluent HT-29 cells. Low dose and the high dose combinations of 3'SL + 6'SL resulted in proliferation rates of 90.1% and 86.6%, respectively, as compared to 0 g/L control. Further, incubation with high dose combinations of 3'SL + 2'FL and 6'SL and 2'FL significantly reduced proliferation ($p<0.05$) to rates to 90.2% and 88.8% , respectively. The low dose combination of the 3'SL + 6'SL + 2'FL did not significantly affect proliferation, however the high dose combination of the three reduced proliferation to 89.1% was significantly different from control ($p<0.01$). The combined high dose combination of the three HMOs (totally 4 g/L) did not have as profound of an effect on proliferation as the total HMO treatment at 4 g/L. Total HMO had the greatest effect, reducing ($p<0.001$) proliferation to 77.0% of the 0 g/L control.

Pre-confluent CaCo-2Bbe cells were less sensitive to HMO treatments (**Table 6.2**). Again, there was no difference in proliferation between 0 g/L control and either the media or the LPS control groups. Proliferation rates were not significantly reduced with individual HMO treatment. Similar to the pre-confluent HT-29 cells, the high dose combinations of 3'SL + 2'FL and 6'SL + 2'FL reduced ($p<0.01$) proliferation rates in pre-confluent CaCo-2Bbe cells to 91.90% and 92.18%, respectively. Again, similar to above, the low dose combination of the 3'SL + 6'SL + 2'FL did not significantly affect proliferation, however the high dose combination of the three reduced proliferation to 89.9% was significantly different from control ($p<0.01$). Interestingly, in these cells, the total pooled HMO (4 g/L) had a similar effect on the proliferation

rate as the 4g/L concomitant high dose group, significantly reducing ($p<0.001$) proliferation to 89.5%.

No changes were detected in cell cycle kinetics in pre-confluent HT-29 cells in any treatment group (**Table 6.3**). In pre-confluent CaCo-2Bbe cells (**Table 6.4**), treatment with 1.0 g/L 6'SL, 0.2 g/L 2'FL and 2.0 g/L 2'FL each resulted in arrest ($p<0.01$) within the G1 phase and a corresponding significant decrease in the percentage of cells in the G2 phase. Both the high and low dose combinations of 3'SL + 6'SL and 3'SL + 2'FL also resulted in arrest within the G1 phase and a subsequent decrease in the percentage of cells in the G2 phase. Only the high dose combinations of 3'SL + 6'SL and 3'SL + 2'FL resulted in a reduced ($p<0.01$) proportion of cells within the S phase. The low dose combination of 6'SL + 2'FL resulted in a significant decrease ($p<0.05$) in the percentage of cells in the G2 phase with no other changes noted in the other phases. The high dose combination of 6'SL + 2'FL has associated with accumulation of cells within the G1 phase. The low dose combination of 3'SL + 6'SL + 2'FL significant decreased ($p<0.01$) in the proportion of cells in the G2 phase; however, no differences were detected in cell cycle kinetics at the high dose level when the three HMOs were combined. Interestingly, the total pooled HMO treatment had the opposite effect of the other HMO treatments in that there was a significant decrease in the proportion of cells in the G1 phase ($p<0.001$) and a corresponding significant increase in the proportion of cells arrested in the S phase ($p<0.001$).

AP activity of pre-confluent HT-29 cells is detailed in **Table 6.5**, pre-confluent CaCo-2Bbe cells in **Table 6.6**, and post-confluent CaCo-2Bbe cells in **Table 6.7**. Of the three cell types, only pre-confluent CaCo-2Bbe cells were affected by treatment. Within pre-confluent CaCo-2Bbe cells, the high dose (2.0 g/L) of 2'FL significantly increased ($p<0.05$) differentiation by 22.6% (SEM=7.4%) as compared to the 0 g/L control. Additionally, the pooled treatment

mean of 0.2 and 2.0 g/L 2'FL was also significantly higher than 0 g/L control (**Figure 6.2**).

Treatment with both the high and low doses of 3'SL + 6'SL tended ($p=0.060$ and $p=0.054$, respectively) to increase differentiation in pre-confluent CaCo-2Bbe cells.

Cellular apoptosis and necrosis was measured in all cell types studied. Both pre-confluent HT-29 cells (**Table 6.8**) and pre-confluent CaCo-2Bbe cells (**Table 6.9**) had exceptionally low levels of apoptosis and necrosis. In pre-confluent HT-29 cells, HMO significantly increased ($p<0.001$) apoptosis by 179.3% with a subsequent decreased in necrosis to 4.0% of that of 0 g/L control cells. In pre-confluent CaCo-2Bbe cells, necrosis was again significantly reduced ($p<0.001$) by the total HMO fraction to 22.8 % of that of the 0 g/L control, but no differences were found in the levels of apoptosis in any treatment group. Post-confluent CaCo-2Bbe cells (**Table 6.10**) treated with the pooled HMO treatment had significant reductions in both apoptosis ($p<0.001$) and necrosis ($p<0.001$), reaching levels of 60.5% and 31.7% of that of the 0 g/L control, respectively. Similar to the total pooled HMO treatment, the matched LPS control also had decreased necrosis ($p<0.01$) as compared to 0 g/L control but to a much lesser extent. The LPS group reduced necrosis by 33.4% versus the 68.3% reduction in necrosis in the total pooled HMO group. Necrosis was also decreased by individual high and low dose of 3'SL and 2'FL. The low dose combination of 3'SL + 6'SL also had a significantly lower ($p<0.05$) proportion of necrotic cells as compared to 0 g/L control while the low dose combination of 3'SL + 2'FL tended to have a lesser proportion of necrotic cells.

Surcrase and lactase activity was also assessed in post-confluent CaCo-2Bbe cells at two different time points, 12 days postconfluence and 21 days post confluence, however no treatment effects were detected (**Table 6.11** and **Table 6.12**). There was a difference in the two time points in their respective disaccharidase activities. Lactase activity was found to be significantly

higher ($p < 0.05$) in cells at day 12 as compared to day 21 and sucrase activity was higher ($p < 0.05$) at day 21 as compared to day 12.

Tables 6.13 and **6.14** provide a summary of the treatment effects following treatment with individual HMOs and concomitant administration of HMOs, respectively.

DISCUSSION

Beginning in the perinatal period and extending through the first year of life, the GI tract undergoes numerous morphological changes that influence its physiological functions (Carver and Barness, 1996). Human milk is known to provide trophic factors that influence maturation of the GI tract. Recently, HMOs have been recognized as potential components within human milk which aid in the promotion of intestinal maturation. In a study by Kuntz and colleagues, as well in the unpublished data from our laboratory group, it was found that neutral and acidic HMO fractions and some individual HMOs increased AP activity in pre-confluent HT-29 cells (Kuntz et al., 2008). Additionally, our lab group has shown that incubation with 2'FL and LNnT enhances specific functional indices relevant to digestion and absorption in post-confluent CaCo-2Bbe cells, a model of the well-differentiated intestinal villus cell. To our knowledge, no data exists on the effects of specific HMO pairings and correlations between enhanced differentiation and digestive and absorptive functionality. As such, these novel assessments will help to fill the gap in knowledge as it relates to understanding functional efficacy of HMO pairings *in vitro*. The present study investigated the effects of individual and concomitant administration of 2'FL, 6'SL, and 3'SL on epithelial cell differentiation utilizing well established intestinal cell lines HT-29 and Caco-2Bbe to model of the crypt-villus axis.

