Impact of Buffering Capacity and Low Dose Galacto-Oligosaccharide on *in-vitro* Infant Fecal Cultures

Thesis

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By

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Abstract

It is generally accepted that human breast milk is the optimal nutrition for infants. This is partly attributed to the microbiota that breast milk encourages. Breast fed infants tend to harbor a high abundance of bifidobacteria while formula fed infants have a more diverse microbiota with clostridia and enteric bacteria in higher abundance. A number of factors present in human milk are responsible for these differences. Recent research and clinical trials indicate that oligosaccharides present in human milk, also known as prebiotics, are the major factor producing this “bifidogenic” effect. Researchers also suggested that the lower buffering capacity of human milk as another factor. However, no research has been reported that tested this premise.

In this study we aim to determine if and to what extent buffering capacity affects infant fecal cultures in vitro. Fresh infant fecal samples collected from seven infants at two different ages were incubated in media with varying buffering capacities (based on NaCHO₃). In a subset of cultures, we included a low dose (0.8g/L) of galacto-oligosaccharide (GOS) to determine if a low buffering capacity can enhance the prebiotic effect of GOS. Quantitative PCR (qPCR) assays were employed to measure microbial shifts among treatments, and short chain fatty acids (SCFAs) were measured by gas
chromatography. pH of the cultures dropped significantly with decreasing buffering capacity, but there were no significant changes in SCFA production. As determined by qPCR assays, bacterial abundance varied considerably among individuals, yet we did find a significant decrease in the *E. coli* population with decreasing buffering capacity, irrespective of GOS addition. However, no significant decrease was found in the abundance of *Clostridium difficile*. The limited amount of substrates available in the cultures might have limited the production of SCFAs and thus the expected decrease in *C. difficile* and *E. coli*. Future studies using higher doses of prebiotics are needed to further evaluate the impact of buffering capacities on inhibition of *C. difficile* and *E. coli*. 
Acknowledgments

I would like to thank my Advisor and Committee members, Dr. Hua Wang, Dr. Zhongtang Yu, and Dr. Mark Morrison for their instruction and support throughout my graduate study. It is a privilege to work with and learn from such an exemplary group of professors. I would especially like to acknowledge Dr. Yu, for giving so much of his time and attention to my project.

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Chapter 1

Literature Review

It is well understood that breast feeding is the healthiest source of nutrition for human infants. The World Health Organization (WHO) recommends exclusively breast feeding for the first 6 months of life while continuing to breast feed as a supplement for the first 2 years (Kramer, 2002). One major reason breast milk is considered the “gold standard” for infant health is its obvious effect on the infant’s gastrointestinal microbiota. Breast-fed infants exhibit higher abundance of *Bifidobacterium* and *Lactobacilli* while formula-fed infants have a more diverse intestinal microbiota with a greater abundance of *Enterobacteriaceae* and *Clostridiales*. Because of the correlation between species presence and health, it is generally accepted that a higher abundance of *Bifidobacterium* and *Lactobacillus* indicates a healthier gut microbiota, and studies aiming to improve infant formula generally target these species in particular. The “Bifidogenic Effect” is a recent term coined to describe the health benefits associated with an increase in *Bifidobacterium*. Additionally, lower or zero counts of opportunistic pathogens such as *E. coli* and *C. difficile* are considered beneficial to health.
Because the infant gastrointestinal (GI) tract and immune system are still developing, early colonization is thought to be crucial to overall GI health. Research indicates that as the infant GI microbiota matures it becomes less amenable to dietary changes, thus early age provides the best opportunity to modulate the GI microbiota.

Breast-fed infants have lower incidence of mortality, gastrointestinal infection, allergy, and dermatitis (Siggers, 2010; Dong, 2010). Gut colonization can also have a major impact on health into adulthood (Palmer 2007). Despite this, it is estimated that only 35% of infants, ages 0-6 months, are exclusively breast fed (WHO). This indicates that there is a great need for alternative infant nutrition that more closely resembles that of breast milk.

**Intestinal colonization and intestinal microbial ecosystem**

The intestinal tract of a fetus is sterile and initial inoculation of the infant GI tract occurs at birth (Palmer, 2007). For a vaginal birth the infant initially comes in contact with the mother’s vaginal and fecal bacteria. Births by cesarean section result in loss of this initial inoculation from the mother and the neonatal GI tract is more likely inoculated from the environment and medical staff (Bezirtoglou, 1997). There is a microbial shift as the infant GI tract is initially colonized by facultative anaerobes. Facultative anaerobic bacteria such as enterobacteria, streptococci and staphylococci consume oxygen and lower the redox potential, thus making a more hospitable environment for obligate anaerobes which are then able to thrive and grow. In breast-fed infants *Bifidobacterium is*
often the predominant species while enterobacteria, *Clostridiales*, and *Bacteroides* are present but in lower numbers. Formula-fed infants on the other hand have a more diverse microbiota and can exhibit higher numbers of enterobacteria and clostridia (McCartney and Gibson, 2006). At the introduction of solid foods (weaning) these bacterial differences between breast-fed and formula-fed infants begin to diminish. The amenability and instability of the GI microbiota also begin to decline.

