The Evolution of the Intestinal Microbiome of Premature Infants and Piglets Receiving Probiotics and Lactoferrin

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ABSTRACT

Probiotics and lactoferrin are currently being used in neonatal intensive care units in the hopes of reducing rates of sepsis and necrotizing enterocolitis. While studies have shown that these measures can be clinically beneficial to premature babies, little is known about their effect at the level of the intestinal microbiome. We performed a prospective study describing the composition and evolution of the microbiota of premature babies receiving probiotics with and without lactoferrin supplementation. Furthermore, we compared our findings to a piglet model. We found that the addition of lactoferrin did not lead to a distinct microbiome using UniFrac analysis, nor increased diversity or richness as compared to probiotics alone. The relative abundance of probiotic bacterial strains was approximately 3%. At the phylum level Firmicutes and Proteobacteria predominated, while overall *Enterobacteriaceae* was the most abundant family. We remain the first to describe the microbiome of premature infants receiving lactoferrin.

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List of abbreviations

C-section= Cesarean Section

CFU= Colony Forming Units

DGGE= Denaturing Gel Gradient Electrophoresis

H_2S= Hydrogen sulfide

HCAI=Health Care Associated Infection

HMO=Human Milk Oligosaccharides

LGG= *Lactobacillus rhamnosus*

LOS= Late Onset Sepsis

NEC= Necrotizing Enterocolitis

NICU= Neonatal Intensive Care Unit

NPO=nil per os (fasting)

OTU=Operational Taxonomic Unit

P = Probiotic Alone

PCA= Principle Component Analysis

PCoA= Principle Coordinate Analysis

PCR =Polymerase Chain Reaction

PL = Probiotic + Lactoferrin

RCT= Randomized Control Study

rRNA=Ribosomal RNA

TCAG= The Center for Applied Genomics

VLBW= Very Low Birth Weight

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CHAPTER 1

Introduction

1.1 Intestinal microbiota and their role in health and disease:

Interactions between the resident intestinal microbiota with luminal contents and the mucosal surface play important roles in normal intestinal development, nutrition and immunity. The intestine has a large and delicate surface area covered by a thin monolayer of multifunctional epithelial cells that overlies a highly immunoreactive submucosa. Interactions in the lumen between resident microbes, nutrients and the intestinal mucosa promote the development of the intestine as well as the maturation of the immune system (Hill and Artis, 2010). Disruption of these interactions may have numerous detrimental consequences, including systemic inflammation, autoimmune and allergic disorders (Indrio and Neu, 2011). Infants born prematurely are a very fragile population and are at risk for late onset sepsis, bacterial translocation and necrotizing enterocolitis (NEC) compared to their full term peers (Kosloske, 1994; Vergnano et al., 2011). Probiotics, prebiotics and other supplements, such as lactoferrin are being introduced in the hopes of reducing the prevalence of these disorders (Deshpande et al., 2010; Sharma and Shastri, 2015; Srinivasjois et al., 2013).

1.2.1 The role of molecular-based techniques in the evaluation of the intestinal microbiota:

The human intestinal microbiota has in recent years become easier to study with the advent of new sequencing technologies that allow an in-depth characterization of bacterial communities. Adult studies have shown that 60-80% of the gut microbiota cannot be

cultivated (Langendijk et al., 1995; Wilson and Blitchington, 1996) and this fact emphasizes the importance of molecular-based techniques in the evaluation of intestinal microbiota composition. Woese discovered that a small ribosomal subunit of RNA, the 16S ribosomal RNA (rRNA), contains nucleotide sequences that are highly conserved across bacterial species (Woese and Fox, 1977). A 2.5-3% difference between two 16S rRNA sequences can be used as a threshold to distinguish different bacterial species (Stackebrandt and Goebel, 1994). This property can be used to identify a bacterial species and in combination with next-generation sequencing approaches to characterize a bacterial population. Bacterial DNA can be extracted from fecal specimens, with subsequent PCR amplification of the 16S rDNA gene. The 16S rDNA has become the most frequently used gene, however other bacterial targets exist such as the ribosomal 23S subunit as well as the internally transcribed spacer, 16S-23S spacer region (Gonzales-Marin et al., 2013; Turroni et al., 2009). The *16S rRNA* gene contains conserved regions, which are unique to bacteria and flank one of 9 hypervariable regions (V1-V9) (Figure 1.1) that help differentiate between bacterial species (Arrieta et al., 2014; Chakravorty et al., 2007). A number of different primers are available to amplify specific regions of the *16S rRNA* gene. The amplification process involves the use of primers that specifically target the conserved regions of the gene (Suau et al., 1999). The specific primers used as well as the extraction protocol can influence the resulting microbial composition. Bias can be introduced early on during DNA extraction, most frequently at the lysis step, since the efficiency of the lyses can lead to a preference towards particular taxa (Wang et al., 2004). Primer selection can also lead to bias, for instance, it is widely known that *Bifidobacterium* is poorly amplified by targeting the V1 or V2 region (Arrieta et al., 2014). Chakravorty et al studied the V1-V8 regions of 110 bacterial species including common blood born pathogens (Chakravorty et al., 2007).

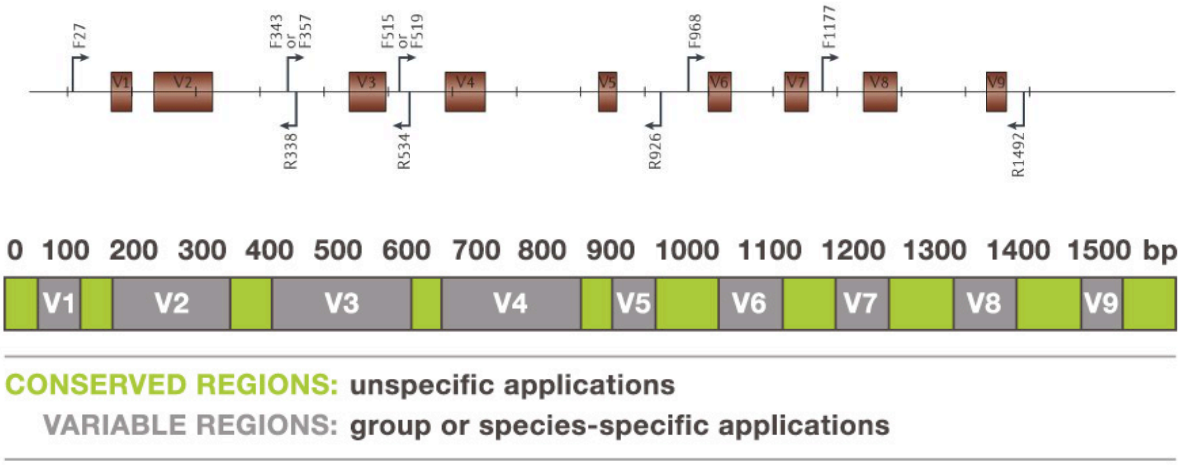

Figure 1.1: *16S rRNA* gene

The conserved regions are highlighted in green and hypervariable regions in grey.

F: refers to forward primers and R, reverse primers

Images taken from (Kuczynski et al., 2012) and http://www.alimetrics.net/en/index.php/dna-sequence-analysis

They concluded that regions V2-V3 were most appropriate for bacterial classification at the genus level, except in the case of closely related *Enterobacteriaceae*. These findings are in keeping with other authors (Liu et al., 2008). Chakravorty et al. also reported that V6 was able to differentiate between most bacterial species except *Enterobacteriacea,* however these results were not replicated in the works of Liu et al who used pyrosequencing analysis. Lastly the authors concluded that targeting V4, V5, V7 and V8 is less helpful at identifying bacteria at the genus or species level (Chakravorty et al., 2007). Unfortunately, there is no real consensus on which hypervariable region to target, and the decision should be influenced by which level of taxonomic identification is required as well as the specific bacterial genera that one wishes to identify.

The resulting PCR amplicons can subsequently be sequenced using next-generation sequencing platforms. Large databases are currently available (e.g. Greengenes (DeSantis et al., 2006)) and can be used for the taxonomic assignment of 16S sequences. The majority of microbiome research employs one of two sequencing platforms, Roche 454 pyrosequencing (GS FLX and FLX Titanium), or Illumina (MiSeq and HiSeq2000, Illumina Inc. San Diego, CA, USA) (Arrieta et al., 2014). The advantages of the 454 Pyrosequencer are its longer reads of up to 500 bp, thereby covering multiple hypervariable regions. Conversely Illumina produces shorter sequences that are 100 to 150 bp in length, but it is capable of sequencing 10-100 more samples and has the added advantage of a lower cost as compared to 454 (Arrieta et al., 2014). Specifically, pyrosequencing is capable of sequencing thousands of reads per sample and hundreds of samples per run, while Illumina can generate millions of reads per sample and thousands of samples per run (Kuczynski et al., 2012). While molecular-based methods are becoming the gold standard in microbiome research, there may

be some advantages to culture based techniques. The risk of contamination and amplification of "foreign" DNA can have serious consequences in molecular-based methods and care needs to be taken to work under strict sterile conditions (Kuczynski et al., 2012). Furthermore the downstream analysis used to identify bacterial taxa can be quite complex and time consuming (Kuczynski et al., 2012).

1.2.2 Natural progression of the neonatal intestinal microbiota and differences in premature infants:

In recent years, scientists have begun to refute the long held belief that the womb is a sterile environment. In fact bacteria have been isolated from placenta, umbilical cord blood, amniotic fluid, and fetal membranes of healthy full term infants (Bearfield et al., 2002; Jimenez et al., 2005; Rautava et al., 2012). A recent study examined human placenta specimens collected under sterile conditions and found that the placental microbiome most resembled the human oral microbiome (Aagaard et al., 2014). Even the first stool produced by infants, known as meconium, which is occasionally excreted *in utero* has been found to contain bacteria (Jimenez et al., 2008). Funkhouser *et al*. have reviewed evidence demonstrating that many different species within the animal kingdom may vertically transmit their microbiota to their offspring (Funkhouser and Bordenstein, 2013). Current evidence therefore suggests that the fetus might be exposed to bacteria even prior to passage through the birth canal, although direct experimental proofs of live microbes are still missing (e.g. bacterial culture from fetus).

Studies have shown that there is an evolution of bacteria in full term neonatal intestines, which follows successive steps (Fanaro et al., 2003; Favier et al., 2002; Mackie et

al., 1999). The first colonizers are facultative anaerobes like Enterobacteria, Enterococci, Lactobacilli and Streptococci, followed by strict anaerobes such as Bifidobacteria, Bacteroides, Clostridia and Eubacterium (Palmer et al., 2007). During the first 6 months of life, *Bifidobacterium* remains one of the most predominant bacteria and is usually detected within the first week of life (Balmer and Wharton, 1989; Hudault, 1996; Kleessen et al., 1995; Palmer et al., 2007; Roberts et al., 1992; Stark and Lee, 1982; Yoshioka et al., 1983). Many changes occur in the first year of life followed by a stabilization of the microbiota, which parallels the transition to solid food, and the weaning of breast milk (Palmer et al., 2007; Stark and Lee, 1982).

1.2.3 Prematurity:

The major consequence of premature birth is a delayed and less diverse microbiota, which has been shown in many studies (Kosloske, 1994; Millar et al., 1996; Orrhage and Nord, 1999; Sakata et al., 1985; Schwiertz et al., 2003). There is also a different pattern of microbial colonization in the gut, specifically with lower concentrations of beneficial bacteria such as the *Bifidobacteriaceae*, which are normally dominant in full-term babies (Hallstrom et al., 2004; Millar et al., 1996; Sakata et al., 1985; Schwiertz et al., 2003) and a higher level of potential and known pathogens like *Enterobacteria, Bacteroides, Clostridia, Staphylococci, Pseudomonas*, and *Klebsiella*. Some studies have shown higher rates of *Clostridium difficile* (Penders et al., 2006), while one prospective study of 29 very preterm infants showed high rates of *Staphylococcus*, lower rates of *Bifidobacterium* and a complete absence of *Lactobacillus* (Jacquot et al., 2011). A study that stratified premature infants based on post conceptual age found that the overall bacterial composition of their gastrointestinal tract is limited to three classes of bacteria; Bacilli, Gammaproteobacteria and

Clostridia (La Rosa et al., 2014).

The commensal microbiota is known to be involved in important functions, including postnatal intestinal development, maintenance of the mucosal barrier, nutrient absorption, and maturation of the immune system (Guarner and Malagelada, 2003). The impaired intestinal colonization observed in preterm infants, is thought to affect intestinal homeostasis (Hsiao et al., 2008; Mai and Draganov, 2009) and to increase the risk of developing gastrointestinal diseases, such as NEC and late onset sepsis (Caicedo et al., 2005). Intestinal colonization in neonates is influenced by various factors such as mode of delivery, type of milk ingested, antibiotic therapy, and hospitalization (Biasucci et al., 2010; Dominguez-Bello et al., 2010; Fanaro et al., 2003; Gronlund et al., 1999; Harmsen et al., 2000; Huurre et al., 2008). In fact the gastrointestinal tract of premature infants are frequently colonized with bacteria present in the neonatal intensive care unit (NICU) environment such as sinks and tubing (Brooks et al., 2014). In contrast the gut of full term vaginally delivered infants is colonized with bacteria such as *Enterococcus* and *Escherichia,* frequent residents of the adult gastrointestinal tract (Gosalbes et al., 2013; Jimenez et al., 2008). They likely arrive in the infants gut through ascension from the vagina (Funkhouser and Bordenstein, 2013). A murine model showed that labeled strains of *E. fecium* can be recovered in the meconium of mice who's mothers were orally inoculated with these strains (Jimenez et al., 2008) lending further support to the theory that infants are in part colonized by bacteria that reside in the maternal gut. Thus many of the risk factors for a less desirable microbiota are unavoidable in premature babies since they are more likely to be born via cesarean section (C-section), delayed with enteral feeds, exposed to antibiotics, and hospitalized for prolonged periods. Interestingly a recent article by La Rosa *et al* suggests that gestational age impacts the pace

of progression of the intestinal microbiota more than exogenous factors like mode of delivery, antibiotics, or type of feeds (La Rosa et al., 2014). These findings are at once reassuring and disconcerting for the clinician. Reassuring in that medical interventions such as prescribing antibiotics or performing a C-section have less of an impact on the progression of the microbiome than what was once believed. At the same time these are worrisome results since medicine has made little progress at decreasing the rates of extremely premature infants in the last 10 years (CDC Wonder online database), and future efforts should focus on delivering premature babies at the latest possible gestational age.