A cell culture model was the most appropriate model to assess the effects of HMOs on cellular kinetics along the crypt-villus axis. Further, it allowed us to overcome the obstacle of limited availability of the HMOs of interest. Although human milk contains as many as 200 different oligosaccharides, one-tenth of those seem to make up nearly the entire 10-15g/L concentration present in human milk (Thurl et al., 2010). The HMOs investigated in this trial were selected based on their predominance in human milk, with 2'FL being a dominant neutral oligosaccharide, and 6'SL and 3'SL comprising a large proportion of the acidic fraction.

In this trial, we found that both individual and concomitant administration of HMOs inhibited proliferation in pre-confluent HT-29 cells. Additionally, concomitant administration of the individual HMOs reduced proliferation in pre-confluent CaCo-2Bbe cells. In both cell types, the total pooled HMO treatment at 4g /L reduced proliferation to levels equal to or slightly greater than the high dose concomitant group which also provided 4 g/L with 1 g/L from 3'SL, 1g/L from 6'SL, and 2 g/L from 2'FL. Overall, the reductions in proliferation were found to be additive when HMOs were added concomitantly. Additionally, the high dose combination of the three individual HMOs (3'SL + 6'SL + 2'FL) affected proliferation to a similar extent of total pooled HMO sample. This suggests that the three HMOs investigated in this trial were able to achieve close to the same effects of what was likely an excess of 100 different oligosaccharides in the total pooled HMO treatment group. This novel finding is promising as it is very unlikely that infant formula can be supplemented with all oligosaccharides found in human milk, instead it is much more clinically and commercially feasible to determine a select few oligosaccharides that are able to promote close to the same effects as the whole.

Interestingly, the inhibition of proliferation in the HT-29 cells was not associated with cell cycle arrest nor was differentiation enhanced as in our previous trial or that of Kuntz et. al

(2008). AP activity, a marker of cell differentiation, was highly variable in HT-29 cells and no changes in cell cycle were noted between treatment groups. Several factors should be considered in light of this. First, research has shown that conditions such as limiting glucose and supplementing galactose promote HT-29 cell differentiation (Lindley et al., 2012) as does supplementation of HMOs (Kuntz et al., 2008). It is possible that exposure of the 0 g/L control medium to the endotoxin removal columns may have introduced low levels of polymyxin B from the column resin into the media. The presence of low levels of polymyxin B and/or the slight dilution of the culture media may have stimulated enhanced AP activity in the 0 g/L group. As the 0g/L control received the same volume of medium passed through the columns as the 4 g/L pooled HMO group, it may be that the effects are more pronounced than the our previous HMO trial when the 0 mg/L controls received a volume matched to the 154 mg/L energy controls. In this instance, utilizing the media group at the control instead of the 0 g/L group may add clarity to discrepancy between the current HMO trial and the previous trial in our laboratory. Second, as there were no changes in the cell cycle kinetics in this trial, it may also be possible that the HMOs simply did not enhance differentiation in the pre-confluent HT-29 cells in this trial as they did previously. As the levels of AP activity are 10 fold higher in in pre-confluent CaCo-2Bbe cells and 40 fold higher in post-confluent CaCo-2Bbe cells, AP activity in the HT-29 cells may not be the best marker to assess differentiation within our *in vitro* model. Translation into preclinical animal models is warranted to determine if HMO promote maturation within the GI tract.

Similar to our previous finding, pre-confluent Caco-2Bbe cells were less sensitive to inhibition of proliferation with only concomitant administration of HMOs having a significant effect. Differentiation was however enhanced in the pre-confluent Caco-2Bbe cells with an

associated G0/G1 arrest. These results are opposite those of Kuntz et al., where neutral and acidic HMO fractions arrested pre-confluent CaCo-2 cells in the G2/M phase. However, it should be noted that they did not detect increased AP activity in pre-confluent CaCo-2 cells as compared to control. Our results are in agreement with other trials in that the enhancement of differentiation in Caco-2Bbe cells is often associated with cell cycle arrest within the G0/G1 phase (Houde et al., 2001). As further evidence of enhanced differentiation occurring following G0/G1 arrest, we saw that the total pooled HMO sample significantly inhibited proliferation with associated cell cycle arrest in the S phase but there was not an associated enhancement of AP activity within the total HMO treatment group.

Although 2'FL tended to enhance AP activity in 21 days post-confluent Caco-2Bbe cells in our previous trial, no effect was observed with any treatments in this trial. Mirroring this, disaccharidase activities were also unchanged due to treatment. Sucrase and lactase activity were however affected by time in culture. Similar to other trials, it was found that lactase activity is higher in 12 day post-confluent cells as compared to 21 days and the opposite for sucrase activity (Olsen et al., 1996).

Finally, we reported that that treatment with HMOs did not increase apoptosis as has been previously reported by our laboratory and Kuntz and co-workers. On the contrary, total HMO at 4g/L decreased apoptosis as compared to control. Necrosis was also decreased in the total HMO group. In light of this, the means by which apoptosis and necrosis were quantified in each trial should be discussed. In both our lab and that of Kuntz and co-workers, caspase-3 was utilized as a marker of apoptosis in previous trials. In this study, we quantified histone-complexed DNA fragments from cell cytoplasm both before and after cell lysis to quantify both apoptosis and necrosis within the same cell population. As genomic DNA fragmentation is

considered the hallmark of apoptosis, it is possible that utilizing caspase-3 activity as a marker of apoptosis in these post-confluent CaCo-2Bbe cells may have resulted in a falsely inflated proportion of apoptotic cells. Or, it may also be possible that in this trial the lack of activation of apoptosis explains why there was no enhancement of functional indices, i.e. sucrase activity, as was observed in our previous trial.

In summary, we observed that individual and concomitant administration of 2'FL, 3'SL and 6'SL inhibited proliferation in pre-confluent transformed small intestinal cell lines HT-29 and Caco-2Bbe. In the pre-confluent CaCo-2Bbe cells, the inhibition of proliferation was associated with cell cycle arrest within the G0/G1 phase and an enhancement of differentiation. No enhancement in differentiation was found in the pre-confluent HT-29 cells nor were there any changes in cell cycle. Further, treatment did not affect post-confluent CaCo-2Bbe differentiation. The combination of inhibition of proliferation and induction of differentiation suggests that HMOs promote maturation of the GI tract. However, the physiological relevance of these maturational changes should be investigated within *in vivo* models. As the availability of synthetic human milk oligosaccharides has improved over the last few years, pre-clinical and clinical trials utilizing targeted oligosaccharides are becoming more feasible.