Stecher et al. (2010) found that the presence of one species in the gut can improve the ability of closely related species to colonize therein. In a murine model, they showed that closely related phylotypes exhibited significant co-occurrence. For example, mice with high titers of lactobacilli were effectively colonized by *L. reuteri* while mice with high *E. coli* abundance were more susceptible to enterocolitis infection by *S. enterica* (Stetcher, 2010). Thus, individuals with higher abundance of opportunistic bacteria actually are at an increased risk of infection by not only the opportunistic bacteria but also by related species. Alternatively, those individuals with higher abundance of *Bifidobacterium* and *Lactobacillus* are more likely to benefit from probiotic therapies. Additionally, the native gut microbiota acts as a defense barrier against pathogenic invasion. Because gut amenability decreases as the GI tract matures, early age is a window of opportunity for improving lifelong health with changes to infant diet.
**Intestinal microbiota and health**

The microbial ecosystem in the GI tract plays an important role in human health. The microbiota in the gut has been implicated in immune system development, improved breakdown and absorption of nutrients, and barrier against pathogenic infection. Breast-fed infants have lower risk of gastrointestinal infection, mortality, and allergies. Breast milk has long been implicated in the immune function of infants due to the antigens present in breast milk but now it is also determined that the oligosaccharides in breast milk can foster a bacterial community highly adept at influencing and modulating the immature immune system of the infant.

Due to difficulties and limitations in studies involving infants, most data regarding immune system function is obtained through studies on germ free and gnotobiotic animal models. Germ free mice exhibit an underdeveloped immune system including poorly developed Preyer’s patch, unbalanced Th1/Th2 cells, and decreased Ig-A secreting cells (Moreau, 1978), indicating that the intestinal bacteria aid in the immune systems development and balance. Studies also indicate that dendritic cells mature as an inflammatory response and thus depend on stimulation from bacterial colonization (Michelson, 2001).

Intestinal bacteria ferment undigested fibers and oligosaccharides in the colon producing SCFAs and vitamins B and K, which can be absorbed by the intestinal epithelial cells. SCFAs allow for energy salvaging, increase cell turnover, and may aid in glucose metabolism and lipogenesis. Acetic acid, the major SCFA produced in the colon
(approximately 70% of total SCFAs), allows the epithelial cells to salvage energy otherwise lost in the feces and is a major energy source to muscle cells (Hooper, 2002).

**Impact of feeding on intestinal microbiota**

Achieving an infant formula that mimics the effects of breast milk is the ultimate goal of the infant formula industry. Human breast milk is a complex mix of proteins, lipids, enzymes, nutrients, bioactive compounds, and immunoglobulins which is impossible to replicate. Researchers aim to target specific components of breast milk to close the gap between breast-fed and formula fed infant health. Recent studies implicate oligosaccharides (prebiotics) as being a major factor contributing to gut dynamics and the bifidogenic effect. Oligosaccharides are the third largest solid constituent of breast milk while cow’s milk is virtually free of oligosaccharides (Bode, 2006).

Infant formula is generally composed of cow’s milk with whey and casein added for protein, lactose added for carbohydrate, and vegetable oils for fat, and a mix of vitamins. To improve infant formula, manufacturers look to human breast milk to determine what components are needed to optimize infant health. Major advancements in research and development have improved and continue to improve infant formula. Oligosaccharides are touted as being a major contributor to the gastrointestinal health of infants. Prebiotic studies have shown that oligosaccharides such as galacto-oligosaccharide and fructo-oligosaccharide (FOS) increase bifidobacteria, increase SCFA production, and decrease pathogenic bacteria such as clostridia.
It is well documented that breast milk has a lower buffering capacity than infant formula but the effects this has on infant health has not been elucidated (Jiang, 1997). There are very few studies examining the effect of buffering capacity on human health. Most of these studies were conducted decades ago and did limited analysis on the effect of the major groups of bacteria (Jiang 1997, Edwards 1985). Additionally, most knowledge elucidated is related to pH effects as opposed to buffering capacity. Berger et al. (1978) showed that not only is the buffering capacity of human milk considerably lower than cows milk and infant formula but that buffering capacity of fecal emulsions of formula-fed infants were two to three times higher than that of fecal emulsions of breast-fed infants. It remains unexplored if and to what extent fecal pH and buffering capacity affect GI microbiota.

From understanding of microbial tolerance to pH we might expect that the lower buffering capacity will further encourage the growth of acid tolerant species such as bifidobacteria and lactobacilli while inhibiting other bacterial species. Many studies have measured pH tolerance of major bacterial species of the GI tract. Pathogenic bacteria, such as C. difficile and E. coli thrive at neutral pH. Alternatively, acid producing bacteria such as bifidobacteria and lactobacilli thrive at pH as low as 5.5 (Edwards, 1985).

Because the pH differs significantly between breast-fed and formula-fed fecal samples, it is hypothesized that this pH difference may have a major impact on the commensal species found within an individual. Bullen and Willis (1971) measured the fecal pH of seven day old breast-fed infants to be pH 5.1 compared to that of formula-fed
infants at 6.5. Combined with other studies, the average pH values increase somewhat with age but the differences between the two feeding groups remain.

**Modulation of intestinal microbiota by prebiotics**

Even long before the first commercially developed infant formula in 1867, mothers who were unable or chose not to breastfeed had prepared their own infant formulas consisting primarily of cow’s milk. Since that time, the improvement of infant formula has been a major industrial focus. Most recently, prebiotics are at the forefront of this research effort because of their ability to induce the “bifidogenic effect” associated with breastfed infants.

Prebiotics are defined as “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health” (Roberfroid, 2007). Although there are a number of oligosaccharides described in the literature as prebiotic, galacto- and fructo-oligosaccharides have been studied more frequently in infants due to their impact as well as tolerance (Ziegler, 2007; Knol 2005).