1.2.4 NEC

Necrotizing enterocolitis (NEC) is a devastating disease that overwhelmingly affects preterm infants (Neu and Walker, 2011). Long term morbidity is significant and mortality rates are as high as 20-30% (Fitzgibbons et al., 2009). The pathophysiology of NEC is multifactorial and is hypothesized to include an altered intestinal microbiota coupled with an excessive immature inflammatory response leading to necrosis (Neu and Walker, 2011). Interestingly no single bacterial species has consistently been isolated as the culprit. A recent prospective study of premature babies less than 32 weeks gestation by Mai *et al.* reported an increase (34%) of Proteobacteria and a decrease (32%) in Firmicutes in the stool samples of NEC cases collected between 1 week and 72 hours prior to the development of NEC, using a16S rDNA based sequencing approach (Mai et al., 2011). Another study by Wang *et al.* showed decreased diversity and once again an increased abundance of γ-Proteobacteria in the stools of patients with NEC (Wang et al., 2009). These studies add weight to the argument that NEC is a consequence of dysbiosis. Unfortunately the literature is conflicting on this topic and Mshvildadze *et al.* did not show a difference in the microbial profiles of babies

with NEC compared to controls (Mshvildadze et al., 2010). It is therefore important to gain a better understanding of the processes involved in the colonization and establishment of the gut microbiota in premature infants.

1.2.5 Mode of Delivery and Diet:

Mode of delivery plays an important role in the initial establishment of an infant's intestinal microbiota. A study that examined the meconium of infants within 24 hours of birth as well as the vaginal, oral and skin microbiota of mothers right before delivery, found that the bacterial communities of the infants born vaginally most resembled their mothers vaginal microbiota with high levels of *Lactobacillus, Prevotella* and *Sneathia* (Dominguez-Bello et al., 2010). Conversely those born via caesarean (C-section) exhibited bacterial communities found on their mother's skin with higher levels of *Staphyloccocus, Corynebacterium*, and *Propionibacterium* (Dominguez-Bello et al., 2010). Furthermore at day 3 of life the microbiota of neonates delivered via C-section has been reported to be less diverse than their vaginally delivered counterparts (Biasucci et al., 2008). This same study also demonstrated an absence of *Bifidobacterium* in the babies born via C-section compared to an abundance of *Bifidobacterium longum* and *B. catenulatum* in the stools of those who were vaginally delivered (Biasucci et al., 2008). Penders *et al.* also reported lower levels of *Bifidobacterium* along with less *Bacteroides fragilis* and higher levels of *Clostridium difficile* in C-section babies (Penders et al., 2006). The differences found persisted for months and perhaps even longer as shown in one Canadian study that examined the intestinal microbiota of 24 healthy infants at 4 months of age using fecal samples (Azad et al., 2013). Their findings were as follows; less *Escherichia* and *Shigella* in infants delivered via C-section, as well as undetectable levels from the Bacteroidetes phylum. Unlike other studies

(Bezirtzoglou et al., 2011; Biasucci et al., 2008; Penders et al., 2006; Roger et al., 2010) Azad et al. showed no difference in *Bifidobacterium* levels based on mode of delivery or diet. Furthermore levels of the *Peptostreptococcacea* family, which includes *C. difficile*, were not different between vaginally and C-section delivered infants. However in keeping with the current literature, bacterial richness and diversity were lowest for babies delivered via C-section at 4 months of age. Interestingly, one small study of 21 premature infants showed no differences between vaginally (8 infants) and C-section delivered infants (Arboleya et al., 2012) suggesting that the above-mentioned differences in the microbiota based on mode of delivery may not be applicable to premature infants.

Breast fed infants will ingest between 10^5-10^7 bacteria while nursing (Heikkila and Saris, 2003). *Staphylococcus epidermis*, *Streptococcus salivarius* and S*treptococcus mitis* have been shown to be the most abundant species in breast milk. These same species have also been identified from stool samples of breast-fed infants (Favier et al., 2002; Kirjavainen et al., 2001). Martin *et al*. went a step further and found identical bacterial strains in the breast milk and feces of mother-infant pairs (Martin et al., 2012). The aforementioned studies thereby suggest that the intestinal microbiota of infants are colonized in part from bacteria present in ingested milk. While it is logical that many of the bacteria that colonize the mother's skin will be transferred to the infant during nursing, the origin of these intestinal bacteria in breast milk is not completely understood. Nevertheless, there is evidence to support an entero-mammary pathway whereby bacteria from the maternal gastrointestinal tract may pass into the breast milk via maternal dendritic cells and macrophages (Jost et al., 2014).

Breast fed babies have lower bacterial richness and diversity, and are dominated by

Bifidobacterium compared to those that are formula fed (Bezirtzoglou et al., 2011). The latter has been previously explained by the presence of prebiotics such as human milk oligosaccharides (HMOs) in breast milk, which favor the growth of specific bacteria (Ward et al., 2006). In contrast, elevated levels of the *Peptostreptococcacea* family, which includes *Clostridium difficile*, as well as higher levels of *Bacteroides*, *Streptococcus*, *Enterobacteria*, and *Veillonella*, were found in babies that were formula fed (Adlerberth and Wold, 2009; Azad et al., 2013; Bezirtzoglou et al., 2011; Fallani et al., 2010).

1.2.6 Effect of Probiotics and Prebiotics:

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (WHO 2001). Gibson and Roberfroid first defined a prebiotic as a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" (Gibson and Roberfroid, 1995).

There is an increasing trend towards administering pro- and prebiotics as supplements to premature infants as a way of replacing the lack of breast milk ingested by this population and promoting a microbiota rich in beneficial Bifidobacteria. Studies have shown that the presence of *Lactobacillus* and *Bifidobacterium* in the bowel plays a role in preventing bacterial translocation into the intestinal bowel wall (Ewaschuk et al., 2008; Qin et al., 2009; Resta-Lenert and Barrett, 2003). Many neonatal intensive care units (NICUs) around the world are using routine probiotic supplementation as a means of decreasing their rates of necrotizing enterocolitis (NEC) (Janvier et al., 2014). This practice is supported by a meta-analysis that showed a relative risk of 0.35 (95% CI 0.23-0.55, 11 RCTs) for the

development of NEC in premature, very low birth weight (VLBW<1500g) infants receiving probiotics versus placebo (Deshpande et al., 2010) as well as a recent Cochrane review (AlFaleh and Anabrees, 2014). The decision to use probiotics in the NICU is not taken lightly in this at risk population especially given the fear of sepsis from probiotic strains, for which there are a few case reports for both *Lactobacillus* (Kunz et al., 2004; Land et al., 2005) and *Bifidobacterium* (Ohishi et al., 2010). Nevertheless, many neonatologists would agree that the benefits of preventing NEC with probiotics outweigh the risk of probiotic strain sepsis (Tarnow-Mordi and Soll, 2014).

The current literature does not support the use of prebiotics alone, such as oligosaccharides in preterm or VLBW babies as a means of reducing late onset sepsis or NEC (Srinivasjois et al., 2013). German *et al.* have proposed that the oligosaccharides in a mother's breast milk confer benefit to her own baby, however past studies argued that the advantages were not reproducible when the oligosaccharide was added to commercial formula (German et al., 2008). In contrast, a recent study (De Leoz et al., 2015) used DNA sequencing to analyze the microbiome of 2 breast fed infants, as well as mass spectrometry to study the HMOs that passed through the gut into the feces. They were able to correlate a decrease in excreted HMOs with a rise in the relative abundance of bacteria that are known to metabolize HMOs such as *Bacteroidaceae* and *Bifidobacteriaceae* thereby suggesting that HMOs can modulate the intestinal microbiome of infants.

It would seem logical that Bifidobacteria is primed to metabolize breast milk in the gut. When comparing the genomes of certain subspecies *B. longum subsp. infantis* contains 7 genes that are involved in HMO metabolism versus only 3 of these genes in *B. longum longum*, a strain more abundant in the gut of adults compared to infants (Lee and O'Sullivan,

2010).

1.2.7 Effect of Antibiotics:

Multiple studies have shown that early antibiotic exposure in neonates greatly influences the intestinal microbiome (Favier et al., 2003; Fouhy et al., 2012; Greenwood et al., 2014; Hussey et al., 2011; Jacquot et al., 2011; Savino et al., 2011; Tanaka et al., 2009). Furthermore the changes that occur can have long lasting effects. A denaturing gel gradient electrophoresis (DGGE) based study performed in Japanese full term infants demonstrated alterations in the microbiome such as; delayed colonization with *Bifidobacterium* and an overgrowth of *Enterococcus* (phylum Firmicutes) and *Enterobacteriaceae* (phylum Proteobacteria) as late as 1 month following antibiotic exposure and a less diverse microbiota at 2 months post exposure (Tanaka et al., 2009). The above results were replicated in a study using high throughput sequencing by Fouhy *et al*. (Fouhy et al., 2012). Full term infants who received early (within 48 hours of birth) intravenous ampicillin and gentamicin showed lower levels of *Bifidobacterium* and *Lactobacillus* at 1-month post treatment, as well as a higher proportion of the Proteobacteria phylum. At 8 weeks after the cessation of antibiotics the latter changes persisted along with lower diversity scores (Fouhy et al., 2012). Changes in relative abundance of commonly pathogenic bacteria, such as *Staphylococcus* and *Enterobacter* and decreased diversity have also been reported in preterm infants receiving antibiotics (Dardas et al., 2014; Greenwood et al., 2014; Jacquot et al., 2011). Several studies have examined the duration of antibiotic exposure (Dardas et al., 2014; Greenwood et al., 2014; Jacquot et al., 2011). Similar to full term babies who receive antibiotics, *Enterobacter* (*Enterobacteriaceae* family) predominated in preemies who received a short course of antibiotics while higher *Enterococcus*, *Clostridium*, *Staphylococcus* and *Escherichia* levels

were found in those who received longer antibiotic therapy (Greenwood et al., 2014). Unlike some of the findings in full term infants where Bifidobacteria levels decreased with antibiotic exposure (Fouhy et al., 2012), all premature infants lacked Bifidobacteria including those that had never received antibiotics. Overall lengthy antibiotic treatments appear to induce long-lasting effects on the microbiome. Indeed, the duration of antibiotic exposure was found to be inversely proportional to the diversity score of the microbiome of premature infants at 6 weeks post antibiotic administration in a study performed by Jacquot *et al*. (Jacquot et al., 2011). A separate study by Greenwood *et al*. (Greenwood et al., 2014) of 74 preterm infants also showed a difference in alpha diversity that was dependent on the duration of antibiotic use. Patients who received a short course (1-4 days) had decreased diversity at week 2, which returned to baseline by week 3 of the study, conversely those who received an intensive course (5-7 days) continued to have a less diverse microbiota at week 3 (end of the study period). Premature babies are at high risk for dysbiosis, since antibiotics are the most frequently prescribed medication in the NICUs (Clark et al., 2006). Furthermore many premature babies receive antibiotics without any accompanying positive culture, known as empiric therapy, since the risk of a possible untreated infection is deemed more significant than unnecessary exposure to antibiotics. Breast-feeding may play a role in re-establishing a normal microbiota following antibiotics exposure (Savino et al., 2011). A culture based study of full term, breast-fed infants treated with ceftriaxone, showed a reduction in bacteria after 5 days of antibiotic administration, but a return to baseline bacterial counts within 15 days following the cessation of antibiotics which suggests that breast milk is speeding up the return to baseline (Savino et al., 2011). In conclusion, while it is obvious that antibiotics perturb the intestinal microbiome of infants, the exact duration of their effect is unknown, but seems to be in the order of months to years according to one study that reported changes

at 1 year following perinatal antibiotic exposure (Persaud et al., 2014). Many have suggested that the impact of early antibiotics may in fact be lifelong since early antibiotic exposure in infancy has been correlated with increased incidence of asthma and other atopic diseases (Celedon et al., 2004; Foliaki et al., 2009; Kozyrskyj et al., 2007). A large prospective study in Copenhagen with 411 pediatric patients established the link with the microbiome by showing that infants with a reduced intestinal bacterial diversity at age 1 and 12 months had a higher risk of developing allergies in early childhood (Bisgaard et al., 2011).

1.3 Lactoferrin can be used as an infant supplement and contains antibacterial properties

Lactoferrin was first isolated from bovine milk in 1939 by Sorenson and Sorenson. It is a glycoprotein member of the transferrin family and binds two Fe^{3+} ions (Baveye et al., 1999). Lactoferrin is the major whey protein in mammalian milk and is important in innate immune host defenses (Lonnerdal, 2003). Human recombinant lactoferrin has been shown to have 69% homology with bovine lactoferrin at the amino acid level (Pierce et al., 1991). The lactoferrin concentration in maternal colostrum of babies born at term is 9 mg/mL but subsequently falls to 1.4-3 mg/mL, in mature breast milk and exists at very low concentrations in commercially available formulas (0.1 mg/mL) (Ronayne de Ferrer et al., 2000) (Figure 1.2). Interestingly, the lactoferrin content in the milk of mothers who deliver prematurely is lower compared to those who deliver at term, however the concentration of lactoferrin decreases at a slower rate (Underwood, 2013). Lactoferrin resists proteolysis in the digestive tract, binds to specific receptors on enterocytes, and is poorly absorbed from the gut. Lactoferrin is of interest as a supplement for premature infants due to its anti-

inflammatory, bacteriostatic and bactericidal properties (Embleton et al., 2013). Due to its ability to bind iron, it supports the growth of beneficial bacteria with lower or nonexistent iron demands such as *Bifidobacterium,* which has special iron acquisition mechanisms that function at a lower pH and *Lactobacillus,* which uses manganese and cobalt instead of iron (Bezkorovainy et al., 1986; Petschow et al., 1999; Sherman et al., 2004; Weinberg, 1997). A culture-based study described changes in the intestinal microbiota of full term infants receiving supplemental lactoferrin in their formula versus breast milk and formula without supplementation (Roberts et al., 1992). At 3 months of age half of the babies receiving lactoferrin supplemented formula had a "bifidus flora" defined as *Bifidobacterium* outnumbering all other components of the fecal microbiota by greater than one $\log_{10}$ CFU/g of feces (Roberts et al., 1992). Lactoferrin also increases the bactericidal activity of neutrophils by providing iron for free radical production (Legrand et al., 2005). To date there has been only one published multicenter prospective randomized trial using bovine lactoferrin and probiotic supplementation in the NICU, and it showed a reduction in sepsis as well as NEC in infants who received lactoferrin (Manzoni et al., 2014; Manzoni et al., 2009). Four hundred and seventy two VLBW infants were randomized to receive lactoferrin alone, lactoferrin and *Lactobacillus rhamnosus* (LGG), or placebo. The two groups receiving lactoferrin had significantly fewer episodes of sepsis, with a relative risk of 0.27 (95% CI, 0.12-0.60) compared to controls (Manzoni et al., 2009). A continuation of this study enrolled a total of 743 infants and showed lower rates of NEC in infants who received lactoferrin alone as well as lactoferrin with LGG 5/247 (2%) and 0/238 (0%), respectively compared to controls 14/258 (5.4%) (Manzoni et al., 2014). A large (2200 infant) clinical study in the UK entitled Enteral Lactoferrin In Neonates (ELFIN) is underway to assess the benefits of lactoferrin supplementation. Despite the unquestionable role of the intestinal microbiota in

human health there is only one human study examining the effects of lactoferrin on the intestinal microbiota of premature infants using culture independent methods (Sherman et al., 2016).