TABLES AND FIGURES

Treatment	3'SL (g/L)	6'SL (g/L)	2'FL (g/L)	Mean (% of control)	SEM (%)
Control: 0 g/L				100.0	4.9
Media				95.7	4.9
LPS				99.4	4.9
4 g/L total HMO				77.0 ****	4.9
3'SL	0.2			95.4	4.9
3'SL	1.0			94.0	4.9
6'SL		0.4		101.0	4.9
6'SL		1.0		91.3 *	4.9
2'FL			0.2	94.9	4.9
2'FL			2.0	91.0 *	4.9
3'SL + 6'SL	0.2	0.4		90.1 *	4.9
3'SL + 6'SL	1.0	1.0		86.6 **	4.9
3'SL + 2'FL	0.2		0.2	95.2	4.9
3'SL + 2'FL	1.0		2.0	90.2 *	4.9
6'SL + 2'FL		0.4	0.2	98.0	4.9
6'SL + 2'FL		1.0	2.0	88.8 **	4.9
3'SL + 6'SL + 2'FL	0.2	0.4	0.2	97.9	4.9
3'SL + 6'SL + 2'FL	1.0	1.0	2.0	89.1 **	4.9

Table 6.1. Effects of treatment on pre-confluent HT-29 cell proliferation. Values represent the percent proliferation as compared to 0 mg/L control (set at 100%). Control (0mg/L) cells: 0.776 ± 0.038 . Values are mean $\pm$ SEM expressed as percentages. Asterisks denote statistically significant decrease in treatment means as compared to 0 mg/L control by one-way ANOVA followed by Dunnett's multiple comparison test (*p<0.05, **p<0.01, ***p<0.001). 2'FL, 2'-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose; LPS, Lipopolysaccharide; HMO, human milk oligosaccharide.

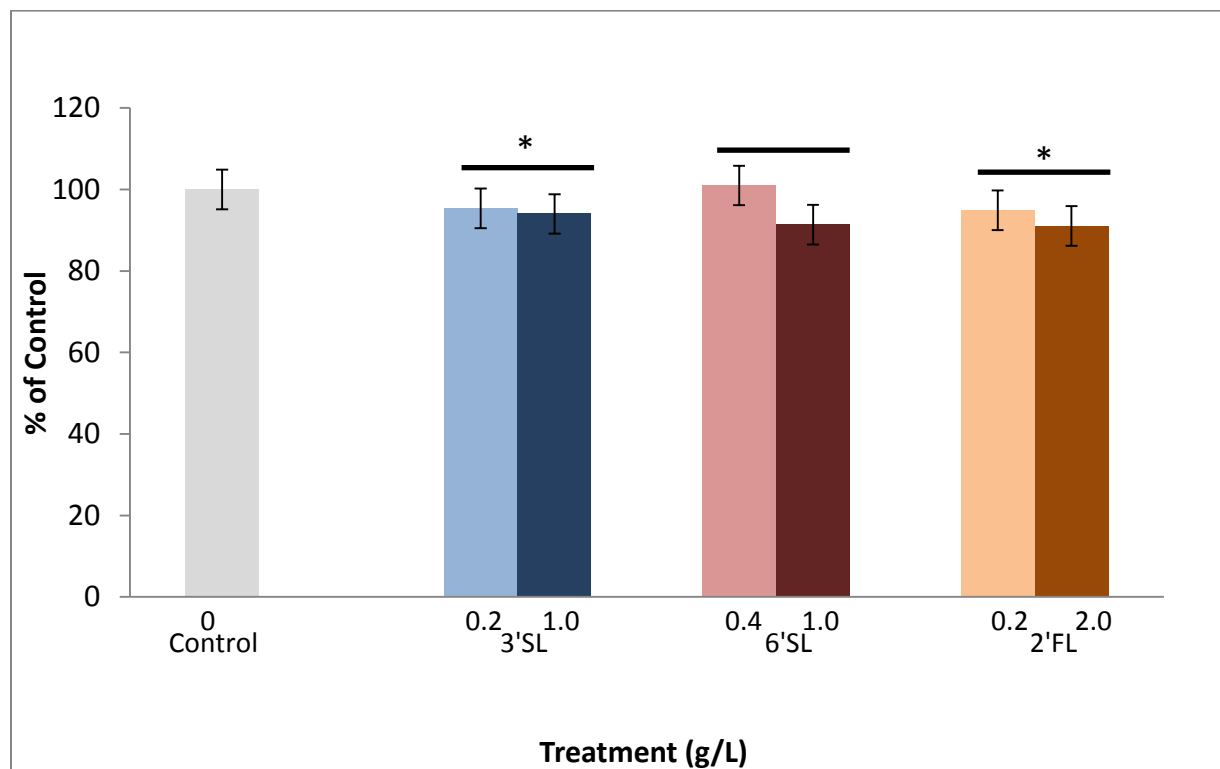


Figure 6.1. Effects of individual HMO on pre-confluent HT-29 cell proliferation. Values represent the percent proliferation as compared to 0 mg/L control (set at 100%). Control (0mg/L) cells: 0.776 ± 0.038 . Values are mean $\pm$ SEM expressed as percentages. Bars with asterisks denote statistically significant decrease in pooled treatment means of the combined high and low treatments of individual HMO compared to 0 g/L control by one-way ANOVA (* $p < 0.05$). Both 3'SL and 2'FL significantly reduced pre-confluent HT-29 cell proliferation. 2'FL, 2'-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose.

Treatment	3'SL (g/L)	6'SL (g/L)	2'FL (g/L)	Mean (% of Control)	SEM (%)
Control: 0 g/L				100.0	3.0
Media				103.5	3.0
LPS				102.9	3.0
4 g/L total HMO				89.5***	3.0
3'SL	0.2			98.9	3.0
3'SL	1.0			97.6	3.0
6'SL		0.4		97.9	3.0
6'SL		1.0		100.6	3.0
2'FL			0.2	103.5	3.0
2'FL			2.0	97.6	3.0
3'SL + 6'SL	0.2	0.4		98.6	3.0
3'SL + 6'SL	1.0	1.0		94.7	3.0
3'SL + 2'FL	0.2		0.2	99.8	3.0
3'SL + 2'FL	1.0		2.0	91.9**	3.0
6'SL + 2'FL		0.4	0.2	96.2	3.0
6'SL + 2'FL		1.0	2.0	92.2**	3.0
3'SL + 6'SL + 2'FL	0.2	0.4	0.2	100.3	3.0
3'SL + 6'SL + 2'FL	1.0	1.0	2.0	89.9**	3.0

Table 6.2. Effects of HMO on pre-confluent CaCo-2Bbe cell proliferation. Values represent the percent proliferation as compared to 0 mg/L control (set at 100%). Control (0mg/L) cells: 0.583 ± 0.0174 . Values are mean $\pm$ SEM expressed as percentages. Asterisks denote statistically significant decrease in treatment means as compared to 0 mg/L control by one-way ANOVA followed by Dunnett's multiple comparison test (**p<0.01, ***p<0.001). 2'FL, 2'-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose; LPS, Lipopolysaccharide; HMO, human milk oligosaccharide.