Galacto-oligosaccharide (GOS) is glycosidic linkages of galactose of varying degrees of polymerization (DP) with one terminal glucose unit. Amaretti et. al.(2007) analyzed the kinetics and metabolism of GOS, lactose, glucose, and galactose by *Bifidobacterium adolescentis* MB 239 and determined that this strain of *Bifidobacterium* grew better on galactose but ATP production was highest with GOS.
Probiotics (live cultures) have also gained a lot of attention recently for their ability to modulate the human GI microbiota and function. Numerous studies demonstrated their ability to effectively modulate the gut microbiota as well as decrease inflammation. These data are largely derived from individual probiotic strains. However, since they are not native to the individual GI tract, they typically only colonize the GI tract transiently. Nevertheless, they have been used in treatment for colonic diseases, such as Crohn’s disease or colitis, but they have yet to be approved for inclusion in infant formulas in the United States. Combinations of pre- and pro-biotics, or synbiotics, are also a real possibility for improved future infant formulas.

Techniques to analyze intestinal microbiota

The human gastrointestinal tract is considered the largest organ in the human body. The GI tract is classified as the entire area from the mouth to the anus and is estimated to have a surface area of 300 m². Because of the ever changing environment due to pH, redox potential, peristalsis, and nutrient availability throughout the GI tract there is a dynamic bacterial community and niches therein. It is important while studying this ecosystem to determine not only bacterial diversity and abundance but also activity. Some bacteria are merely transient and have little to no effect on the host while other species might be specialized to a particular niche within the GI tract.
Most studies of the human GI tract are based on studies of fecal samples because collection of such samples is non-invasive. Some studies have used gastrointestinal fluids or biopsy samples, but these are usually obtained when an individual is having gastrointestinal problems, therefore these studies may represent abnormal GI ecosystems.

Traditionally, bacterial communities were studied by culturing of bacteria in laboratory media. The media preparation and selectivity are key to this technique but it has many drawbacks. Bacterial communities are so complex that media selection will only select for specific bacteria. Other species may require unknown growth factors or may rely on interactions with its host or other bacterial species. Additionally, because gastrointestinal microbiota consists mainly of obligate anaerobes, these species require a strict environment free of oxygen. It is understood that exposure to stress can also limit growth. With the advent and perfection of the anaerobic chamber, anaerobic bacteria are now able to be cultured more readily. Despite this, with all the advancements in bacterial culture methods it is determined that only about 15% of bacterial species can be cultured (Langendijk 1995, Suau 1999).

In addition to the limitations on culturing, another serious drawback to the cultivation-based methods is the accuracy of the data obtained. Firstly, bacterial communities are so highly complex that enumeration of species is not very reliable or accurate. Not only are many species completely unaccounted for, others are grossly under or over estimated. Additionally, species can only be categorized based on their phenotypes, which lead to individual bias based on observation.
More recent molecular approaches make it feasible to analyze complex bacterial communities more accurate and in greater detail than cultivation based methods. Most of these techniques target 16S ribosomal RNA genes, which is a highly conserved genetic marker with both hyper variable and conserved regions, the former of which enables design of primers and probes for analysis of individual species, while the latter of which enables primers and probes for a group of related bacteria. The commonly used methods include community profiling by Denaturing Gradient Gel Electrophoresis (DGGE), quantification of individual bacteria by real-time PCR, and detailed diversity analysis by sequence analysis of 16S rRNA genes recovered from bacterial communities.

Community fingerprinting methodologies such as DGGE generate a visual banding pattern to qualitatively show the bacterial diversity present in individual samples. This method is suitable for assessing community changes between environments or treatments but it cannot be used quantitatively.

Phylogenetic analysis of cloned and sequenced 16S rRNA gene has been the primary technology in detailed description of bacterial communities. In recent studies, researchers turned to pyrosequencing to determine 16S rRNA gene sequences because it is more cost effective and provide deep coverage. However, abundance of individual bacteria identified cannot be determined from pyrosequencing data (Amend et al., 2010; Niu et al., 2010).

Real-time PCR utilizes the technology of traditional PCR along with quantification of PCR products in real-time so that it enables quantification of individual bacteria. The quantities of PCR products generated during PCR are typically measured as
a fluorescent signal, which is produced by using a fluorescent dye or probe. This method is fast, accurate, and cost effective, which makes it a preferred method for quantifying individual bacteria in assessing effect of environments.
Chapter 2

Impact of Buffering Capacity and Low Dose Galacto-Oligosaccharide on \textit{in-vitro} Infant Fecal Cultures

Introduction

Both the World Health Organization (WHO) and the American Association of Pediatrics recommend breast feeding exclusively for the first 6 months of life and as a dietary supplement for up to 2 years of life. Research has shown that breast-fed infants have stronger immune systems resulting in lower risk of enteritis and allergies (Kelly 2007, Siggers 2010). The reason for these health implications is tightly associated with the gastrointestinal microbiota that breast milk encourages. Infants fed breast milk exhibit higher counts of lactobacilli and bifidobacteria as opposed to formula-fed infants, who have a gut microbiota more similar to that of an adult, including increased counts of \textit{E. coli} and clostridia. The WHO currently estimates that only 35\% of infants up to 6 months of age are exclusively breast fed. Thus, it is inherently important to improve infant formulas so that the health of formula-fed infants can be further enhanced.

Most infant formulas are based on cow’s milk and have a number of deficiencies compared to natural human milk. Human milk contains a very complex assortment of
enzymes, oligosaccharides, and immunoglobulins which have varied effects on the GI microbiota. Oligosaccharides in human milk have gained a lot of interest in the last decade for their ability to survive human digestion and to stimulate growth of beneficial bacteria in the GI tract. The bifidogenic effect is becoming a common term to describe the impact of human milk, most likely by these oligosaccharides, to induce an increase in bifidobacteria in the lower GI tract. The bifidogenic effect is considered beneficial because it may modulate GI microbiota and bacteria-host interactions, improving infant health. Additionally, increased population of bifidobacteria can enhance competitive exclusion, and SCFA production, further benefiting infant health.