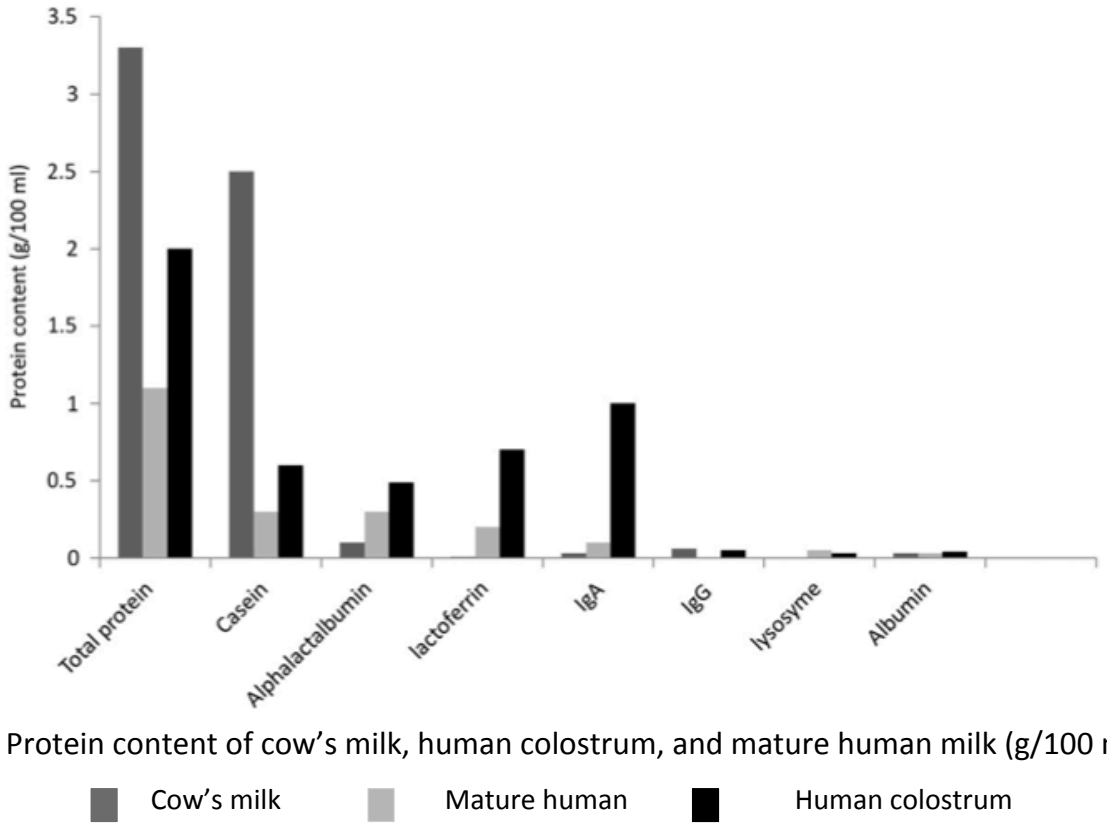

Figure 1.2: Protein content found in cow's milk, colostrum and mature human milk

The lactoferrin content of mature human milk is negligible and highest in human colostrum, and then decreases in more mature breast milk (Figure taken from (Embleton et al., 2013)).

1.4 Piglets as a model for the neonatal intestine

Animal models have allowed for an in-depth look at the pathophysiological mechanisms of lactoferrin in the gastrointestinal tract and specifically its antibacterial activity. Lactoferrin inhibits the attachment of enteropathogenic *E. coli* (EPEC) to intestinal cells (Edde et al., 2001) and a study by Sherman et al showed that administration of LGG and recombinant human lactoferrin led to decreased *E.coli* in the small bowel of rat pups infected with *E. coli* (Sherman et al., 2004). Piglets are an excellent surrogate model for the infant intestine, since they have similar gastrointestinal physiology and intestinal maturation (Guilloteau et al., 2010). Additionally piglet intestines contain lactoferrin receptors (Liao et al., 2007) and neonatal piglets can absorb orally administered lactoferrin (Harada et al., 1999). Hu et al administered human recombinant lactoferrin to piglets and characterized the intestinal microbiome using both culture dependent and independent techniques (Hu et al., 2012). Piglets receiving the recombinant lactoferrin had better weight gain compared to control, a trend towards increasing diversity of the intestinal biota, as well as lower rates of pathogenic bacteria such as *E. coli* and *Salmonella* and higher rates of protective bacteria such as *Bifidobacterium* and *Lactobacillus*. Their study bears repeating with high throughput sequencing since Hu et al relied on low-resolution molecular methods like DGGE to monitor the gut microbiota.

1.5 Hypothesis: As mentioned above there are no reports characterizing the effect of probiotic and lactoferrin supplementation on the development of the gut microbiota communities in preterm babies. Therefore and based on the current knowledge I proposed to test the hypothesis that probiotic and lactoferrin supplementation positively influence the intestinal microbiota.

1.6 Objectives of this study

The following three objectives were designed to test my hypothesis:

1. Perform a prospective study describing the evolution and diversity of the intestinal microbiota of premature infants receiving lactoferrin and probiotic supplementation
2. Correlate clinical information such as; mode of delivery, gestational age, antibiotic use, and type of milk ingested with high and low diversity microbiota
3. Compare the results obtained in objective 1 with an animal model (piglet) supplemented with probiotic and lactoferrin

CHAPTER 2

MATERIALS AND METHODS

2.1 Ethics statement

The sampling protocol was approved by the Research Ethics Board of the Sainte Justine University Hospital Center. Informed consent was signed by the parents of each subject. Ethics approval was also obtained from the University of Ottawa for the animal study. Funding was provided by the Canadian Institute of Health Research (CIHR).

2.2 Intervention

2.2.1 Infants:

Seventy premature infants of gestational age 23 $^{0/7}$ to 30 $^{6/7}$ weeks were recruited within 12 hours of their first enteral feed. All infants were less than 48 hours of age at study inclusion. Exclusion criteria included; infants that were considered non-viable by the neonatology team, infants with dysmorphic features or congenital malformations that considerably affect life expectancy, and babies with serious gastrointestinal malformations. The subjects were randomized to receive a daily dose at once of 0.5 g of FloraBABY^{TM} (Renew Life Canada, Oakville, ON,) alone (control group) or with the addition of 100 mg of bovine lactoferrin (Advanced Orthomolecular Research (AOR), Calgary, AB) (experimental group). The bovine lactoferrin used was approximately 15% iron saturated (personal communication with manufacturer). According to the manufacturer's specifications, 1 g of FloraBABY^{TM} contains 4 billion colony forming units (CFU's); 1.2 billion *Bifidobacterium breve*, 1 billion *Lactobacillus rhamnosus*, 800 million *Bifidobacterium bifidum*, 600 million *Bifidobacterium infantis*, and 400 million *Bifidobacterium longum* (Figure 2.1).

FloraBABY -Each serving (1g) contains

Proprietary Blend	4 Billion CFU
Bifidobacterium breve (HA-129)	1.2 Billion CFU
Lactobacillus rhamnosus (HA-111)	1 Billion CFU
Bifidobacterium bifidum (HA-132)	800 Million CFU
Bifidobacterium infantis (HA-116)	600 Million CFU
Bifidobacterium longum (HA-135)	400 Million CFU

***Note that 500 mg (half a serving) was used in this study

Figure 2.1: Probiotic Used in the Study

FloraBaby™ is produced by Renew Life Canada Oakville, ON. All probiotics used in the study were purchased by Sainte Justine University Hospital Center

The powdered supplements were mixed with water and given prior to receiving milk (breast/formula) and caretakers were blinded to the addition of lactoferrin. This project was part of a prospective pilot study entitled LACUNA (LACtoferrin Use in NeoNAtes) (Trial of lactoferrin for prevention of infections in very premature babies, ISRCTN66482337). LACUNA's primary objective was to determine if lactoferrin and probiotic supplementation increase the rate of feeding tolerance. A secondary outcome that was measured was the incidence of hospital-acquired infections. In fact prophylactic probiotic administration has become the standard of care in the CHU Sainte-Justine's NICU for all infants less than 32 weeks gestation (Janvier et al., 2014).

2.2.2 **Piglets**:

Healthy full-term piglets were obtained from a local farm in the Ottawa area, on day 2 of life and were placed in groups of two. While on the farm, the piglets received their mother's colostrum on the first two days of life and an iron injection. The experiment was performed in 2 batches, the first in January 2013 with 8 piglets and the second in November 2013 with 6 piglets (no lactoferrin alone group). In each case the piglets were taken from the litter of a single sow. Briefly, the piglets were fed Wet Nurse Piglet Milk Replacer [24-24-12] (Prairie Micro-Tech Inc. Regina, SK, Canada) every 5 hours. Starting on day 3 we supplemented the piglets with 0.5 g of FloraBABY^{TM} (Renew Life Canada, Oakville, ON,) and/or 100 mg of bovine lactoferrin (Advanced Orthomolecular Research (AOR), Calgary, AB) diluted in 5ml of Piglet Milk Replacer once daily via syringe. Piglets were randomized in 4 groups; 1: piglets fed milk replacer alone; 2: piglets fed milk replacer supplemented with probiotics; 3: piglets fed milk replacer supplemented with lactoferrin; and 4: piglets fed milk replacer supplemented with probiotics and lactoferrin. Note that supplementation was done

with equivalent dosing to the infant study given the similar weights between the premature infants and newborn piglets.

2.3 Sample collection

2.3.1 Infants:

Whenever possible the infant meconium was collected for each infant enrolled in the study. This was followed by the collection of 1 stool sample per week (+/- 2 days), while the infant remained in the NICU, for 1 month for a total of up to 5 samples per baby. A research nurse collected each stool sample from the infant's diaper. Samples were stored in Eppendorf tubes and frozen immediately after collection at -80°C until further use.

2.3.2 Piglets:

Stool samples were collected once daily and frozen at -80°C until further use. Piglets were housed 2 per cage according to their treatment group. Stool was collected immediately after a feeding at the same time each day, and rectal stimulation with a thermometer was used as needed. Occasionally rectal swabs were used in place of stool samples. Daily weights, and temperature were also recorded. Piglets were sacrificed after 7 days of supplementation (experimental day 10) and mucosal scrapings and luminal samples from various intestinal segments were obtained (Figure 2.2). Briefly, at the time of necropsy 10 cm long intestinal samples were isolated from each piglet. For the small bowel all measurements were taken at the stomach. The duodenum was taken at 30 cm and jejunum at 60 cm from the stomach. The ileum was isolated at 10 cm proximal to the cecum. The cecum was easily identified due to its characteristic thumb-like appearance. Colon samples were measured at fixed distance from the cecum. The ascending, transverse, and descending colon samples were isolated at 5, 10, and 20 cm from the cecum respectively.

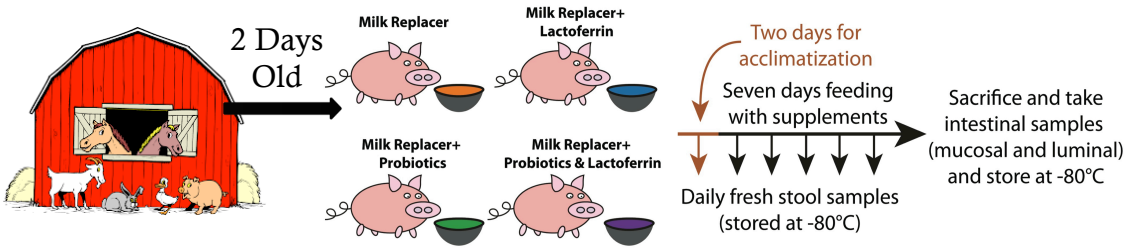

Figure 2.2: Piglet protocol
Piglets were randomized into 4 groups (control, lactoferrin alone, probiotic alone, and probiotic and lactoferrin). All piglets received milk replacer as their food source. Following 2 days of acclimatization piglets received the supplements for a total of 7 days. Figure prepared by Dr. James Butcher

2.4 Clinical Data Collection

Demographic information was collected for all infants such as; date of birth, infant sex, gestational age at birth, presence of multiple gestations, birth weight, mode of delivery (vaginal versus C-section), and feeding methods at the study initiation (breast milk, commercial formula, or both) and at study conclusion. In addition antibiotic use was recorded, as was the occurrence of sepsis defined as: an illness occurring more than 72 hours after birth, with clinical signs and symptoms consistent with sepsis, in association with the isolation of a causative organism from a blood culture. Cases of NEC were diagnosed using the modified Bell's criteria (Bell et al., 1978; Walsh and Kliegman, 1986). Only cases with radiologic confirmation of pneumatosis intestinalis, portal venous air, or perforation were included as cases.

2.5 Extraction of metagenomic DNA.

DNA was extracted from the stool samples using the FastDNA® Spin Kit (MP Biomedicals) according to the manufacturer's instructions. DNA extraction is based on both a chemical and mechanical cell lysis using garnet particles and a ceramic sphere. This included two mechanical lysis cycles in a FastPrep® Instrument (MP Biomedicals) set at a speed of 6.0 for 40 seconds. RNA extraction was minimized by using the patented silica based GENECLEAN® technology. Extracted DNA was quantified using the Qubit fluorometer (Life Technologies), which is selective for DNA, and then stored at -20°C until library construction.

2.6 Preparation of 16S rRNA libraries from metagenomic DNA for deep sequencing

Amplification of the V6 hypervariable region of the *16S rRNA* region was carried out

using a two-step PCR strategy as previously described (Abujamel et al., 2013). All primers used are outlined in Appendix I. Briefly, the first step added sample specific barcodes at each end of the *16S rRNA* V6 region to allow for multiplexing and also added Illumina paired-end sequencing adaptors. The second step added the Illumina flow cell adaptors for cluster generation. In the 1st step, 50 ng of extracted DNA was used in a 50 µL PCR reaction containing 10 µL of water, 25 µL 2x Phusion High-Fidelity PCR Master Mix (New England BioLabs Inc.), 2.5 µL each of the forward and reverse barcode primers (10 µM), and 10 µL of template DNA (concentrated to 5ng/uL). This allowed for a unique set of barcodes for each sample. Amplification started with an initial enzyme activation step at 98°C for 30 sec. Then, amplification was carried out using cycles of 94°C (5 seconds), 61°C (15 seconds), and 72°C (15 seconds). Then a touch down of 1°C each cycle from 61°C until a temperature of 51°C was reached. Amplification was continued with an additional 15 cycles using 51°C as an annealing temperature. PCR was terminated with a final elongation at 72°C for 5 minutes. The setup for the second PCR was similar to the first PCR except that 10 µL of product from the first PCR reaction was used as a template and a universal primer set was used in place of the barcoded primers. The reaction started at 98°C for 30 seconds with amplification proceeding for 15 cycles at 98°C for 5 seconds, 65°C for 15 seconds, 72°C for 15 seconds and a final extension step at 72°C for 5 minutes. Following amplification, PCR products were inspected on a 1.5% agarose gel, and purified using Montage PCR_{96} Cleanup Kit (Millipore) following the manufacturer's protocol with an additional 100 µL sterile water wash in each well prior to DNA reconstitution. DNA from each well was quantified using the Qubit fluorometer (Life Technologies). Fifty ng of DNA from each well was pooled together into a single tube and inspected on a 1.5% agarose gel. Gel extraction was then performed with a 2% agarose gel (Qiagen gel purification) and the 300bp band was targeted

to avoid sequencing primer dimers.