Treatment	3'SL (g/L)	6'SL (g/L)	2'FL (g/L)	% G1 Mean	% G1 SEM	% S Mean	%S SEM	%G2 Mean	%G2 SEM
Control: 0 g/L				65.0	3.0	26.9	4.1	8.1	1.5
Media				68.2	4.6	17.7	6.2	14.1	2.4
LPS				67.3	4.6	18.2	6.2	14.5	2.4
4 g/L total HMO				73.2	2.9	18.4	4.0	8.4	1.5
3'SL	0.2			66.3	3.0	24.0	4.1	9.7	1.5
3'SL	1.0			65.9	3.0	24.6	4.1	9.6	1.5
6'SL		0.4		66.6	3.0	23.4	4.1	10.0	1.5
6'SL		1.0		66.4	3.0	24.4	4.1	9.2	1.5
2'FL			0.2	65.5	3.0	24.7	4.1	9.8	1.5
2'FL			2.0	67.0	3.0	24.0	4.1	9.0	1.5
3'SL + 6'SL	0.2	0.4		67.9	3.0	22.7	4.1	9.4	1.5
3'SL + 6'SL	1.0	1.0		67.8	3.0	23.9	4.1	8.2	1.5
3'SL + 2'FL	0.2		0.2	67.6	3.0	23.6	4.1	8.8	1.5
3'SL + 2'FL	1.0		2.0	67.6	3.0	24.8	4.1	7.7	1.5
6'SL + 2'FL		0.4	0.2	68.2	3.0	22.7	4.1	9.1	1.5
6'SL + 2'FL		1.0	2.0	66.8	3.0	24.5	4.1	8.6	1.5
3'SL + 6'SL + 2'FL	0.2	0.4	0.2	67.7	3.0	22.9	4.1	9.4	1.5
3'SL + 6'SL + 2'FL	1.0	1.0	2.0	68.1	3.0	24.9	4.1	7.0	1.5

Table 6.3. Effects of treatment on pre-confluent HT-29 cell cycle kinetics. Treatment did not affect cell cycle kinetics compared to control (0 mg/L) as analyzed by one-way ANOVA followed by Dunnett's multiple comparison test. Values are mean $\pm$ SEM. 2'FL, 2'-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose; LPS, Lipopolysaccharide; HMO, human milk oligosaccharide.

Treatment	3'SL (g/L)	6'SL (g/L)	2'FL (g/L)	% G1 Mean	%G1 SEM	% S Mean	%S SEM	%G2 Mean	%G2 SEM
Control: 0 g/L				51.4	1.5	34.2	1.4	14.4	0.4
Media				59.4*	2.0	29.7	1.9	10.9****	0.5
LPS				60.5*	2.0	28.1	1.9	11.4**	0.5
4 g/L total HMO				46.1****	1.5	37.7****	1.4	16.2	0.4
3'SL	0.2			52.7	1.5	34.5	1.4	12.8	0.4
3'SL	1.0			54.0	1.5	33.8	1.4	12.2**	0.4
6'SL		0.4		52.9	1.5	34.6	1.4	12.6*	0.4
6'SL		1.0		54.7**	1.5	34.2	1.4	11.2****	0.4
2'FL			0.2	55.1**	1.5	33.5	1.4	11.4****	0.4
2'FL			2.0	57.1****	1.5	32.3	1.4	10.1****	0.4
3'SL + 6'SL	0.2	0.4		54.3*	1.5	34.0	1.4	11.7**	0.4
3'SL + 6'SL	1.0	1.0		55.4**	1.5	32.2*	1.4	12.3*	0.4
3'SL + 2'FL	0.2		0.2	54.6*	1.5	33.3	1.4	12.2**	0.4
3'SL + 2'FL	1.0		2.0	55.7**	1.5	32.0*	1.4	12.3**	0.4
6'SL + 2'FL		0.4	0.2	53.9	1.5	33.5	1.4	12.6*	0.4
6'SL + 2'FL		1.0	2.0	54.5*	1.5	32.5	1.4	13.1	0.4
3'SL + 6'SL + 2'FL	0.2	0.4	0.2	53.8	1.5	33.5	1.4	12.6*	0.4
3'SL + 6'SL + 2'FL	1.0	1.0	2.0	53.5	1.5	32.7	1.4	13.7	0.4

Table 6.4. Effects of treatment on pre-confluent CaCo-2Bbe cell cycle kinetics. Values are mean $\pm$ SEM. Asterisks denote statistically significant differences in treatment means as compared to 0 mg/L control by one-way ANOVA followed by Dunnett's multiple comparison test (* p <0.05; ** p <0.01; **** p <0.0001). 2'FL, 2'-fucosyllactose; 3'-SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose; LPS, Lipopolysaccharide; HMO, human milk oligosaccharide.

Treatment	3'SL (g/L)	6'SL (g/L)	2'FL (g/L)	Alkaline Phosphatase Activity (U/mg protein)	SEM
Control: 0 g/L				1.32	0.99
Media				0.36	0.04
LPS				0.34	0.04
4 g/L total HMO				0.47	0.06
3'SL	0.2			0.90	0.29
3'SL	1.0			0.64	0.12
6'SL		0.4		0.55	0.09
6'SL		1.0		0.58	0.10
2'FL			0.2	0.56	0.09
2'FL			2.0	0.53	0.08
3'SL + 6'SL	0.2	0.4		0.52	0.08
3'SL + 6'SL	1.0	1.0		0.46	0.06
3'SL + 2'FL	0.2		0.2	0.51	0.07
3'SL + 2'FL	1.0		2.0	0.51	0.07
6'SL + 2'FL		0.4	0.2	0.53	0.08
6'SL + 2'FL		1.0	2.0	0.44	0.06
3'SL + 6'SL + 2'FL	0.2	0.4	0.2	0.42	0.05
3'SL + 6'SL + 2'FL	1.0	1.0	2.0	0.43	0.05

Table 6.5. Effects of treatment on pre-confluent HT-29 cell differentiation. Treatment did not increase differentiation compared to control (0 mg/L) as analyzed by one-way ANOVA followed by Dunnett's multiple comparison test. Values are mean $\pm$ SEM. 2'FL, 2'-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose; LPS, Lipopolysaccharide; HMO, human milk oligosaccharide.

Treatment	3'SL (g/L)	6'SL (g/L)	2'FL (g/L)	Alkaline Phosphatase Activity (U/mg protein)	SE
Control: 0 g/L				2.57	0.19
Media				2.47	0.22
LPS				2.30	0.22
4 g/L total HMO				2.56	0.22
3'SL	0.2			2.92	0.20
3'SL	1.0			2.77	0.20
6'SL		0.4		2.78	0.22
6'SL		1.0		2.98	0.22
2'FL			0.2	3.05	0.20
2'FL			2.0	3.15 *	0.19
3'SL + 6'SL	0.2	0.4		3.15 #	0.20
3'SL + 6'SL	1.0	1.0		3.13 #	0.19
3'SL + 2'FL	0.2		0.2	2.93	0.20
3'SL + 2'FL	1.0		2.0	2.72	0.19
6'SL + 2'FL		0.4	0.2	2.97	0.20
6'SL + 2'FL		1.0	2.0	2.79	0.20
3'SL + 6'SL + 2'FL	0.2	0.4	0.2	2.72	0.19
3'SL + 6'SL + 2'FL	1.0	1.0	2.0	2.56	0.19

Table 6.6. Effects of treatment on pre-confluent CaCo-2Bbe cell differentiation. A 22.6% increase increase ($p=0.049$) in AP activity occurred in cells treated with 2.0 g/L 2'FL as compared to 0 g/L control. The combinations of 3'SL and 6'SL tended (#) to increase differentiation ($p=0.0595$ and $p=.0535$). Values are mean $\pm$ SEM. Asterisks denote statistically significant difference in treatment means as compared to 0 mg/L control by one-way ANOVA followed by Dunnett's multiple comparison test (# $p<0.01$, * $p<0.05$). 2'FL, 2'-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose; LPS, Lipopolysaccharide; HMO, human milk oligosaccharide.