Human milk has a lower buffering capacity than cow’s milk (almost twice as high as human milk), and the buffering capacity of infant formula falls between the two. The lower buffering capacity of human milk is probably responsible for the lower pH observed in the GI tract of breast-fed infants. The lower pH in the GI tract of breast-fed infants than in the GI tract of formula-fed infants is likely attributable to the greater abundance of bifidobacteria and lactobacilli found in breast-fed infants. We hypothesize that a lower buffering capacity of culture media will support the growth of acid-tolerant species such as bifidobacteria and lactobacillus while reducing the abundance of species such as *E. coli* and *C. difficile* which thrive at a neutral pH. Because a study in a murine model showed that HMO’s can stimulate the excretion of intestinal bifidus factors when supplementing a low buffering capacity diet, yet failed to do so on a high buffering capacity diet (Romond, 1989), we also expect that a low dose oligosaccharide and low buffering capacity might have a synergistic effect on modulating GI bacterial
communities. These hypotheses were tested using in vitro cultures of feces collected from infants (both breast-fed and formula-fed) and galacto-oligosaccharides.

Materials and Methods

Sampling protocol

An institutional review board reviewed and approved this study protocol and consent forms. Informed consent was obtained from the infant’s mother prior to first fecal collection. Seven term infants, free of antibiotic use, were recruited. Infant fecal samples were collected from each infant at 30 and 45 days of age, aside from two infants who were only sampled once each at 32 or 56 days of age.

Information on mode of delivery and infant diet were collected (Table 2). The feeding methods were chosen by the mothers. In the case where infants were fed an infant formula the brand and type of formula was noted if the infants were breast fed with infant formula supplemented into the diet (referred to as mixed feeding), a rough percentage of daily intake of formula was noted.

Media and Cultivation

A basal medium was prepared with decreasing concentrations of sodium bicarbonate (2g/L, 1g/L, 0.5g/L and 0.25g/L) to produce a series of media with decreasing buffering capacity (designated as 1x, 0.5x, 0.25x, and 0.125x) with all other
ingredients remaining the same. The basal medium contained (grams per liter) peptone, 2.0; yeast extract, 2.0; NaCl, 0.1; K2HPO4, 0.04; KH2PO4, 0.04; MgSO4.7H2O, 0.01; CaCl2.2H2O, 0.01; cysteine-HCl, 0.5; bile salts, 0.5; Tween 80, 2.0 mL; phylloquinone, 10 μL; hemin solution, 1.0 mL; and Na-resazurin.

To minimize differences in medium preparation, the media were prepared together in one media bottle, without addition of different amounts of NaHCO3. The media were mixed with slight heating to dissolve all the ingredients into solution then separated into 4 media bottles. NaHCO3 was then added as described above and autoclaved. The media were made anaerobic by purging with nitrogen gas prior to transferring into an anaerobic chamber where it was held overnight. Cyctein-HCl, hemin, phyloquinone and Tween-80 were added after autoclaving and once media cooled to about 55°C to avoid inactivation by heat. The pH of the media was adjusted after becoming anaerobic to reduce any pH variation between the four buffering capacities. This was done by aseptically transferring a small amount to a 50 ml flask and testing pH with a battery powered pH probe. The pH was then adjusted to pH7 using 1M NaOH.

The relative buffering capacity of the media was determined by titration of 50ml media using 1M HCl (Fig. 1). Buffering capacity was determined by the volume of 0.01M HCl needed to lower the pH of one liter of the medium by one unit.
**Infant fecal sample collection and culturing**

Infant fecal samples were collected directly from the diaper into a 15ml bottle using a sterile wooden stick. The 15ml collection bottle was prepared with roughly 8 sterile beads which were used to aid in sample homogenization. The collection bottle was then filled with anaerobic peptone water containing 0.1% peptone, 0.85% NaCl, and 0.5% cystein-HCl (pH 7). The sample bottle was immediately sealed with a rubber stopper and packed on ice. The sample was taken to the laboratory and inoculation of the media was carried out within 1 hour of the initial bowel movement. Prior to inoculations, the sample was shaken and vortexed to homogenize the fecal sample into buffer. The sample bottle was then taken into the anaerobic chamber where all further inoculations were carried out.

A 1:10 dilution of the fecal sample was prepared in the same anaerobic peptone buffer and homogenized by vortexing. One ml of the dilution was subsequently inoculated into the eight varieties of media in triplicate. These tubes were then transferred to a 37°C water bath and incubated for 24 hours.

Subsamples (4ml total) were collected from each culture at 0, 12, and 24hr post incubation for further analysis for pH, SCFAs, and for DNA extraction. Zero hr subsample was collected directly from the 10x fecal dilution while 12 and 24 hour samples were collected from their respective incubation tubes. All samples collected were kept at -80°C until being analyzed.
**Community DNA Extraction**

One aliquot (1.5 ml) subsample was centrifuged at 13,000 rpm for 5 min to harvest the microbial biomass. The supernatant was removed by aspiration and 1 ml lysis buffer was added. The solution was mixed by pipeting and transferred to a 2ml tube and metagenomic DNA extraction was carried out using the RBB+C method (Yu, 2005). The DNA quality was evaluated using agarose gel (1.0%) electrophoresis and DNA yield was quantified using the Quant-it™ dsDNA Broad Range Kit (Invitrogen Corporation, Carlsbad, CA).