The nucleic acid concentration A260/A280 which is a representation of the ratio of nucleic acid to protein concentration was then measured using NanoDrop (Thermo Scientific).

The 16S rDNA library was sequenced at The Centre for Applied Genomics (TCAG) at the Hospital for Sick Children in Toronto (Canada) using an Illumina HiSeq 2500 platform to generate paired-end reads of 2x100 bases (see Figure 2.3 for workflow). Infants and piglets samples were sequenced on four different sequence runs AS27 (mixed infant and piglet samples), AS13 (infants alone) AS07 (piglets) and AS18 (piglets).

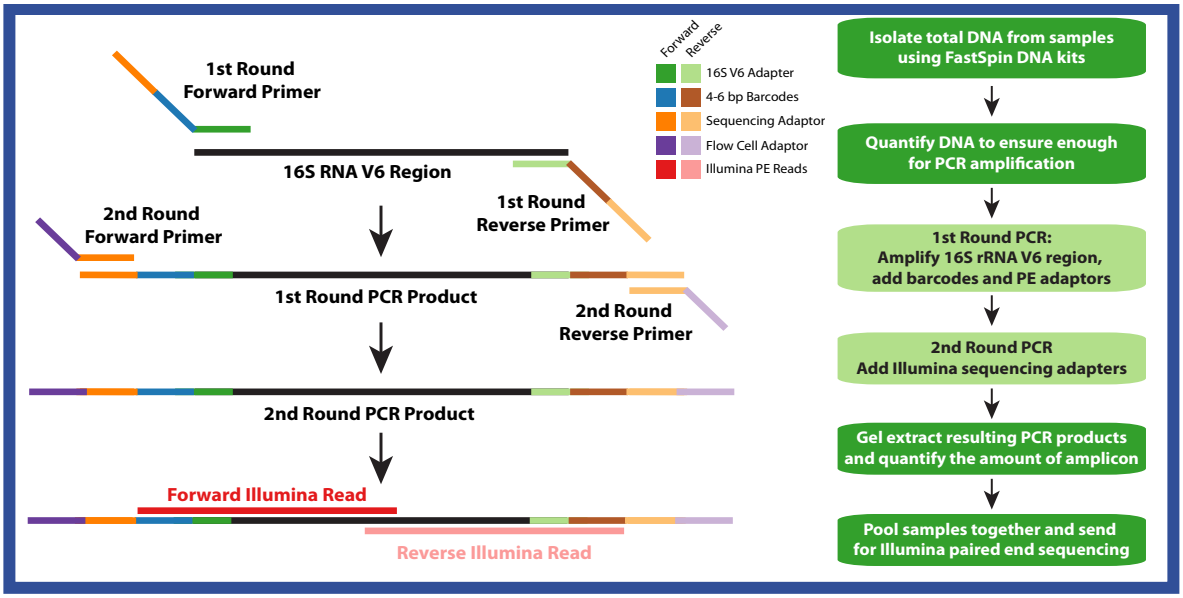

Figure 2.3: DNA extraction and sequencing strategy

Genomic DNA from samples stored at -80°C was extracted using FastDNA spin kits with bead beating. Isolated DNA was quantified using a Qubit fluorometer from Invitrogen and subjected to two rounds of PCR amplification. The first round added barcodes at each end of the 16S rDNA V6 region to allow for multiplexing and also added Illumina paired-end sequencing adaptors. The second round added the Illumina flow cell adaptors for cluster generation. The resulting amplicons were then gel purified to remove primer dimers/non-specific amplicons and quantified using a Qubit fluorometer. Samples were then pooled together in equal amounts and sent for paired-end sequencing at the Center of Applied Genomics in Toronto, Canada on an Illumina HiSeq. Note that this approach results in the Illumina paired reads overlapping in the 16S rDNA V6 region (Figure prepared by Dr. James Butcher).

2.7 Sequence analysis and characterization of microbiota diversity and composition

The resulting sequencing reads were analyzed as follows. Paired-end reads were first merged together using the FLASh algorithm (Magoc and Salzberg, 2011) and poor quality reads (defined as reads with a quality score <20 across >10% of the read) were filtered out using the FASTX toolkit (version 0.0.13.1) (http://hannonlab.cshl.edu./fastx_toolkit/index.html). Quality scores for each of the sequence runs can be seen in Appendix II. There were greater than 80,000,000 reads in each sequence run, with a raw read length of 101 bases. After merging the reads, the average read length varied from 101-197 bases, and >99% were considered good sequences. Reads were then demultiplexed and barcodes removed with FastqMultX (using *ea-utils* : "Command-line tools for processing biological sequencing data"; http://code.google.com/p/ea-utils) . Reads were then renamed and unknown bases were removed with PReprocessing and Information of SEQuence data (PRINSEQ) (http://edwards.sdsu.edu/prinseq/) (Schmieder and Edwards, 2011).

Quantitative Insights Into Microbial Analysis (QIIME) version 1.8 was used to cluster the reads into operational taxonomic units (OTUs) by using a closed reference OTU picking process known as uclust (unpublished), against the Greengenes database (version 13.5) at 97% sequence identity (Caporaso et al., 2010; DeSantis et al., 2006) generating a biom file. Samples with <100,000 reads after the removal of singleton and doubleton OTUs (defined as OTUs with <2 counts across all samples) were excluded from subsequent analysis. This led us to remove; 3555, 2806, 4130, and 4187 OTUs from each of the sequence runs. The relative abundance of taxa, from the phylum to the species level (when available), along with alpha diversity (diversity within a sample) and beta diversity (diversity

between samples) was also computed using QIIME (Figure 2.4). Additional analysis was done using the Metagenassist server, (www.metagenassist.ca/METAGENassist/faces/Home.Jsp) (Arndt et al., 2012). All biom files were rarified to 100,000 reads per sample in QIIME based on the chao1 score. This allowed us to compared the observed richness among treatment groups and correct for the possibility that they were unequally sampled. Chao1 uses non-parametric parameters, and as such we used non-linear statistics. The rarefied biom file was then uploaded into Metagenassist. In Metagenassist OTUs with the same taxonomic assignment were grouped together and unassigned/unmapped reads were not removed. No data filtering was done on the taxonomic counts and a generalized $\log_2$ transformation (Arndt et al., 2012) was used for column-wise normalization. Metagenassist was used for fold change analysis, pie chart construction of relative abundance at various taxa levels, principle component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) which was cross validated and then tested with 2000 permutations (Arndt et al., 2012). The principle coordinate analysis was based on the phylogenetic relatedness of the sample using a UniFrac distance.

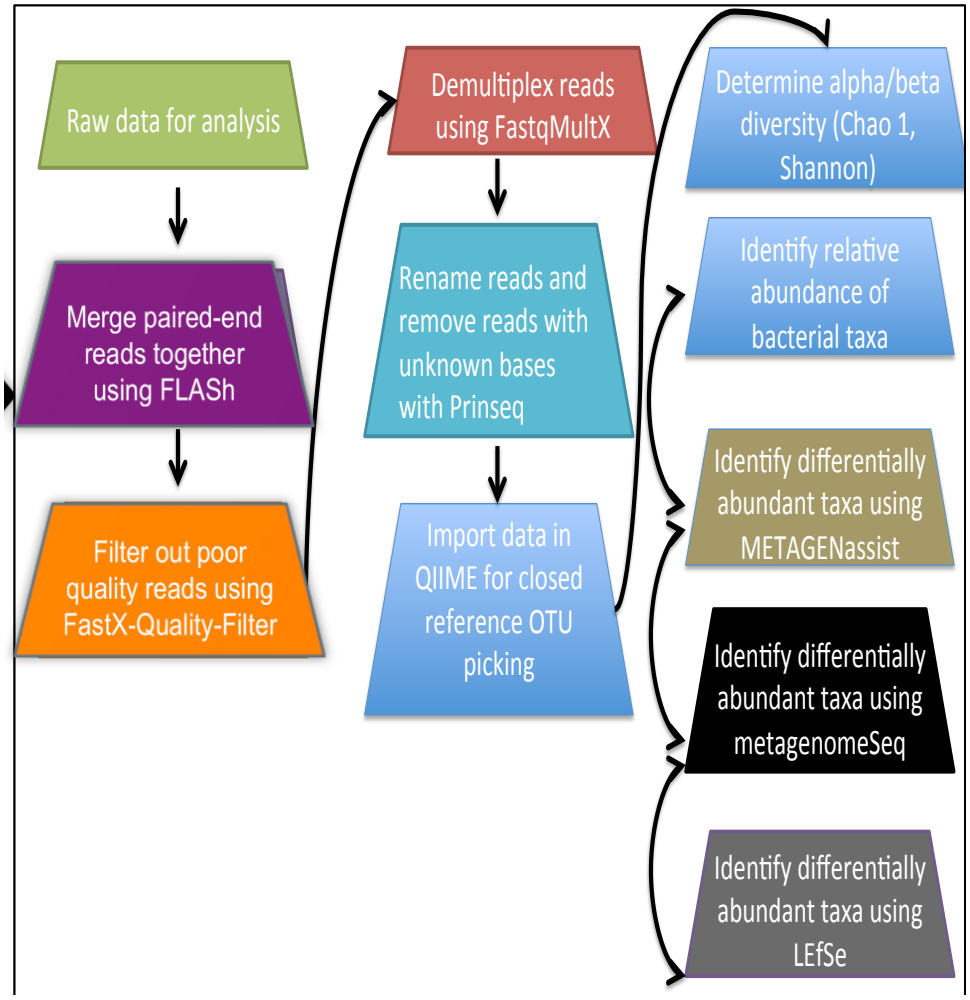

Figure 2.4: Bioinformatics Workflow

Schematic summary of the various bioinformatics steps required to process and analyze the resulting Illumina sequencing data. Different programs are highlighted with colors and a brief summary of each step is outlined. Several steps were automated and/or done in parallel using custom in-house scripts. Raw reads were also trimmed when deemed necessary to remove excess bases with poor quality scores.

2.8 Differential abundance analysis

Differential abundance analysis was done using both LEfSe and metagenomeSeq (Paulson et al., 2013; Segata et al., 2011). LEfSe identifies taxa exhibiting significant differential representation between groups by linear discriminant analysis (LDA) (Segata et al., 2011). MetagenomeSeq (Paulson et al., 2013) version 1.8.3 was used to determine differentially abundant OTUs as it implements a zero-inflated Gaussian linear mixed model that accounts for under sampling and confounding covariates. The following variables were chosen as covariates; sequencing run, gestational age at birth (<26 weeks, 26-28 weeks and >28 weeks), delivery method (vaginal, C-section), type of milk (breast, formula, or both), and post conceptual age at time of sample collection (<30 weeks, 30-33 weeks, >33 weeks). Cutoff values for significant fold changes in relative abundance were ≥ 2 fold difference, FDR corrected *p* value <0.05 with an effective sample proportion >0.75.

2.9 Statistical Analysis

Unless otherwise specified, results from the qPCR and sequencing were analyzed using two-tailed Mann-Whitney test comparing the probiotic group to the probiotic+lactoferrin group. When applicable, Kruskal Wallis test was used for multiple comparisons with a Dunn's correction. A p-value less than 0.05 was considered significant.

CHAPTER 3

RESULTS FROM THE INFANT STUDY

3.1 Demographics and stool sample information

Our project was part of a larger clinical study of 80 patients in which the ability of lactoferrin supplementation to reduce health care associated infections was studied. We enrolled patients for microbiome stool analysis while the clinical study was already underway and recruited 70 of the 80 patients. Thirty-five infants were randomized to the probiotic alone group (P) and 35 received probiotic with lactoferrin supplementation (PL). Average gestational age was 29 weeks and the majority of subjects were males. The study was limited to the duration of hospitalization, and stools were not collected if the infant was discharged home or transferred to an outside facility. Tables 3.1 gives a detailed account of all demographic information for all infants enrolled (Table 3.1A) as well the subset of those analyzed (Table 3.1B). Fifty-nine infants (29 P, 30 PL) were included in the final analysis. No statistically significant difference was observed between treatment groups for any of the demographic information listed using T-tests for numerical data and Fisher exact tests for categorical data. Type of milk ingested (breast milk, formula or a combination) reflected feeding practices at the start of the study since this information was not collected on a daily basis for the duration of the study. Note that genetic testing is not routinely performed in the CHU Sainte Justine NICU, to determine whether twin siblings are identical or not. As such we cannot be certain of how may identical or fraternal twin pairs we had. We did include one family of monochorionic monoamniotic (identical) twins (Table 3.3).

Overall 127 unique stool samples were analyzed in our study (63 in the P group and

64 in the PL group; occasionally duplicate samples were sent for sequencing). Some of the factors impeding the targeted stool collection are outlined in Figure 3.1. Furthermore premature infants did not always stool every day and our dedicated research nurses collected stools only during regular office hours. A minimum amount of 50 ng of DNA was required for PCR. Whenever possible numerous attempts were made to extract sufficient DNA for sequencing.

The vast majority of infants included for analysis received antibiotics at some point during the study (79% P and 80% PL) (Table 3.1B). Of the 48 and 52 samples in each group that were exposed to antibiotics, ampicillin and gentamicin were the most commonly used antibiotics (34/48 in P group, 40/52 in PL). The next most commonly prescribed antibiotics in our cohort were vancomycin (8P/5PL samples), linezolid (6P/5PL samples), meropenem (0P/3 PL samples), piperacillin+tazobactam (0P/2PL samples), and cefazolin (1P/0PL samples). Of note, many of the above-mentioned antibiotics were used in combination.

3.2 Rarefaction

In order to assess the depth of sequencing, rarefaction curves were constructed using the chao 1 index. The shape of the curve (Figure 3.2) suggests that deeper sequencing would not have identified significantly more OTUs. I therefore chose 100,000 sequences per sample as a cut-off. The average number of reads per sample was 701,623.427 +/- 278,876.998.