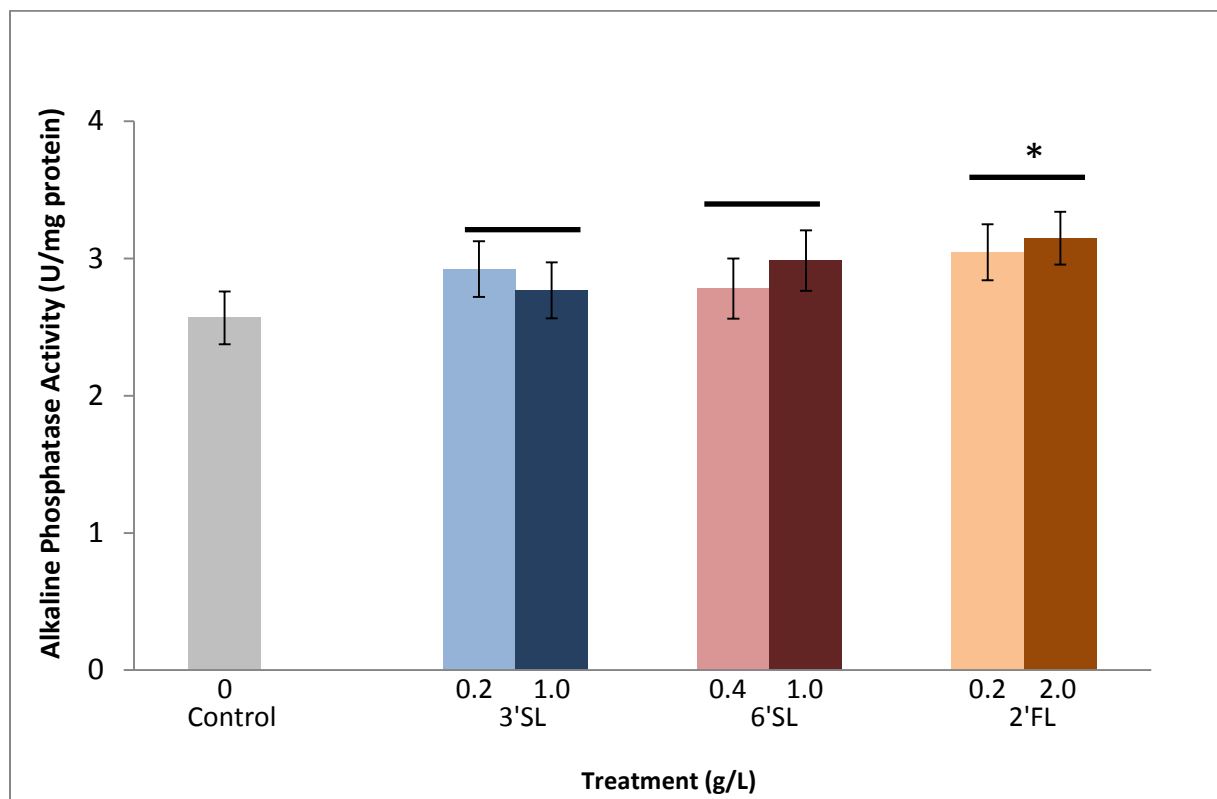


Figure 6.2. Effects of individual HMO on pre-confluent Caco-2Bbe cell differentiation.

Values are mean $\pm$ SEM. Bars with asterisks denote statistically significant increase in pooled treatment means of the high and low dose treatments of individual HMO as compared to 0 mg/L control by one-way ANOVA (* p <0.05). 2'FL significantly increased pre-confluent Caco-2Bbe cell differentiation. Pooled treatments of 3'SL, 6'SL, and 2'FL vs 0g/L control; * p =0.0059. 2'FL, 2'-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose.

Treatment	3'SL (g/L)	6'SL (g/L)	2'FL (g/L)	Alkaline Phosphatase Activity (U/mg protein)	SEM
Control: 0 g/L				12.04	1.36
Media				10.69	1.31
LPS				8.37	1.36
4 g/L total HMO				10.12	1.27
3'SL	0.2			13.59	1.27
3'SL	1.0			12.65	1.27
6'SL		0.4		13.27	1.44
6'SL		1.0		13.42	1.31
2'FL			0.2	11.32	1.31
2'FL			2.0	12.82	1.27
3'SL + 6'SL	0.2	0.4		13.75	1.27
3'SL + 6'SL	1.0	1.0		12.89	1.27
3'SL + 2'FL	0.2		0.2	13.40	1.27
3'SL + 2'FL	1.0		2.0	12.67	1.27
6'SL + 2'FL		0.4	0.2	14.27	1.27
6'SL + 2'FL		1.0	2.0	11.92	1.31
3'SL + 6'SL + 2'FL	0.2	0.4	0.2	11.94	1.27
3'SL + 6'SL + 2'FL	1.0	1.0	2.0	11.77	1.31

Table 6.7. Effects of treatment on post-confluent CaCo-2Bbe cell differentiation.

Treatment did not increase differentiation compared to control (0 mg/L) as analyzed by one-way ANOVA followed by Dunnett's multiple comparison test. Values are mean $\pm$ SEM. 2'FL, 2'-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose; LPS, Lipopolysaccharide; HMO, human milk oligosaccharide.

Treatment	3'SL (g/L)	6'SL (g/L)	2'FL (g/L)	Apoptosis (% of control)	SEM (%)	Necrosis (% of control)	SEM (%)
Control: 0 g/L				100.0	13.1	100.0	8.2
Media				121.7	10.1	85.9	8.2
LPS				138.2	13.1	69.5*	8.2
4 g/L total HMO				279.3***	10.7	4.0***	7.0
3'SL	0.2			118.1	13.1	105.6	8.2
3'SL	1.0			86.7	13.1	102.1	8.8
6'SL		0.4		111.5	13.1	116.2	8.2
6'SL		1.0		82.6	13.1	99.7	8.2
2'FL			0.2	91.1	13.1	95.7	8.2
2'FL			2.0	94.4	13.1	71.1 #	8.2
3'SL + 6'SL	0.2	0.4		123.1	13.1	96.8	8.2
3'SL + 6'SL	1.0	1.0		132.4	13.1	87.1	8.2
3'SL + 2'FL	0.2		0.2	87.5	13.1	96.6	8.2
3'SL + 2'FL	1.0		2.0	94.6	13.1	84.4	8.2
6'SL + 2'FL		0.4	0.2	89.9	13.1	100.5	8.2
6'SL + 2'FL		1.0	2.0	110.0	13.1	77.8	8.8
3'SL + 6'SL + 2'FL	0.2	0.4	0.2	129.4	13.1	102.3	8.2
3'SL + 6'SL + 2'FL	1.0	1.0	2.0	130.8	13.1	75.8	8.2

Table 6.8. Effects of treatment on pre-confluent HT-29 Cell Apoptosis and Necrosis. Values represent the percent apoptosis or necrosis as compared to 0 mg/L control (set at 100%). Control (0mg/L) cell apoptosis: 0.127 ± 0.0166 . Control (0mg/L) cell necrosis: 0.145 ± 0.0118 . Values are mean $\pm$ SEM expressed as percentages. Asterisks denote statistically significant differences either apoptosis or necrosis in treatment means as compared to 0 mg/L control by one-way ANOVA followed by Dunnett's multiple comparison test (# $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). There was a significant increase in apoptosis in the HMO treatment group ($p < 0.001$). There was a significant reduction in necrosis compared to the 0g/L control for HMO ($p < 0.001$) and LPS ($p = 0.0439$) and a trend for 2g/L of 2'FL ($p = 0.065$). 2'FL, 2'-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose; LPS, Lipopolysaccharide; HMO, human milk oligosaccharide.