**Bacterial Quantification**

Quantification of bifidobacteria, *E. coli*, *C. difficile*, and lactobacilli was carried out using quantitative PCR. Table 1 lists the primers and conditions used to prepare each of the standards. The quantitative PCR conditions were pre-optimized for each species or genus. Standards were sample-derived from pooled DNA and quantitative PCR was carried out using a Stratagene MX3000P real-time PCR machine. Standards ($10^1 – 10^7$ copies per reaction) were included on each 96 well plate in triplicate to reduce plate to plate variation. Because samples were already experimentally in triplicate and the large number of samples to analyze, each DNA sample was run in a single replicate. A no template control was run in triplicate to detect any contamination errors on each plate.
**SCFA analysis**

Samples were thawed at 4°C. Then 350µl of each sample was combined with 200µl formic acid, 350µl distilled water, and 100µl ethylbutyric acid (1.25g/L, internal standard). The solution was then shaken by hand and centrifuged for 5min at 13,200 rpm. Supernatant was transferred to a GC vial, capped with a rubber septum, and crimp sealed with an aluminum cap. The prepared SCFA samples were stored at -20°C until GC analysis. Gas chromatography was carried out on a Supelco column (15%SP 1220/1%H₃PO₄ on 100/120 Chromosorb WAW. 6 ft x 2 mmID). The initial oven temperature was at 113°C and ramped up at a rate of 2°C/min to reach an oven temperature of 120°C in 15 minutes. The injector temperature was maintained at 150°C while the FID detector temperature was maintained at 180°C. The carrier gas was nitrogen gas with a column head pressure of 36 psi. A standard was run after every 5 samples to calibrate the GC and was quantified to be within 5% of standard error.

Because lactic acid cannot be detected by GC, it was analyzed biochemically. The samples were centrifuged for 5min at 13,200 rpm. The supernatant was collected and heated to 100°C for 10 minutes to inactivate enzymes (knoll, 2004). The Biovision lactate assay kit for L-lactate (K607) was used to measure lactate in the samples. Absorbance was measured at 490nm using a Spectramax plus 384 spectrometer.
Table 1  The PCR Primers and Probe Used in this Study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-&gt;3')</th>
<th>Annealing position</th>
<th>Target regions</th>
<th>Annealing temperature</th>
<th>Amplicon length</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>36f</td>
<td>CAG GCC TAA CAC ACA TGC AAG TC</td>
<td>36-59</td>
<td>Nearly complete rrs</td>
<td>55°C</td>
<td>1364 bp</td>
<td>Marchesi 1989</td>
</tr>
<tr>
<td>1389r</td>
<td>ACG GGC GGT GTG TAC AAG</td>
<td>1374-1389</td>
<td></td>
<td></td>
<td></td>
<td>Osborn 2000</td>
</tr>
<tr>
<td>340f</td>
<td>TCC TAC GGG AGG CAG CAG T</td>
<td>340-258</td>
<td>V3-V4</td>
<td>60°C</td>
<td>467 bp</td>
<td>Nadkarni 2002</td>
</tr>
<tr>
<td>806r</td>
<td>GGA CTA CCA GGG TAT CTA ATC CTG TT</td>
<td>781-806</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bif164-f</td>
<td>GGG TGG TAA TGC CGG ATG</td>
<td>164-181</td>
<td>V2-V4</td>
<td>60°C</td>
<td>530 bp</td>
<td>Satokari 2001</td>
</tr>
<tr>
<td>Bif662-r</td>
<td>CCA CCG TTA CAC CGG GAA</td>
<td>676-693</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cdif-706f</td>
<td>ATT AGG AGG AAC ACC AGT TG</td>
<td>706-725</td>
<td>V5</td>
<td>54°C</td>
<td>307 bp</td>
<td>Williams 2010</td>
</tr>
<tr>
<td>Cdif-994r</td>
<td>AGG AGA TGT CAT TGG GAT GT</td>
<td>994-1012</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECA75f</td>
<td>GGA AGA AGC TTT CTT CTT TGCT GAC</td>
<td>75-99</td>
<td>V1-V3</td>
<td>56°C</td>
<td>545 bp</td>
<td>Sabat 2000</td>
</tr>
<tr>
<td>ECR619r</td>
<td>AGC CCG GGG ATT TCA CAT CTG ACT TA</td>
<td>594-619</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lac1</td>
<td>AGC AGT AGG GAA TCT TCC A</td>
<td>352-370</td>
<td>V3-V4</td>
<td>60°C</td>
<td>345 bp</td>
<td>Walter 2001</td>
</tr>
<tr>
<td>Lac2</td>
<td>ATT YCA CCG CTA CAC ATG</td>
<td>679-696</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taqman</td>
<td>6-FAM-5'-CGT ATT ACC GCG GCT GCT GGC AC-3'-TAMRA</td>
<td>515-537</td>
<td></td>
<td>70°C (Tm)</td>
<td></td>
<td>Nadkarni 2002</td>
</tr>
</tbody>
</table>

*a* Numbering according to the *rrs* gene of *E. coli.*

*b* Calculated from *rrs* of *E. coli* with primers included.
**Statistical Analysis**

Concentrations of SCFAs and pH were compared for each treatment and time point using One Way Analysis of Variance (ANOVA) and buffering concentrations were compared pair-wise using Tukey-Kramer adjustment for p-values. A log\(_{10}\) transformation was performed on the SCFA data to account for variation among groups. For quantitative PCR data Bartlett’s test of equal variance indicated that variance between individuals was significant. To account for such variation among individuals the PROC MIXED® procedure of SAS (SAS Institute, Inc., Cary, NC) was used to fit a Randomized Complete Block ANOVA for each treatment and buffering capacity and between the two time points. The model contained a fixed effect for GOS presence and buffering capacity and a random effect for subjects. The least square means (LSM) were compared using Tukey-Kramer pair-wise comparison. Again data was transformed by log\(_{10}\) prior to analysis. Significance was determined at p-value ≤ 0.5.