A

	Probiotic	Probiotic+Lactoferrin
Number of subjects	35	35
Gestational Age in weeks at birth, mean (SD)	29 (2.6)	29 (2.6)
Males (%)	21 (60)	23 (66)
Birth Weight g, mean (SD)	1123 (327)	1105 (264)
Multiple n infants	9	9
Vaginal Delivery, n (%)	18 (51)	14 (40)
Breast Milk Use n (%)	27 (77)	27 (77)
Formula Use n (%)	4 (11)	1 (3)
Combination Feeds n (%)	4 (11)	7 (20)
Infants wth post natal use of antibiotics n (%)	28 (80)	26 (74)
Necrotizing Enterocolitis	1	2
Sepsis	7	9
Death	3	4

B

	Probiotic	Probiotic+Lactoferrin
Number of subjects (n stool samples)	29 (63)	30 (64)
Gestational Age in weeks at birth, mean (SD)	28.6 (2.2)	28.1 (1.8)
Males (%)	18 (62)	21 (70)
Birth Weight g, mean (SD)	1138 (311)	1127 (248)
Multiples n Infants	8	7
Vaginal Delivery, n (%)	17 (59)	13 (43)
Breast Milk Use n (%)	23 (79)	24 (80)
Formula Use n (%)	4 (14)	6 (20)
Combination Feeds n (%)	2 (7)	0
Infants with Post natal use of antibiotics n (%)	23 (79)	24 (80)
Number of samples with antibiotic exposure	48	52
Number of days since antibiotic use, mean (SD)	11 (8)	10 (12)
Range of days since antibiotic use[a]	0-30	0-56
Necrotizing Enterocolitis	1	2
Sepsis	5	7
Death	1	1

Table 3.1: Summary of patient demographics

(A) Demographic information of all infants enrolled in the study
(B) Demographic information of subset of infants analyzed
[a] 0 indicates that subject was on antibiotics at time of sample collection

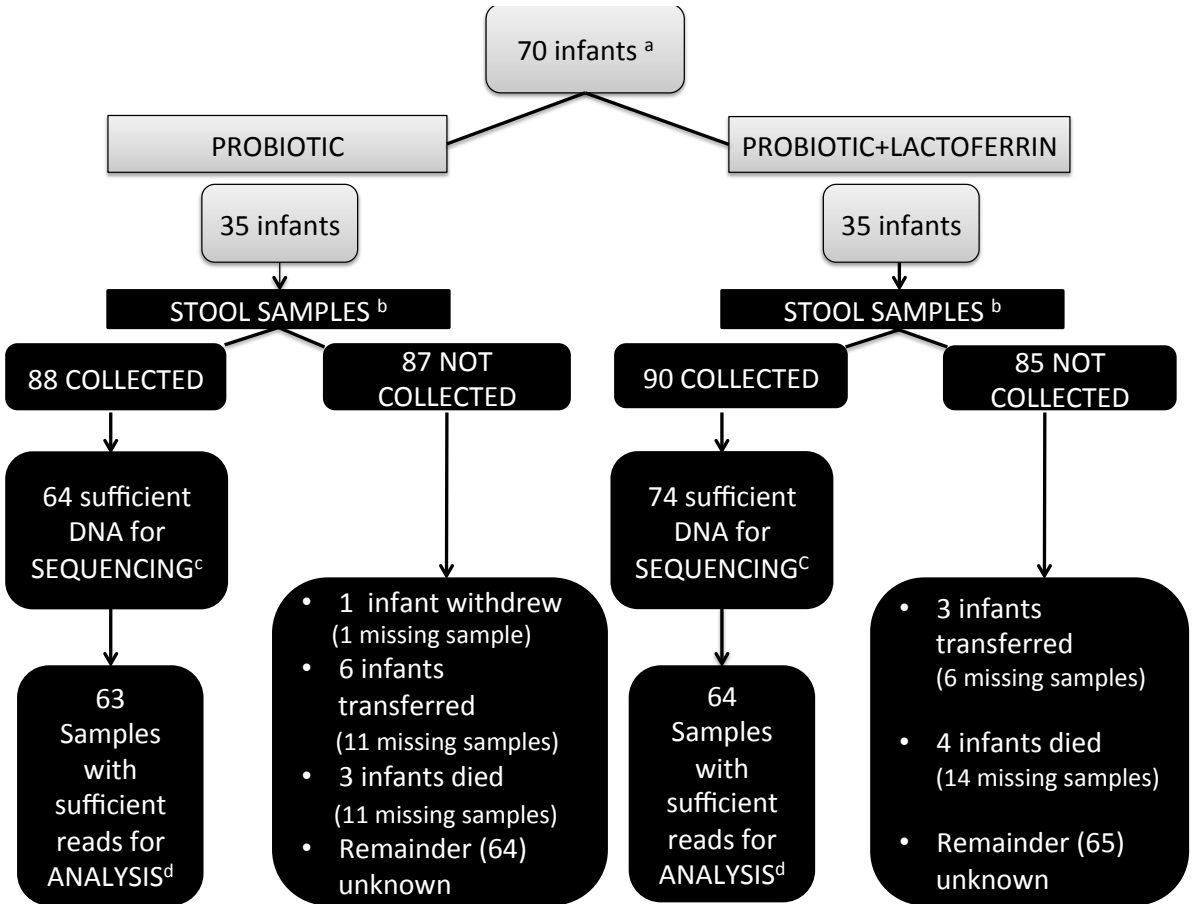

Figure 3.1: Stool sample summary

The figure outlines all unique stool samples that were analyzed. Dedicated research nurses collected all stool samples in a sterile fashion. A weekly stool sample was collected from each infant for the first month of life.

[a]: 70 infants were included in the study with a goal of collecting 350 stools total (5 samples per subject)

[b]: The objective was to collect 175 stool samples per treatment group however this was not feasible for numerous reasons.

[c]: Some of the samples did not contain enough DNA upon extraction to be sent for sequencing

[d]: Following sequencing on the Illumina HiSeq some samples were removed prior to analysis due to an insufficient number of reads

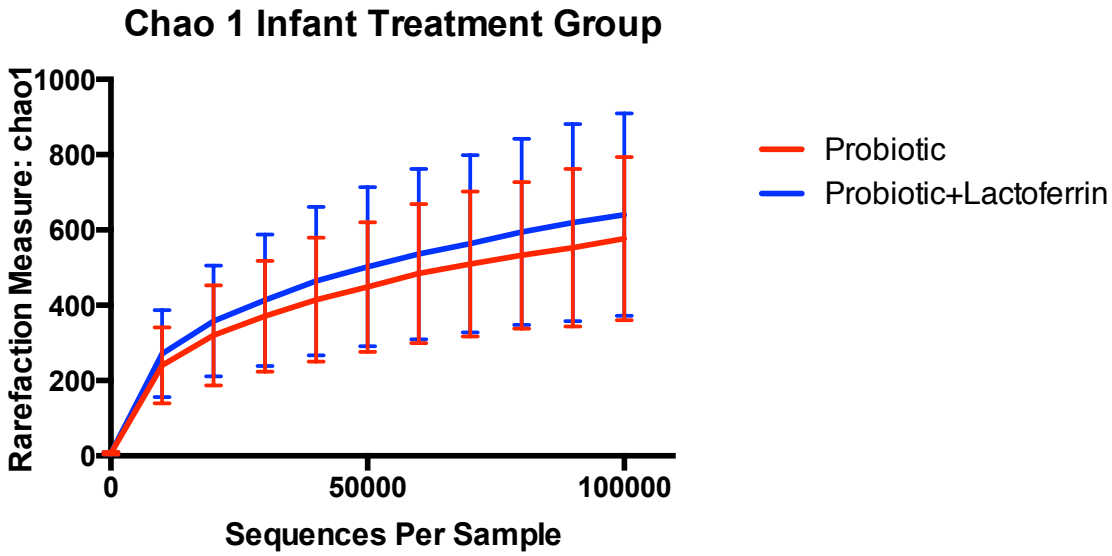

Figure 3.2: Rarefaction Curves

Rarefaction curves for our two treatment groups (Probiotic n=66 samples, Probiotic+Lactoferrin n=65 samples). Bars indicate SE.

3.3 The effect of sequence run and treatment group on phylogenetic relatedness using principal coordinate analysis (PCoA)

UniFrac distance evaluated the phylogenetic relatedness between OTUs and was visualized using PCoA (Lozupone and Knight, 2005). In addition, weighted UniFrac distance took into account the relative abundance of each OTU. The infant stool samples were analyzed in two different sequence runs entitled AS13 and AS27, and as such I wanted to explore if this influenced my results. Principal coordinate analysis (PCoA) plots showed a distinct separation as a function of the sequencing run based on unweighted UniFrac, but this separation disappeared with the weighted UniFrac (Figure 3.3 a,b), implying that only a few low abundant bacterial taxa are driving the separation. The Bray-Curtis dissimilarity (Bray, 1957) is another measure used to compare the bacterial composition and relatedness of 2 groups. The separation based on sequencing run seen using UniFrac distance was absent when the samples are analyzed using Bray-Curtis (Figure 3.4a). The observed separation did not significantly affect our results; moreover, in our metagenomSeq analysis we analyzed sequence run as a covariate. Using these two measures of relatedness I also explored whether the addition of lactoferrin to probiotic supplementation influenced bacterial similarity. Treatment group (P versus PL) did not show any separation on PoCA plots using the UniFrac distance (Figure 3.3c,d) or the Bray-Curtis dissimilarity (Figure 3.4b).

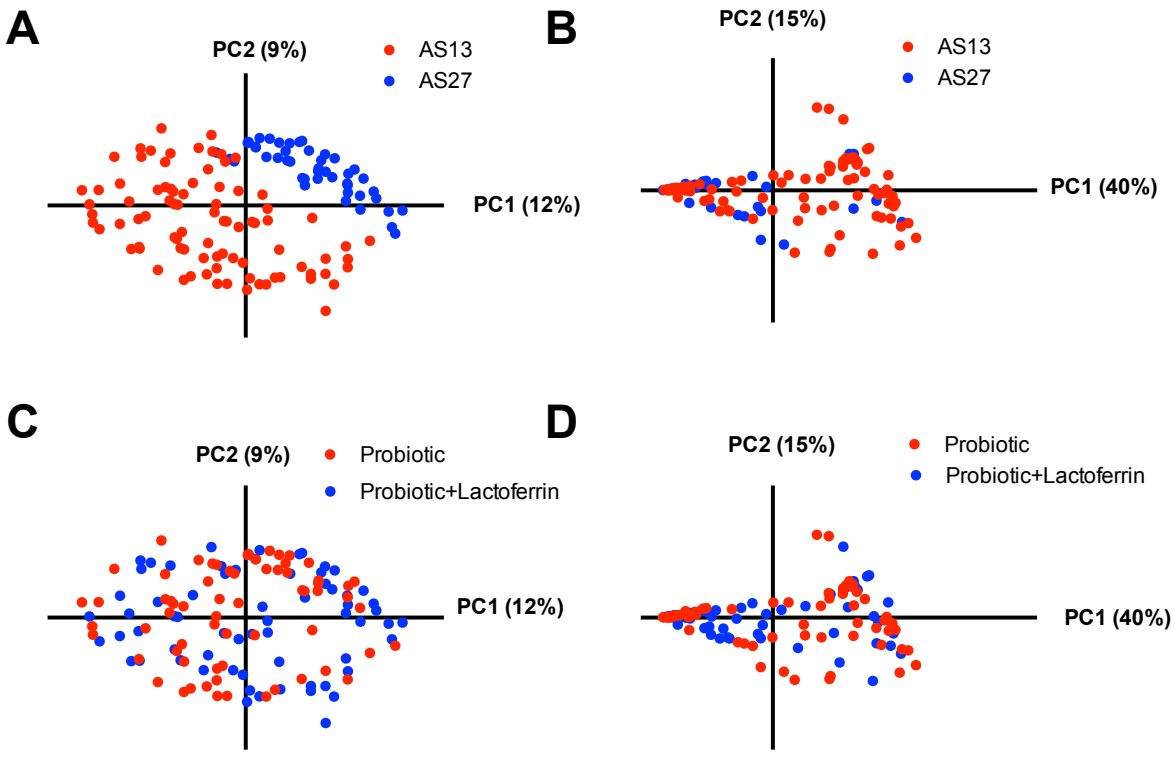

Figure 3.3: Principle Coordinate Analysis (PCoA) plots based on Unweighted UniFrac (A,C) and Weighted UniFrac distance (B,D), and colored by the sequencing run (A,B) or infant treatment group (C,D)

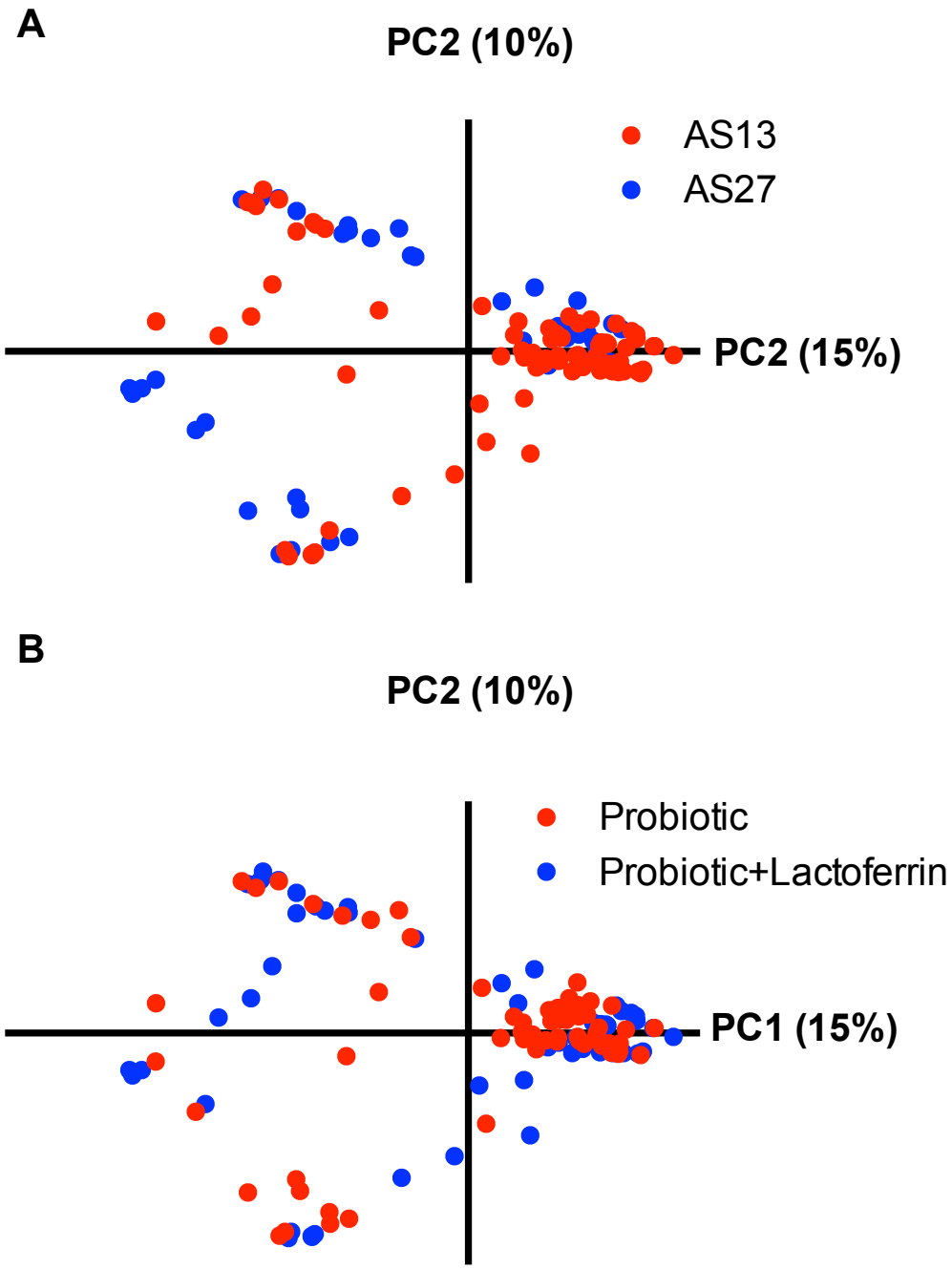

Figure 3.4: Bray-Curtis Plots of Sequence run (A) and Infant treatment group (B)