Treatment	3'SL (g/L)	6'SL (g/L)	2'FL (g/L)	Apoptosis (% of Control)	SEM (%)	Necrosis (% of Control)	SEM (%)
Control: 0 g/L				100.0	17.7	100.0	22.7
Media				153.2	17.7	119.5	22.7
LPS				144.9	17.7	109.6	22.7
4 g/L total HMO				68.5	14.4	22.8***	21.3
3'SL	0.2			108.1	17.7	137.2	22.7
3'SL	1.0			92.4	17.7	111.7	22.7
6'SL		0.4		121.6	17.7	109.3	22.7
6'SL		1.0		109.0	17.7	117.1	22.7
2'FL			0.2	154.1	17.7	89.7	22.7
2'FL			2.0	103.6	17.7	108.1	22.7
3'SL + 6'SL	0.2	0.4		128.6	17.7	100.8	22.7
3'SL + 6'SL	1.0	1.0		100.7	17.7	103.5	22.7
3'SL + 2'FL	0.2		0.2	121.8	17.7	102.2	22.7
3'SL + 2'FL	1.0		2.0	109.8	17.7	98.6	22.7
6'SL + 2'FL		0.4	0.2	86.7	17.7	107.3	22.7
6'SL + 2'FL		1.0	2.0	153.7	17.7	92.1	22.7
3'SL + 6'SL + 2'FL	0.2	0.4	0.2	118.8	17.7	114.6	22.7
3'SL + 6'SL + 2'FL	1.0	1.0	2.0	126.7	17.7	94.2	22.7

Table 6.9. Effects of treatment on pre-confluent CaCo-2Bbe cell apoptosis and necrosis. Values represent the percent apoptosis or necrosis as compared to 0 mg/L control (set at 100%). Control (0mg/L) cell apoptosis: 0.0645 ± 0.0114 . Control (0mg/L) cell necrosis: 0.0899 ± 0.0204 . Values are mean $\pm$ SEM expressed as percentages. Asterisks denote statistically significant differences in apoptosis or necrosis in treatment means as compared to 0 mg/L control by one-way ANOVA followed by Dunnett's multiple comparison test (***p<0.001). There was a significant decrease in necrosis in the HMO treatment group (p<0.001). 2'FL, 2'-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose; LPS, Lipopolysaccharide; HMO, human milk oligosaccharide.

Treatment	3'SL (g/L)	6'SL (g/L)	2'FL (g/L)	Apoptosis (% of Control)	SEM (%)	Necrosis (% of Control)	SEM (%)
Control: 0 g/L				100.0	9.4	100.0	28.2
Media				91.5	8.7	65.7**	18.6
LPS				94.2	9.1	67.6**	19.1
4 g/L total HMO				60.5****	5.8	31.7****	8.9
3'SL	0.2			105.4	10.0	76.7	21.6
3'SL	1.0			109.3	10.4	80.9	22.8
6'SL		0.4		105.8	10.0	72.8*	20.5
6'SL		1.0		96.9	9.2	69.4**	19.6
2'FL			0.2	104.6	9.9	68.9**	19.4
2'FL			2.0	104.8	9.9	71.1**	20.0
3'SL + 6'SL	0.2	0.4		106.0	10.0	75.4*	21.2
3'SL + 6'SL	1.0	1.0		107.2	10.2	85.7	24.1
3'SL + 2'FL	0.2		0.2	103.0	9.8	77.2#	21.7
3'SL + 2'FL	1.0		2.0	106.7	10.1	82.9	23.4
6'SL + 2'FL		0.4	0.2	100.1	9.5	80.8	22.8
6'SL + 2'FL		1.0	2.0	103.0	9.8	85.2	24.0
3'SL + 6'SL + 2'FL	0.2	0.4	0.2	104.4	9.9	80.6	22.7
3'SL + 6'SL + 2'FL	1.0	1.0	2.0	105.5	10.0	85.4	24.1

Table 6.10. Effects of treatment on post-confluent CaCo-2Bbe cell apoptosis and necrosis. Values represent the percent apoptosis or necrosis as compared to 0 mg/L control (set at 100%). Control (0mg/L) cell apoptosis: 1.11 ± 0.105 . Control (0mg/L) cell necrosis: 0.561 ± 0.158 . Values are mean $\pm$ SEM expressed as percentages. Asterisks denote statistically significant differences in apoptosis or necrosis in treatment means as compared to 0 mg/L control by one-way ANOVA followed by Dunnett's multiple comparison test ($p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). There was a significant decrease in necrosis in the HMO treatment group ($p < 0.001$). 2'FL, 2'-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose; LPS, Lipopolysaccharide; HMO, human milk oligosaccharide.

Treatment	3'SL (g/L)	6'SL (g/L)	2'FL (g/L)	Sucrase Activity (10⁻² U/mg protein) Day 12	SEM	Sucrase Activity (10⁻² U/mg protein) Day 21	SEM
Control: 0 g/L				1.78	0.13	2.53	0.22
Media				1.82	0.25	1.95	0.33
LPS				1.71	0.19	1.92	0.33
4 g/L total HMO				1.58	0.10	1.96	0.22
3'SL	0.2			1.93	0.15	2.47	0.22
3'SL	1.0			1.85	0.14	2.29	0.21
6'SL		0.4		1.91	0.15	2.37	0.23
6'SL		1.0		1.75	0.13	2.39	0.23
2'FL			0.2	1.72	0.12	2.28	0.22
2'FL			2.0	1.61	0.11	2.43	0.22
3'SL + 6'SL	0.2	0.4		1.86	0.14	2.37	0.22
3'SL + 6'SL	1.0	1.0		1.67	0.12	2.51	0.22
3'SL + 2'FL	0.2		0.2	1.85	0.14	2.49	0.22
3'SL + 2'FL	1.0		2.0	1.68	0.12	2.40	0.22
6'SL + 2'FL		0.4	0.2	1.58	0.10	2.42	0.22
6'SL + 2'FL		1.0	2.0	1.49	0.09	2.31	0.23
3'SL + 6'SL + 2'FL	0.2	0.4	0.2	1.98	0.16	2.46	0.22
3'SL + 6'SL + 2'FL	1.0	1.0	2.0	1.70	0.12	2.45	0.23

Table 6.11. Effects of treatment on post-confluent CaCo-2Bbe cell sucrase activity at day 12 and day 21. Treatment did not increase sucrase activity compared to control (0 mg/L) as analyzed by one-way ANOVA followed by Dunnett's multiple comparison test. Values are mean $\pm$ SEM. 2'FL, 2'-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose; LPS, Lipopolysaccharide; HMO, human milk oligosaccharide.