**Results**

Fecal samples were collected from seven infants in total (Table 2). Of the seven infants, five were sampled at two different ages. The purpose of this was to determine if modulation of the gut microbiota at different buffering capacities differs with age. Fecal samples were collected from different feeding groups to account for variation in the microbiota between breast-fed and formula-fed infants. A mixed feeding group was also measured, which included two infants fed a mixed diet of breast milk supplemented with formula.
Table 2    Demographic Information of Infants

<table>
<thead>
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<th>Variable</th>
<th>Feeding Group</th>
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<tr>
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<td>FF</td>
<td>HM</td>
<td>Mixed</td>
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<tr>
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<td>2</td>
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<tr>
<td>Method of Delivery</td>
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*a  Feeding groups: FF=milk-based infant formula; HM=human milk; mixed=human milk supplemented with formula*
**Buffering Capacity of the Media**

Buffering capacity of the media is shown in Fig. 1. It is evident that with reduced concentration of bicarbonate, the buffering capacity of the media decreased, with a larger decrease being seen from 1x to 0.5x. The buffering capacity of human milk was determined by the same methods (Hedge, 2007) as used in this study. The buffering capacity of human milk falls between the media with 1x and 0.5x buffering capacity.
Fig. 1. Buffering capacity of the media and human milk. The buffering capacity was expressed as the volume of 0.01 M HCl used to lower one liter of the media by one pH unit. The buffering capacity of human milk was from (Hedge et al. 2007).
Bacterial Abundance

Abundance of Bifidobacteria determined by qPCR

At time zero, the average abundance of bifidobacteria was $2.02 \times 10^6$ copies of 16S rRNA genes/ml culture. At 12 hour and 24 hour, the bifidobacterial abundance was still approximately $10^6$ copies of 16S rRNA genes/ml culture (Fig. 2). There appeared to be a slight increase in bifidobacteria as buffering capacity decreased but this increase was not statistically significant. At 24 hours, the abundance of bifidobacteria appeared to decrease slightly as buffering dropped. In the cultures that received the addition of GOS, the abundance of bifidobacteria reached roughly $10^7$ copies of 16S rRNA genes/ml culture at 12 hours, which is numerically higher than that observed in the cultures that did not receive the GOS addition. The abundance of bifidobacteria varied considerably among the samples from different infants. Such large variations make it impossible to ascertain the differences in bifidobacteria among the cultures with different buffering capacities.
Fig. 2. Abundance of bifidobacteria (copies of 16S rRNA genes/ml culture) determined by quantitative PCR. Each dot represents an individual sample and the horizontal line represents the mean.
**Abundance of *C. difficile* Determined by qPCR**

At zero hour, the average abundance of *C. difficile* was $2.15 \times 10^7$ copies of 16S rRNA genes/ml culture. At 12 hours, the *C. difficile* abundance reached almost $10^8$ copies of 16S rRNA genes/ml culture, and no significant variation was observed among the treatments (Fig. 3). However, large variations among the samples were evident, with 5 samples having about 4 logs more *C. difficile* than the rest of the samples. Samples collected at age 30 and 45 days for individual infants are depicted as two separate dots. Because of this, 4 of the 5 samples with high *C. difficile* come from two infants at the two ages of collection and the fifth is from an infant that was only sampled at one age.

The addition of GOS did not seem to have an effect on *C. difficile* abundance irrespective of buffering capacity or incubation time.
Fig. 3. Abundance of *C. difficile* (copies of 16S rRNA genes/ml culture) determined by quantitative PCR. Each dot represents an individual sample and the horizontal line represents the mean.
**Abundance of *E. coli* determined by qPCR**

The abundance of *E. coli* averaged $1.74 \times 10^7$ copies of 16S rRNA genes/ml culture at time zero. Each dot represents an individual sample, infants that were sampled at 30 and 45 days of age have two dots. At 12 hours, the average *E. coli* abundance reached about $10^8$ copies of 16S rRNA genes/ml culture (Fig. 4). The *E. coli* abundance was significantly lower in the media with 0.25x and 0.125x buffering capacity than in the medium with 1x buffering capacity, though there was no significant differences among the treatments at 24 hours. With the addition of GOS there was a significant decreased in *E. coli* abundance at 12 hours between 1x and the other three lower buffering capacities and a significant decrease between 0.5x and 0.125x buffering capacity. At 24 hour these differences decreased but the medium with 1x buffering capacity still had significantly more *E. coli* than the media with 0.25x or 0.125x buffering capacity. The abundance of *E. coli* also significantly decreased when the buffering capacity was reduced from 0.5x to 0.125x (Fig. 4)
Fig. 4. Abundance of *E. coli* (copies of 16S rRNA genes/ml culture) determined by quantitative PCR. Each dot represents an individual sample and the horizontal line represents the mean.
**Abundance of *Lactobacillus* Determined by qPCR**

At time zero, *Lactobacillus* abundance average approximately $10^3$ copies of 16S rRNA genes/ml culture. The abundance did not change significantly over time (Fig. 5). None of the treatments, either buffering capacities or GOS addition, significantly affected the abundance of *Lactobacillus*. Although not statistically significant, lactobacillus appears to be most abundant at 0.25x buffering concentration at both 12 and 24 hour time points and with and without GOS. Large variations in abundance were also observed among samples collected from different infants.
Fig. 5. Abundance of *Lactobacillus* (copies of 16S rRNA genes/ml culture) determined by quantitative PCR. Each dot represents an individual sample and the horizontal line represents the mean.
**Culture pH**