3.4 Alpha Diversity is the same between treatment groups

Intestinal bacterial diversity has been shown to be a marker of good health; conversely, a state of decreased bacterial gut diversity has been associated with serious illness (Zaborin et al., 2014). Furthermore, animal models have shown a trend towards increased bacterial diversity in piglets receiving lactoferrin (Hu et al., 2012). Based on this observation, we hypothesized that the addition of lactoferrin to probiotic supplementation would increase bacterial diversity. The chao 1 is a measure of bacterial richness while the Shannon index measures bacterial diversity. Richness describes the number of different bacterial taxa within a sample, while diversity accounts for the relative abundance and looks at how evenly distributed the taxa are. For example a high diversity score implies that all the different bacterial taxa are present in similar quantities. However, neither the chao 1 nor the Shannon ecological indexes were found to be increased in infants receiving PL as compared to infants receiving P (Figure 3.5). Additionally no difference in diversity was observed when the samples were categorized by week of collection (Figures 3.6-3.7). In doing so we introduce a bias by assuming that richness scores at each week were independent from one another. However if the same baby had been sampled repeatedly (stools collected from week 1-week 5) the chao 1 scores would not independent. That said, on average we only had 2 samples per baby, and due to clinical research constraints the weeks differed between infants (e.g. infant 1 sampled at week 1 and 2, and infant 2 sampled at week 3 and 5) and we therefore pooled samples from each week together.

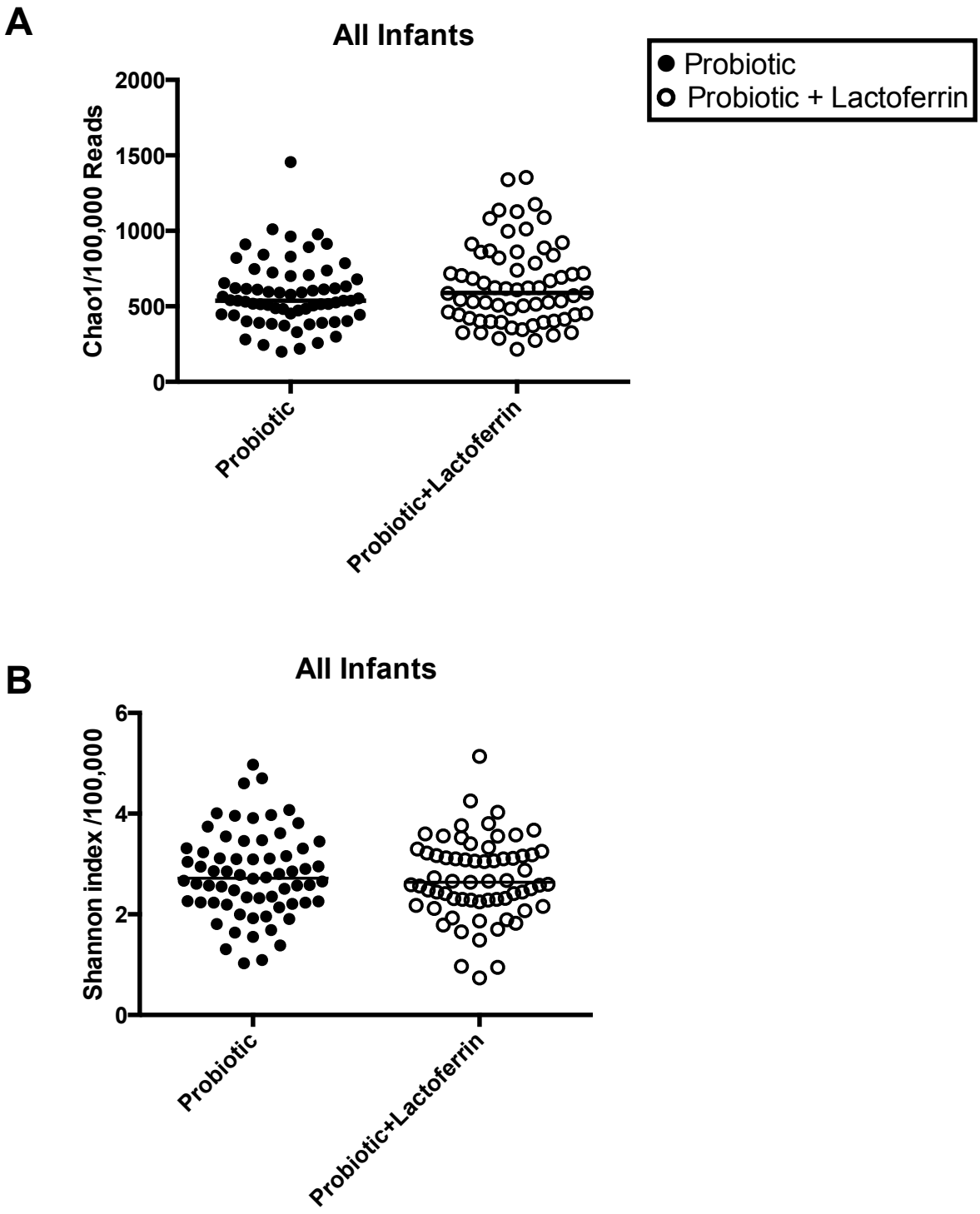

Figure 3.5: Alpha diversity of treatment groups

No significant difference is observed in the alpha diversity of each treatment group (probiotic, n=66 versus probiotic+lactoferrin, n=65) using 2 different ecological measures A: Chao 1 score and B: Shannon index.

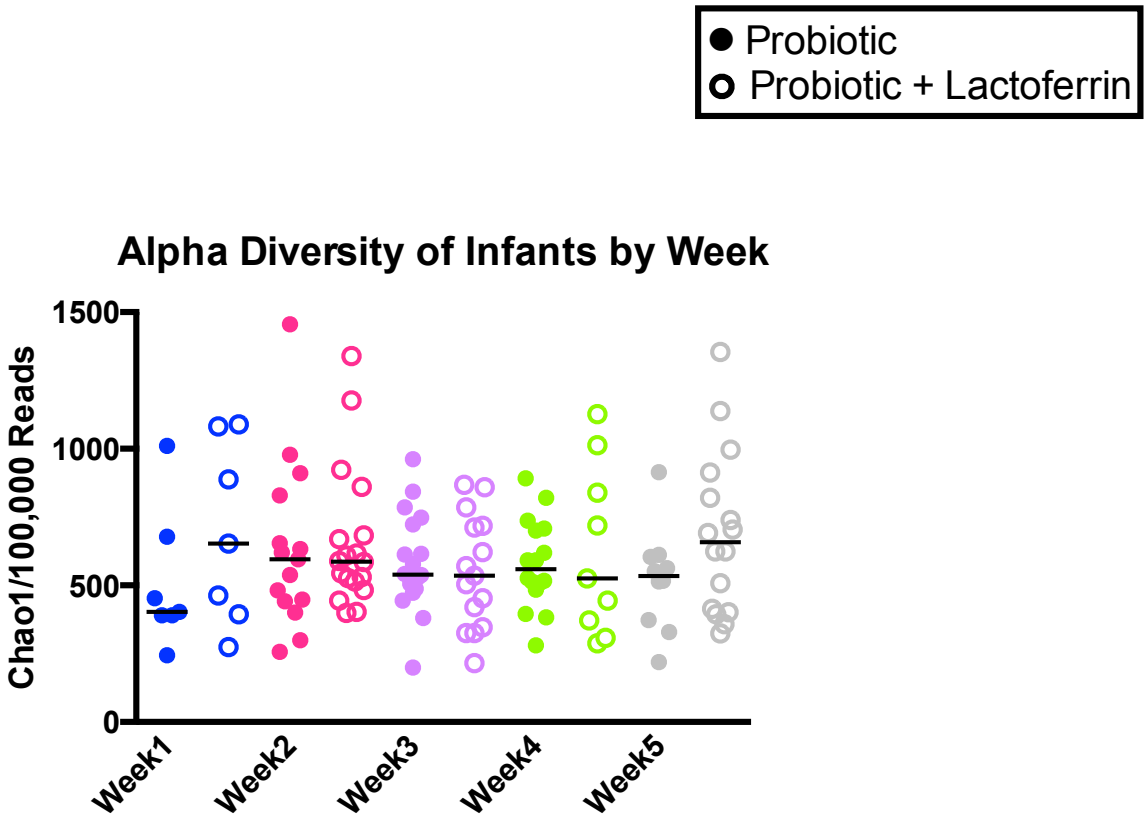

Figure 3.6: Chao 1 score by week Chao 1 score was calculated per treatment group (probiotic versus probiotic + lactoferrin) for each week of life that samples were collected. None of the results were found to be statistically significant using a Mann-Whitney test for each week and Kruskal-Wallis test with Dunn's multiple comparison's tests for all weeks.

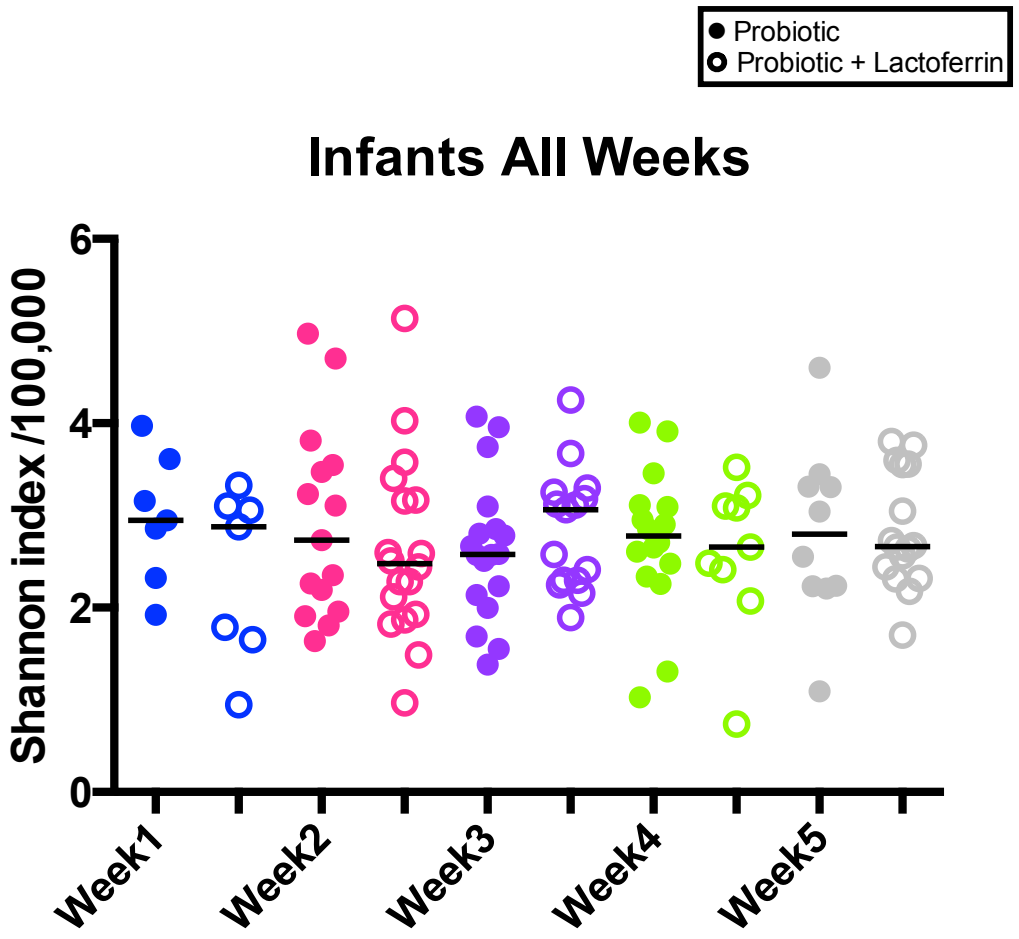

Figure 3.7: Shannon Index by week

Shannon index was calculated per treatment group (probiotic versus probiotic + lactoferrin) for each week of life that samples were collected. None of the results were found to be statistically significant using a Mann-Whitney test for each week and Kruskal-Wallis test with Dunn's multiple comparison's tests for all weeks

3.5 Gestational age at birth affects diversity

Many studies have demonstrated that the intestinal microbiome of premature babies differs significantly from that of their full term counterparts (Kosloske, 1994; Millar et al., 1996; Orrhage and Nord, 1999; Sakata et al., 1985; Schwiertz et al., 2003). The work of La Rosa et al. suggests that the development of the intestinal microbiome of premature infants follows a specific pattern of bacterial classes (La Rosa et al., 2014). They also argue that the pace of progression is influenced by gestational age at birth, with the slowest rate seen in infants with a greater degree of prematurity. I therefore analyzed my diversity scores by stratifying infant stool samples into 3 gestational age brackets (<26 weeks, 26-28 weeks, and >28 weeks). An increased richness (Chao1 index) was observed for infants born between 26-28 weeks gestation receiving PL as compared to those receiving P alone (Figure 3.8A) ($p=0.0482$ using ANOVA with Holm-Sidak's multiple comparison test). An increased diversity (using the Shannon index) was seen for babies of older gestational age brackets (Figure 3.8B). An increased diversity was observed for infants born at >28 weeks gestation receiving P compared to those <26 weeks receiving PL ($p=0.0401$). Increased diversity was also seen for the 26-28 week age bracket compared to <26 weeks gestation ($p=0.0081$). All statistical tests on the Shannon index were performed using the Kruskal-Wallis test with a Dunn's multiple comparison test.

3.6 Clinical factors appear to have little impact on overall diversity

In order to assess the combined impact of age at time of stool collection as well as degree of prematurity on diversity, samples were stratified into 3 age categories (<30 weeks, 30-33 weeks, >33 weeks) based on the post-conceptual age at the time of stool collection. Post-conceptual age was calculated by adding the gestational age of the infant at birth in weeks to the age in weeks of the infant at the time of stool collection. For example if a baby was born

at 25 weeks and the sample was taken at day of life 14 the post-conceptual age would be 27 weeks. No statistically significant difference was observed in richness or diversity between post-conceptual age groups using both the chao1 and Shannon indices (Figure 3.9A-B).