Treatment	3'SL (g/L)	6'SL (g/L)	2'FL (g/L)	Lactase Activity (10⁻³ U/mg protein) Day 12	SEM	Lactase Activity (10⁻³ U/mg protein) Day 21	SEM
Control: 0 g/L				7.59	0.67	6.86	0.55
Media				9.72	2.13	6.22	0.70
LPS				9.54	2.04	6.22	0.64
4 g/L total HMO				7.98	0.74	6.78	0.51
3'SL	0.2			7.39	0.63	7.01	0.55
3'SL	1.0			7.32	0.62	6.61	0.55
6'SL		0.4		8.06	0.75	6.40	0.60
6'SL		1.0		7.91	0.73	5.17	0.60
2'FL			0.2	7.32	0.62	5.41	0.55
2'FL			2.0	6.84	0.54	6.94	0.55
3'SL + 6'SL	0.2	0.4		8.49	0.84	6.23	0.55
3'SL + 6'SL	1.0	1.0		8.10	0.76	6.56	0.55
3'SL + 2'FL	0.2		0.2	8.76	0.90	7.01	0.55
3'SL + 2'FL	1.0		2.0	9.64	1.09	6.93	0.55
6'SL + 2'FL		0.4	0.2	7.04	0.57	6.78	0.55
6'SL + 2'FL		1.0	2.0	8.05	0.75	6.79	0.60
3'SL + 6'SL + 2'FL	0.2	0.4	0.2	9.62	1.09	5.85	0.55
3'SL + 6'SL + 2'FL	1.0	1.0	2.0	9.20	0.99	7.71	0.60

Table 6.12. Effects of treatment on post-confluent CaCo-2Bbe cell lactase activity at day 12 and day 21. Treatment did not increase lactase activity compared to control (0 mg/L) as analyzed by one-way ANOVA followed by Dunnett's multiple comparison test. Values are mean $\pm$ SEM. 2'FL, 2'-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose; LPS, Lipopolysaccharide; HMO, human milk oligosaccharide.

	3'SL	6'SL	2'FL
Pre-confluent HT-29			
Proliferation		Decreased	Decreased
Cell Cycle			
Differentiation			
Apoptosis			
Necrosis			Decreased
Pre-confluent CaCo-2Bbe			
Proliferation			
Cell Cycle			
Differentiation			Increased
Apoptosis			
Necrosis			
Post-confluent CaCo-2Bbe			
Differentiation			
Apoptosis			
Necrosis		Decreased	Decreased
Sucrase			
Lactase			

Table 6.13. Summary of individual HMO effects in each cell type. 2'FL, 2'-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose.

	3'SL + 6'SL	3'SL + 2'FL	6'SL + 2'FL	3'SL + 6'SL + 2'FL	HMO
Pre-confluent HT-29					
Proliferation	Decreased	Decreased	Decreased	Decreased	Decreased
Cell Cycle					
Differentiation					
Apoptosis					Increased
Necrosis					Decreased
Pre-confluent CaCo-2Bbe					
Proliferation		Decreased	Decreased	Decreased	Decreased
Cell Cycle					
Differentiation	Increased	Increased			
Apoptosis					
Necrosis					Decreased
Post-confluent CaCo-2Bbe					
Differentiation					
Apoptosis					Decreased
Necrosis	Decreased	Decreased			Decreased
Sucrase					
Lactase					

Table 6.14. Summary of concomitant HMO effects in each cell type. 2'FL, 2'-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose.

CHAPTER 7

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

It is well established that human milk is the optimal source of nutrition for the developing infant. Human milk contains both microbes and oligosaccharides. Research has shown that bifidobacteria predominate the microbiota of breast-fed infants. Human milk oligosaccharides contribute to the bifidogenic properties of human milk, provide immune enhancing effects, and may enhance intestinal maturation. However, research is still lacking on specific ingredients which may be added to infant formula to impart some of the benefits of breast feeding onto the formula fed infant. Therefore, the specific aim of this thesis research was to assess 1) the effects of the probiotic *Bifidobacterium animalis* subspecies *lactis*, 2) the prebiotics galacto- and fructo-oligosaccharides, and 3) the individual and combined effects of specific human milk oligosaccharides (HMOs) as means to narrow the compositional gap between human milk and infant formula. This was explored through two clinical trials investigating the individual effects of the addition of a pro- or prebiotic to infant formula as well as through *in vitro* studies on the effects of human milk oligosaccharides on intestinal maturation.

Hypothesis 1: Consumption of an infant formula containing *Bifidobacterium animalis* subspecies *lactis* for 6 weeks will positively impact the intestinal-associated immune system and resident microbiota of formula-fed infants.

Human milk contains a multitude of components including bacteria, nutrients that stimulate colonization of bifidobacteria within the intestine, and immunoglobulins (Goldman et

al., 1982; Heikkila and Saris, 2003; Martín et al., 2003; German et al., 2008a). Thus, we investigated the effects of infant formula containing *B. lactis* and found it to be well tolerated by health infants and to stimulate intestinal immunity. We found both enhanced fecal secretory IgA (sIgA) and vaccine-specific IgA following immunization in supplemented infants.

Although the mechanism of the stimulation of sIgA by bifidobacteria remains largely unknown, there is speculation that the Gram-positive cell walls, which are rich in peptidoglycans, may have a role in the immune stimulation (Bakker-Zierikzee et al., 2006). Research from our lab group as well as others, suggests that induction of inflammation may play a role in immune maturation and enhanced IgA production (Kukkonen et al., 2010; Holscher et al., 2012a). In addition to stimulating antibody production from B cells, probiotic supplementation has also been shown to induce regulatory dendritic cells and T cells (Kwon et al., 2010). Bifidobacteria in particular have been found to stimulate maturation of dendritic cells, characterized by the release of tumor necrosis factor- α , interleukin (IL-10) and IL-12 (Hart et al., 2004). Overall, the low level production of cytokines stimulated by probiotic supplementation is thought to be beneficial as it is an immunoregulatory response, rather than an aggressive immune response. It seems likely that a combined response from dendritic cells, T and B cells is necessary for induction of IgA production. Further research is needed to clarify the mechanism by which *B. lactis* supplementation stimulates antibody production.

While we have shown that *B. lactis* supplementation results in detectable levels of fecal *B. lactis* and increases fecal immunoglobulins with an enhanced effect in cesarean-section delivered infants, it would be beneficial to further investigate the composition of the fecal microbiota utilizing more sophisticated techniques such as pyrosequencing. Specifically, investigation of the effects of supplementation on bifidobacteria species would be of interest due

to their association with enhanced sIgA. A recent study by Hascoet and coworkers showed that a whey/casein ratio of 70/30 along with supplementation of *Bifidobacterium longum* BL999 resulted in similar fecal bifidobacteria counts between formula fed infants and breast fed infants along with a numeric increase in fecal secretory IgA in the formula fed groups (Hascoët et al., 2011). As the probiotic formula utilized in our study was whey based and enhanced fecal sIgA, it is possible that it also stimulated an increased proportion of fecal bifidobacteria. However, in light of the findings by Hascoet and colleagues, it is possible that the lower phosphate, whey based formulas we investigated promoted bifidobacteria regardless of probiotic supplementation. Even so, it remains likely that delivery method in combination with feeding method resulted in differences in the respective microbiota of each group thus further study will better our understanding of the influence of diet and delivery method on the microbiota.

Further, assessment of the fecal microbiota of our infant stool samples may add clarity to the effects seen in infants fed probiotic formula in our research trial. Utilizing pyrosequencing to assess the fecal microbiota would allow for quantification of bacteria at the species and subspecies level. These data are needed so that we may better understand the compositional differences between breast-fed and formula-fed infants as well as the impact of delivery method and probiotic feeding.