The average pH of the cultures at time zero was 6.66. The pH rose in all the cultures irrespective of buffering capacity by 12 hours, but smaller pH increases were found in the media with decreased buffering capacity (Fig. 6). In cultures supplemented with GOS, the culture pH significantly decreased with decreasing buffering capacity, with the pH reaching 6.26 in the 0.125x buffering cultures at 12 hour. Greater decrease in pH was seen in the cultures that received the GOS addition than in the cultures that received no GOS addition.
Fig. 6. The pH values in the cultures at 12 and 24 hours. Different letters on top of bars denote significance (P<0.05).
**Lactate Concentration**

Lactate concentration was not significantly changed among the cultures with different buffering capacities. However, lactate concentration in the GOS-supplemented cultures was higher, though not significantly (Fig. 7). Although not statistically significant, lactate appears to increase slightly at 0.25x buffering concentration for both time points and both with and without GOS. This appears to be correlated to the increase seen in lactobacillus at this buffering concentration.
Fig. 7. Lactate concentration (mM) in the cultures with different buffering capacity.
**Total SCFA production**

The cultures were subjected for analysis for acetic, butyric, propionic, iso-butyric, iso-propionic, and valeric acids. There was no significant difference in concentrations of these SCFA (data not shown). Thus, the concentrations of all the above SCFAs were combined and shown as Total SCFAs. In the media without GOS, total SCFA concentrations increased with the increase in buffering capacity but not significantly. With the addition of GOS, SCFA concentration was significantly higher in the higher buffering capacity in the 12 hour samples (Fig 8). Although this trend is similar in the 24 hour cultures with GOS there is no significant difference.
Fig. 8. Total SCFA concentration (mM) determined by gas chromatography. Different letters denote significance (p<0.05).
Discussion

The main objective of this study was to determine the impact dietary buffering capacity has on the bacterial community of the infant GI tract. It is known that the buffering capacity of human milk is far less than that of infant formula. Surprisingly, few studies have been reported that investigated if and to what extent buffering capacity affect GI microbiota of infants. Furthermore, most of the early studies were conducted prior to the advent of 16s rRNA techniques and relied solely on culture-based methods for bacterial enumeration which do not support accurate or precise measurements of bacterial abundance of individual species.

We hypothesize that lowering buffering capacity of the media could effectively lower culture pH, which in turn increases predominance of acid-tolerant species such as Lactobacillus and Bifidobacterium. In doing so, lowered pH coupled with competitive exclusion by these beneficial bacteria would inhibit potentially pathogenic bacteria such as E. coli and C. difficile. Additionally, we chose to add a low dose of galacto-oligosaccharide to determine if this component of breast milk works in synergy with buffering capacity to achieve the above bifidogenic effects. Previous studies have shown that prebiotics added to infant formula indeed increase bifidobacteria and lower pH but the doses used in these studies are much higher than that used here (Knol 2005, Bakker-Zierkzze 2005). We chose this low dose because, although prebiotics have been touted as inducing a bifidogenic effect and is the newest addition to infant formulas, the
fermentation in the gut can often lead to gas and discomfort. By lowering the prebiotic
dose while conserving the bifidogenic effects, we hope to show that prebiotics can be
used minimally while retaining their beneficial effects.

Because bacterial abundance varied considerably by individual and even between
the two collections ages, data for all infants was combined for analysis. Three infants in
this study had relatively high abundance of *C. difficile*. Two of these infants were from
the formula fed group and one was from the mixed feeding group. This follows the
results of similar studies which found higher abundance of *C. difficile* in formula fed
infants (Balmer, 1994). *Bifidobacterium* also followed the trends described by the
literature and was consistently higher in breast fed infants. However, significant
differences in *E. coli* abundance was not observed between the feeding types. Being an
exploratory study, there was little control over infants sampled, and as a result the
demographics of infants sampled are quite skewed. Additionally, the formula and mixed
feeding groups have a very diverse formula type which might have added to the
complexity of analyzing the microbiota based on feeding type. Future studies might
benefit from larger sample size as well as more control of infant feeding.

*E. coli* abundance decreased significantly as buffering capacity decreased in the
cultures irrespective to the addition of GOS. This effect was observed more so in the 12
hour time point. This indicates a bacterial shift in growth by 24 hours, most likely caused
by the depletion of carbohydrate. Cultures receiving the GOS supplementation showed a
greater decrease in *E. coli*, and the magnitude of the decrease was negatively associated
with the buffering capacity of the media. These results suggest that even at extremely low
doses, GOS can have a positive impact on gut health. These results are encouraging, as lower counts of *E. coli* correspond with lower risk of gastrointestinal infection (Kelly 2007).

The culture pH increased during the incubation in all the treatments without GOS, yet they showed a significantly lowered culture pH in a buffering capacity-dependent manner. These results suggest that reduced buffering capacity can help lower culture pH. The pH increase observed in the course of the incubation suggests production of base and/or utilization of SCFA. The peptone included in the dilution buffer can result in release of ammonia, an alkaline compound. Additionally, as the media contained very little fermentable carbohydrates, the bacteria might have utilized the SCFAs carried over with the inoculums. These premises are supported by the increase in pH from 12 to 24 hours post incubation.

In the cultures that received the low dose of GOS, the culture pH decreased significantly when compared to that at time zero, yet the concentration of SCFAs and lactate did not increase significantly. Because the buffering capacity was so low, the small increase in acid production significantly lowered the culture pH. This finding supports our hypothesis.