I also explored the role of other modifiable clinical factors such as type of milk ingested, delivery type, and antibiotic exposure, all of which have been shown to impact microbial diversity (Azad et al., 2013; Bezirtzoglou et al., 2011; Biasucci et al., 2008; Dardas et al., 2014; Dominguez-Bello et al., 2010; Fouhy et al., 2012; Greenwood et al., 2014; Jacquot et al., 2011; Penders et al., 2006; Tanaka et al., 2009). My results showed that the diversity or richness scores were not influenced by milk ingested at the start of the study (breast milk, formula or a combination) (Figures 3.9 C-D), or exposure to antibiotics (Figure 3.10). Note that there were no infants in the PL group who received solely formula. In general we had very high rates (>75% in both treatment arms) of babies receiving breast milk (Table 3.1). There was no difference in bacterial species richness or diversity between infants born via vaginal and C-section except for stool samples taken from the second week of life (Figure 3.9 E). Using the chao 1, samples from week 2 of life for babies born via C-section showed greater bacterial richness ($p=0.0035$) in the PL group, but not the P group. These results were not replicated in samples from other weeks of life (data not shown).

In order to account for the many covariables in our infant study that could influence alpha diversity and richness, we re-analyzed 5 measures of diversity or richness (Shannon index, observed species, chao1, phylogenetic diversity (PD) whole tree, and goods coverage) using linear mixed models implemented in MetagenomeSeq. The following clinical factors were used as covariates; gestational age bracket (<26, 26-28, >28 weeks gestation), post-conceptual age bracket (<30 weeks, 30-33weeks, >33 weeks), delivery method (vaginal/C-

section), milk ingested (breast milk/formula), and sequence run. None of the diversity/richness scores were found to be statistically significant between treatment groups (data not shown).

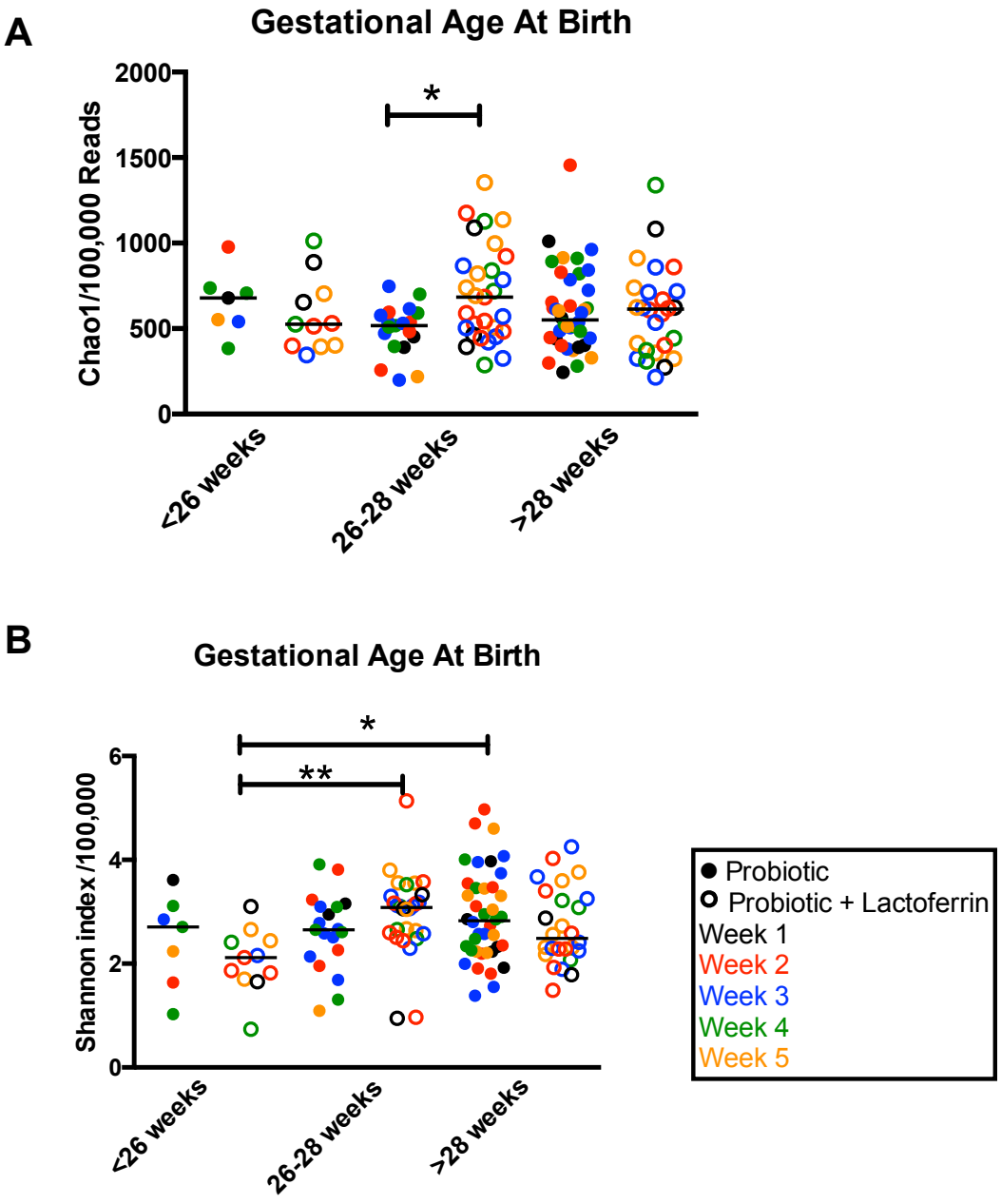

Figure 3.8: Diversity scores for gestational age

A: Chao 1

B: Shannon index

Colors indicate the week of life at which the stool samples were collected

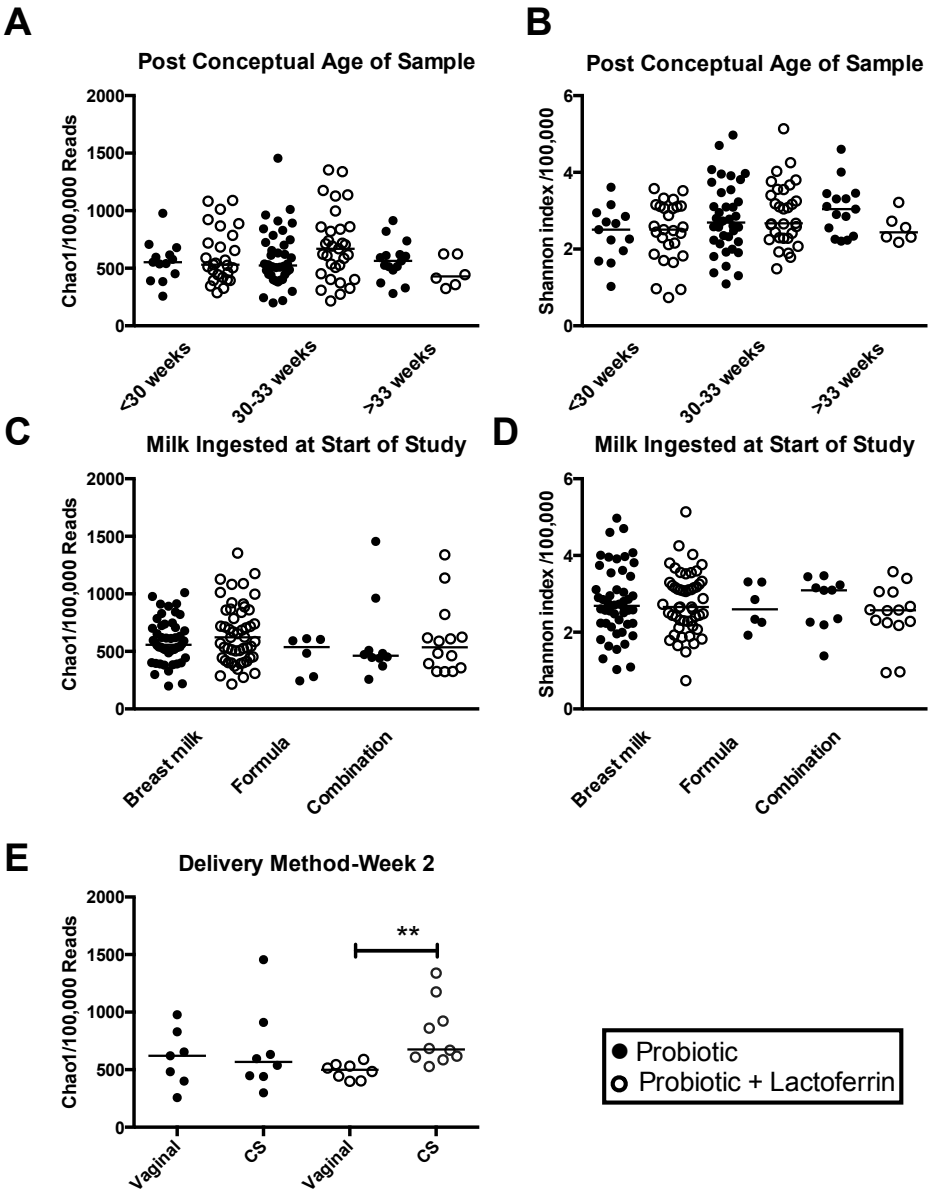

Figure 3.9: Diversity scores for metadata

A: Chao1 as a function of post conceptual age

B: Shannon index as a function of post conceptual age

C: Chao 1 as a function of milk ingested

D: Shannon index as a function of milk ingested

E: Chao1 as a function of delivery method

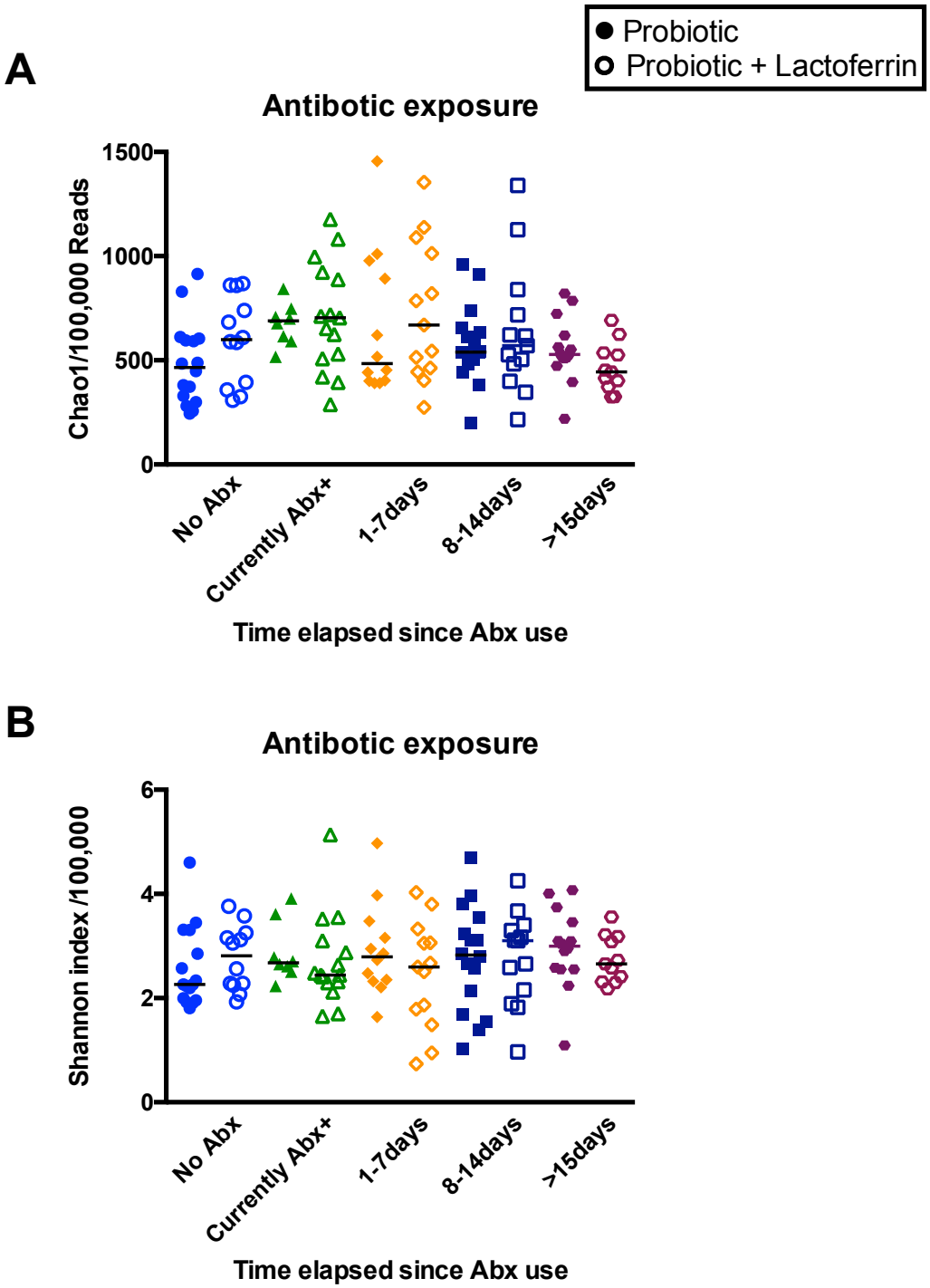

Figure 3.10: Alpha Diversity based on Antibiotic (Abx) Exposure A: Chao 1 and Shannon index (B) did not show a difference in richness or diversity between treatment groups based on whether the infants had previously been exposed to antibiotics or not, or were on antibiotics at the time of stool collection (Currently Abx+).

3.7 Alpha Diversity increases over time for infants born between 26-28 weeks gestation

Since I previously showed that gestational age influenced the richness and diversity scores of premature infants, I determined whether gestational age affected the rate of change of diversity over time. The chao 1 and Shannon index of all infants with 2 or more samples were plotted over time based on the gestational age bracket at birth (>26 weeks, 26-28 weeks and >28 weeks) (Figure 3.11). Appendix III displays the richness of all patient samples in a single figure based on gestational age bracket at birth. These results were subsequently merged together into a single slope (Figure 3.12A,C) and then corrected using week 1 results as a baseline (3.12 B,D). Doing so resulted in a slope that was greater than zero for infants born in the 26-28 week gestational age bracket using a comparison of fits test ($p=0.0385$ and $p=0.0068$) suggesting that infants in the 26-28 week age bracket increased their bacterial diversity over time at a faster rate than the other age brackets.