In addition to exploring the microbial composition, it would also be interesting to follow-up with the infants in the trial to see if the immune enhancing effects persist after cessation of study formula. A study by Kuitunen and co-workers showed that infants supplemented with probiotics until 6 months of age and delivered by cesarean section had lower IgE-associated allergies at 5 years of age (Kuitunen et al., 2009). A follow-up study in our infant population

would allow us to broaden our understanding of the potential long term effects of probiotic supplementation.

Hypothesis 2: Consumption of the galacto- and fructo-oligosaccharide-containing formula for six weeks will be well-tolerated and increase fecal bifidobacteria proportions in formula-fed infants.

Infant formulas are often supplemented with prebiotic galacto- (GOS) and fructo-oligosaccharides (FOS) in an effort to mimic the neutral oligosaccharide content of human milk which is known to contribute to the higher proportion of bifidobacteria within the microbiota of breastfed infants (Sela and Mills, 2010). A large number of clinical trials in infants have shown that supplementation of infant formula with GOS and FOS at varying concentrations and blends increases fecal bifidobacteria (Moro et al., 2002; Moro et al., 2003; Schmelzle et al., 2003; Ben et al., 2004; Boehm et al., 2004; Bakker-Zierikzee et al., 2005; Haarman and Knol, 2005; Knol et al., 2005a; Knol et al., 2005b). Our laboratory has also demonstrated that infant formula containing GOS and FOS was well-tolerated by formula fed infants, increased the abundance and proportion of fecal bifidobacteria, and reduced fecal pH after six weeks of feeding (Holscher et al., 2012b).

To expand on these findings, it would be beneficial to analyze the fecal bacterial populations of our infants using more sophisticated techniques such as pyrosequencing. Much of the research detailing the microbiota composition of infants is based on culture-based techniques. Although we utilized FISH, a culture-independent method, we were unable to discriminate many of the bacteria at the species level. Pyrosequencing would allow us to further characterize the

bacteria present within the infant's microbiota. Assessment of the microbiome along with metagenomics analysis would also help us to better interpret the modifications in the SCFA profiles seen in the formula-fed and breastfed infant groups. Utilizing the most up-to-date technologies to detail both microbes and microbial gene expression will help us to better understand the impact of diet on the human infant's gastrointestinal tract.

In addition to further characterization of the microbiota, the impact of prebiotic supplementation on immunity should also be assessed. Recent infant formula trials have demonstrated that prebiotic supplementation enhances fecal secretory IgA levels, thus further assessment of immune enhancing effects of the GOS/FOS provided in our trial would add to this body of literature (Bakker-Zierikzee et al., 2006; Scholtens et al., 2008).

Hypothesis 3: Three individual HMO, 2'-fucosyllactose (2'FL), lacto-N-neotetraose (LNnT), and 6'-sialyllactose (6'SL), will induce epithelial differentiation in an *in vitro* epithelial model of the crypt-villus axis consisting of pre-confluent HT-29 cells, pre-confluent CaCo-2Bbe cells, and post-confluent CaCo-2Bbe cells.

Human milk has both high structural diversity and concentration of oligosaccharides—HMOs constitute up to 20 g/L of human milk, making them the third largest solid component (Coppa et al., 1999; Chaturvedi et al., 2001a; Ninonuevo et al., 2006; German et al., 2008; Thurl et al., 2010; Wu et al., 2010). Infant formula completely lacks HMOs and milk based formulas provide only negligible amounts of free complex bovine oligosaccharides (Kunz et al., 2000). Previous studies have shown that HMOs influence maturation of intestinal epithelial cells making them an ingredient of interest for supplementation in infant formula (Kuntz et al., 2008).

Although greater than 200 different oligosaccharides structures are present in human milk, the neutral and acidic fractions are primarily made up of fourteen neutral and six acidic oligosaccharides with 2'FL and LNnT being dominant neutral oligosaccharides and 6'SL representing a large proportion of the acidic fraction (Thurl et al., 2010). Thus, using an *in vitro* model of the crypt-villus axis we investigated the effect of the three individual HMO (2'FL, LNnT, and 6'SL) and found that they promoted intestinal cell maturation *in vitro*. HMOs inhibited cellular proliferation, with 2'FL also providing stimulation for associated enhancement of epithelial differentiation in pre-confluent HT-29 cells. Furthermore, this trial demonstrated that well differentiated Caco-2Bbe cells have greater digestive and barrier function when treated with physiological levels of 2'FL and LNnT, respectively. In light of these findings, we investigated the synergistic effects of specific human milk oligosaccharides in our fourth hypothesis.

Hypothesis 4: Concomitant administration of HMO, 2'-fucosyllactose (2'FL), 3'-sialyllactose (3'SL), and 6'-sialyllactose (6'SL), will induce epithelial differentiation and enhance maturation of the small intestine *in vitro* in an epithelial model of the crypt-villus axis consisting of pre-confluent HT-29 cells, pre-confluent CaCo-2Bbe cells, and post-confluent CaCo-2Bbe cells.

In this trial, we investigated the effects of concomitant administration of human milk oligosaccharides 2'FL, 3'SL, and 6'SL within our *in vitro* model of the small intestine. It was again demonstrated that individual HMO inhibit cellular proliferation. Further, combinations of HMO were shown to have with additive effects on the reduction in proliferation in both pre-confluent HT-29 and CaCo-2Bbe cells. Inhibition of proliferation was associated with cell cycle

arrest within the G0/G1 phase and an enhancement of differentiation in pre-confluent CaCo-2Bbe cells, however there was no enhancement in differentiation in the pre-confluent HT-29 cells nor were there any changes in cell cycle contrary to our previous findings. Further, treatment did not affect post-confluent CaCo-2Bbe differentiation. Apoptosis and necrosis were reduced in post-confluent CaCo-2Bbe cells treated with the 4 g/L pooled HMO sample, but this physiological change was not associated with a functional outcome. The combination of inhibition of proliferation and induction of differentiation do suggests that HMOs promote maturation of the gastrointestinal (GI) tract *in vitro*. However, the physiological relevance of these changes should be investigated within *in vivo* models. Pre-clinical animal models, such as the short bowel syndrome (SBS) piglet model utilized by our laboratory group would allow for assessments to be made on structural and functional outcomes associated with enteral supplementation of HMO. The piglet intestinal failure model has been shown to resemble the complications associated with short-bowel syndrome in human infants including the ability of the remaining residual bowel to adapt at both the cellular and tissue level (Heemskerk et al., 1999; Bartholome et al., 2004). Further, it has been shown that piglets recovering from intestinal resection fed polymeric infant formula supplemented with colostrum protein concentrate had improved intestinal adaption and overall growth as compared to control animals (Nagy et al., 2004). Additionally, unpublished data from our laboratory group has demonstrated that piglets provided 20% of their needs as colostrum had enhanced structural and functional outcomes within the GI tract as compared to piglets receiving sow-replacer formula during the first week of life. As colostrum contains the richest source of human milk oligosaccharides (>20 g/L), it is possible that the oligosaccharides also played a role in stimulating the intestinal adaption.

Summary

In summary, this thesis research has provided insight into the roles of probiotics, prebiotics, and HMOs in infant formulae. It further substantiated the safety and efficacy of supplementation of pro- and prebiotics in infant formulae as well as provided valuable insight into novel infant formula ingredients, HMOs. Thus, the information gained from this research strengthens our understanding of specific ingredients which may play a role in narrowing the functional and compositional gap between human milk and infant formula.

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