Although we were able to show that the pH was significantly lower in the cultures with lower buffering capacity, there seemed to be little effect on bifidobacterial abundance. The limited amount of substrates available in the media might have limited any significant bacterial growth. The large variation among the samples of different infants may also be attributable to the lack of significant difference. Some prebiotic
studies have shown that although bifidobacterial abundance does not change, metabolism and bifidogenic factors can increase (Romond, 1989; Edwards, 1985). With this increased activity, there is an increase in SCFA production and a lowering of pH. Despite these reports we did not see conclusive evidence that activity was increased. Furthermore, SCFA concentrations were not significantly changed either by the variation of the buffering capacity or the oligosaccharide addition. Overall, the concentration of SCFAs was higher in the cultures with a higher buffering capacity and high pH. This finding is corroborated by the study by Edwards et. al. (1985) who showed that more acetate was produced in continuous cultures at pH 7 than at pH 6 and pH 5. Although this seems to be consistent with previous findings, it remains to be determined why lower pH leads to lower SCFA production.

In a study by Bullen and Willis (1971), an artificial infant formula modified to have a buffering capacity equal to that of breast milk was prepared and inoculated with infant fecal bacteria. They showed an increase in *Lactobacillus* along with a decrease in *E. coli* (bifidobacteria was not measured). These results may be a factor of the high nutrient content of their growth conditions. We postulate that an increase in carbohydrates in the media might significantly increase bifidobacteria while reducing *E. coli*.

A number of in-vitro studies measuring bacterial growth and activity based on pH or buffering component additions such as citric acid or sodium bicarbonate concur with our results, that a higher pH, and increased buffering capacity result in higher SCFA production (Dolby 1977, Berger 1978, Jiang 1997). These results are confounding to our
understanding of how low buffering capacity of breast milk modulates the GI tract microbiota. Our expectations are that a low buffering capacity diet leads to a decrease in pH in the colon, resulting in an increased abundance of acid producing bacteria and increased SCFA production. This theory is supported by the fact that breast fed infants have higher SCFA concentrations and a much lower fecal pH than formula fed infants.

Future studies will need to address the issue of carbohydrate availability and measurement of ammonia may be beneficial in elucidating metabolic processes occurring in culture. Additionally, individual microbiota is highly variable and increased sample number is merited.
Chapter 3

General Discussion

Although the lower buffering capacity of human milk is often considered as a possible factor in improved health of the breast-fed infant, there is very little data to support this hypothesis. Of the few studies that investigate the effects of buffering capacity on GI microbial species and metabolism, many are based solely on pH in continuous culture or addition of NaHCO$_3$ to increase the buffering capacity further from that of breast milk (Harrison and Peat 1972, Dolby 1977).

This study aims to simulate a buffering capacity within the range of both breast milk and infant formula in batch in vitro cultures of fecal microbiota of both breast-fed and formula-fed infants. We also chose to include a subset of cultures that received a low dose of galacto-oligosaccharide to assess potential additive or synergistic effects. Our results did show that reduced buffering capacity could potentially reduce pathogenic bacteria, particularly 	extit{E. coli}. This finding is important because although it has been shown that infant formula can improve the abundance of bifidobacteria and lactobacilli similar to human milk, to our knowledge no infant formula, irrespective to addition of prebiotics, can reduce the abundance of 	extit{E. coli} (Ito, 1993; Bouhnik 1997) or 	extit{C. difficile}
to abundances similar to the GI tract of human milk-fed infants. The finding of this study can be useful in formulating new infant formula that can reduce abundance of *E. coli*.

In this study, we did not see a significant increase in beneficial bacterial species, *Bifidobacterium* and *Lactobacillus*, with reduced buffering capacity. We expect the abundance of these two species would increase if more carbohydrates were added to provide the substrate for these species to grow. We also expect increased carbohydrate source would yield a greater increase in SCFA production and decrease in abundance of *C. difficile*. This premise can be tested in future studies.

Numerous studies have shown that prebiotics, such as GOS, can effectively increase bifidobacterial abundance in formula-fed infants (Ito, 1993; Bouhnik 1997). In those studies, GOS was included at concentrations much higher than that used in this present study. Based on the results of this study, we hypothesize that if a higher concentration of GOS is included in future studies, we will demonstrate a greater bifidogenic effect. In return, the culture pH may decrease to a larger extent due to the acetic acid and lactic acid produced by *Bifidobacterium*, and the abundance of *E. coli* and *C. difficile* may decrease significantly. With a lower buffering capacity, lower amounts of prebiotics are needed to reduce the culture or GI pH. This is also important because high levels of prebiotics can lead to excessive gas and discomfort, impeding the inclusion of prebiotics to achieve desirable effects.

Greater SCFA concentrations were observed in the cultures with higher buffering capacity than in the cultures with lower buffering capacity. Both the higher pH and higher concentration of bicarbonate present in the cultures with high buffering capacity
can be attributable. Future studies that test these two factors independently can help to identify the responsible factor. It should also be pointed out that the buffering capacity of breast milk is primarily provided by the protein present in breast milk. Different buffering reagents may affect the results. Future studies might benefit from using a buffering system that is similar to that of human milk.

Many studies use *in vitro* cultures to test a proof of concept. However, *in vitro* cultures have limitations to mimic the GI microbiota because of the lack of the interaction between the mucosa and epithelial cells of human GI tract and its microbiota and absorption of fermentation products. Additionally, liquid *in vitro* cultures have different conditions than the GI content found in the colon. Therefore, *in vivo* studies are needed to verify and confirm the results of *in vitro* studies. An animal model should be used before clinical trials using infants.
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