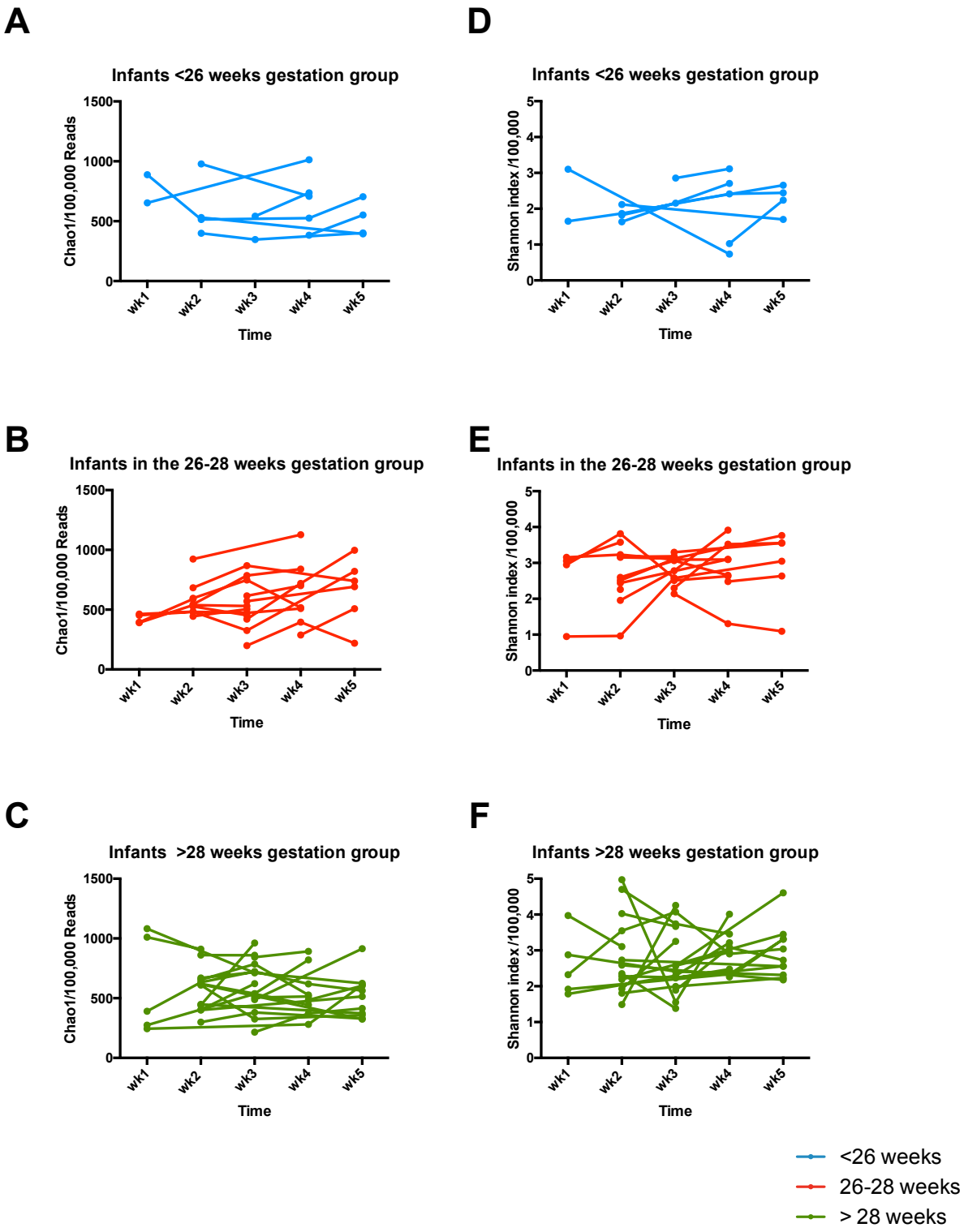

Figure 3.11: Alpha diversity over time per week of gestation

Figures A,B,C show alpha diversity using a Chao1 score. Figures D,E,F depict results with the Shannon Diversity Index.

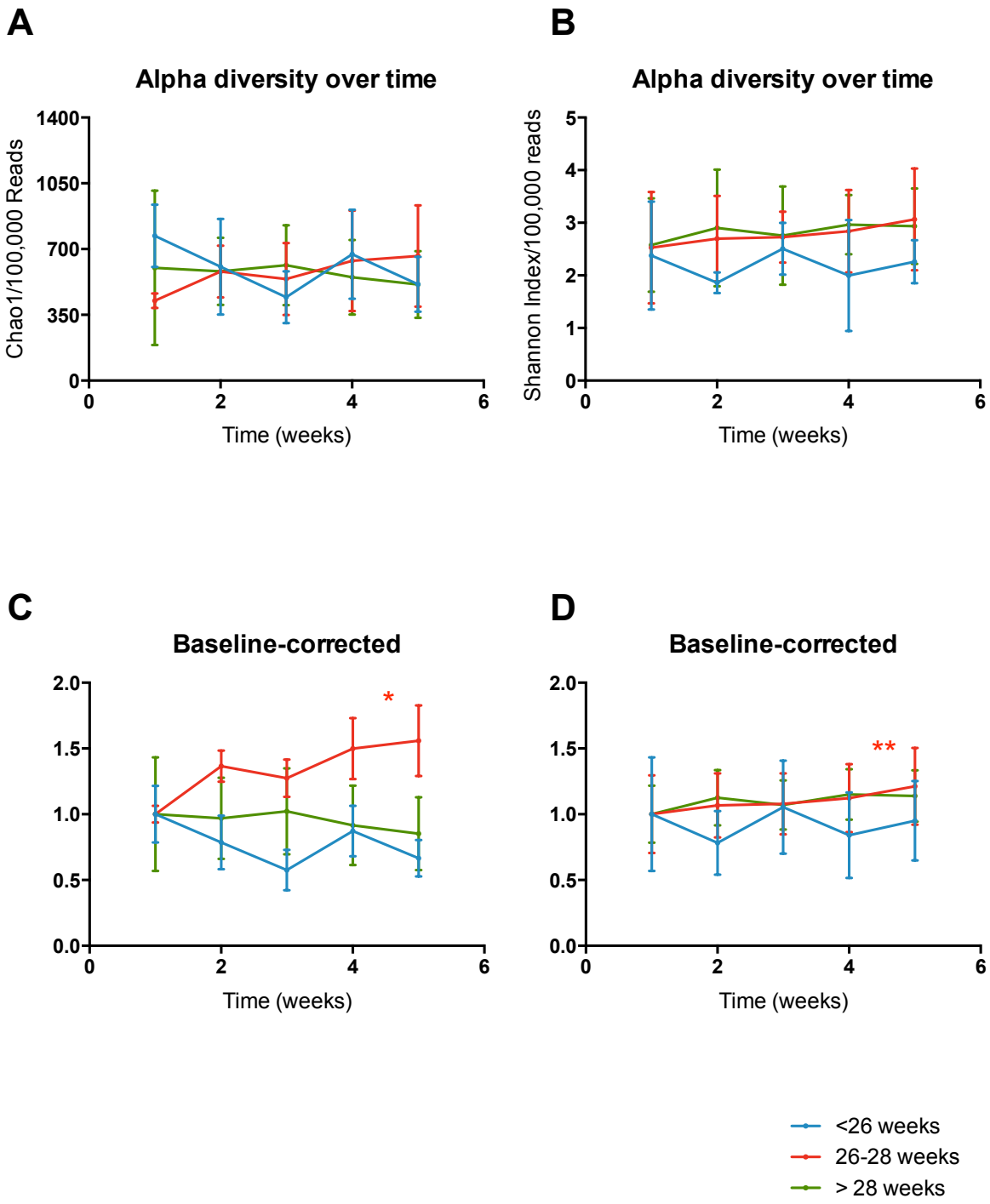

Figure 3.12: Alpha diversity over time per week of gestation. All infants were categorized into 3 age brackets based on week of gestation at birth (<26 weeks, 26-28 weeks completed gestation, >28 weeks). Chao1 score (A,B) and Shannon index C,D) were used to depict alpha diversity. A,C: Samples from the same gestational age bracket were merged together. B,D: Results were corrected using week 1 as a baseline. Slopes for infants born between 26-28 weeks gestation were found to be different than zero using a comparison of fits test (* $p<0.05$, ** $p<0.01$)

3.8 The relative abundance of probiotic strains in infant stool samples is approximately 3%

FloraBABY^{TM} is a probiotic mixture composed of the *Lactobacillus* and *Bifidobacterium* genera, and includes 5 different bacterial species (Figure 2.1). To confirm that probiotic strains can be identified in the stools of premature infants supplemented with FloraBABY^{TM}, I sought to determine the presence of these strains in our samples. As expected, our analysis revealed the presence of Lactobacillus and Bifidobacterium in the infant stool microbiota representing up to 3.23% of the microbial community (Figure 3.13). However, while three of the 5 species in FloraBABY^{TM} were readily detected in the stool samples, namely *B. bifidum*, *B. breve* and *B. longum,* the two other expected species, *L. rhamnosus* and *B. infantis*, could not be identified.

It is possible that OTU 484304 (corresponding to *Bifidobacterium*; s_) may in fact have represented *B. infantis* and that the OTU 806179 (*Lactobacillus*; s) may have represented *LGG*. Among the probiotic strains *Lactobacillus* OTU 806179 was most abundant with 0.93% (+/-4.8%) abundance in the P group and 1.25% (+/- 3.2%) in the PL group (Figure 3.13). The least abundant among the probiotic strains was *Bifidobacterium bifidum* in the P and PL groups ($3.6x10^{-5}$ % +/- $1.6x10^{-4}$ % and $1.9x10^{-5}$ % +/-$8.5x10^{-5}$ % respectively). In all, the probiotic strains accounted for 2.9% and 3.2% of the total relative abundance. Unfortunately, this number may not have been accurate since it has previously been shown that Bifidobacterium can be difficult to amplify by PCR and thereby to detect with16S rDNA-based sequencing approaches as was performed here (Gill et al., 2006).

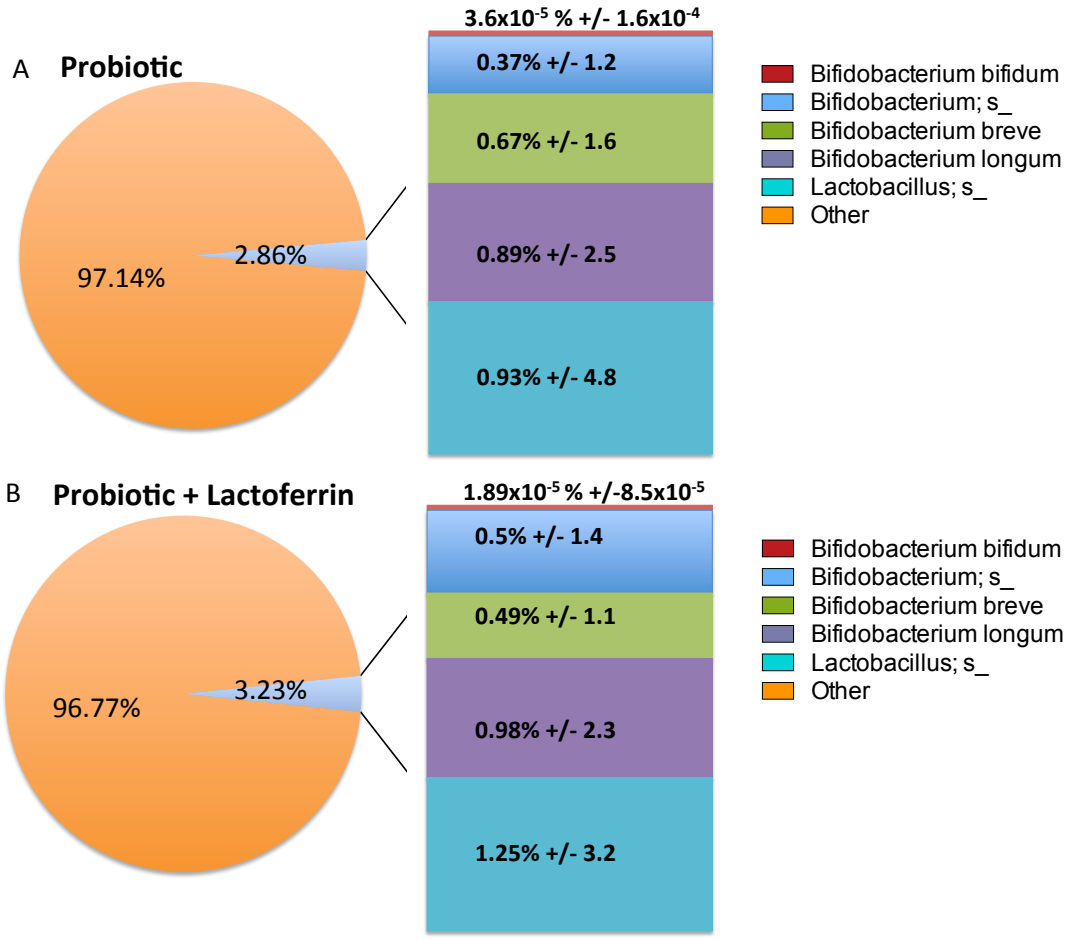

Figure 3.13: Relative abundance of probiotic strains

Overall the probiotic strains accounted for approximately 3% of the relative abundance of bacteria found in the stool samples of both treatment groups. A: Probiotic alone B: Probiotic+Lactoferrin

3.9 Firmicutes and Proteobacteria were the most abundant phyla in premature infant stool samples

Analysis of the microbiota composition at the phylum level revealed a preponderance of the Firmicutes and Proteobacteria phyla in both treatment groups. The Firmicutes was the most abundant phylum in the P group (51.7% +/-38%), while the Proteobacteria was the most abundant phylum in the PL group (51.3% +/- 36.9%) (Figure 3.14 A,B). Bacteroidetes, Actinobacteria, and Fusobacteria were also present, but represented less than 4% of the total taxa identified. A detailed list of the relative abundance of all taxa present in our samples down to the lowest possible level is depicted in Figures 3.14 C-D and in Table 3.2. Taxa with less than 0.1% relative abundance were grouped together in the "other" category. The family *Enterobacteriaceae*, which includes many pathogenic bacteria such as *Shigella, E. coli, Salmonella*, and *Klebsiella* was by far the most abundant taxa in both groups (30.3 ± 33.1% and 44.2 ± 36.1% in the P and PL group respectively) (Figure 3.14 C,D and Table 3.2). The second, third, and fourth highest taxa were *Clostridium perfringens* (8.3± 18.4% and 7.3 ±15.5%), *Veillonella dispar (*7.61% ±17.88%), and OTU 928538 (*Staphylococcus* (species unknown)); (7.2 ±15.8% and 7.1 ± 13%). Once again these are known pathogens and *Staphylococcus* and specifically *S. aureus* (relative abundance 1.1 ±4.5% in P group) is an especially frequent pathogen in the NICU setting (Vergnano et al., 2011). It should be noted that these potentially pathogenic taxa displayed a relative abundance with a standard deviation that was larger than the mean. This implies that *S. aureus* was only detected in a subset of the samples or perhaps each infant only has a short and transient period of colonization with *S. aureus.*

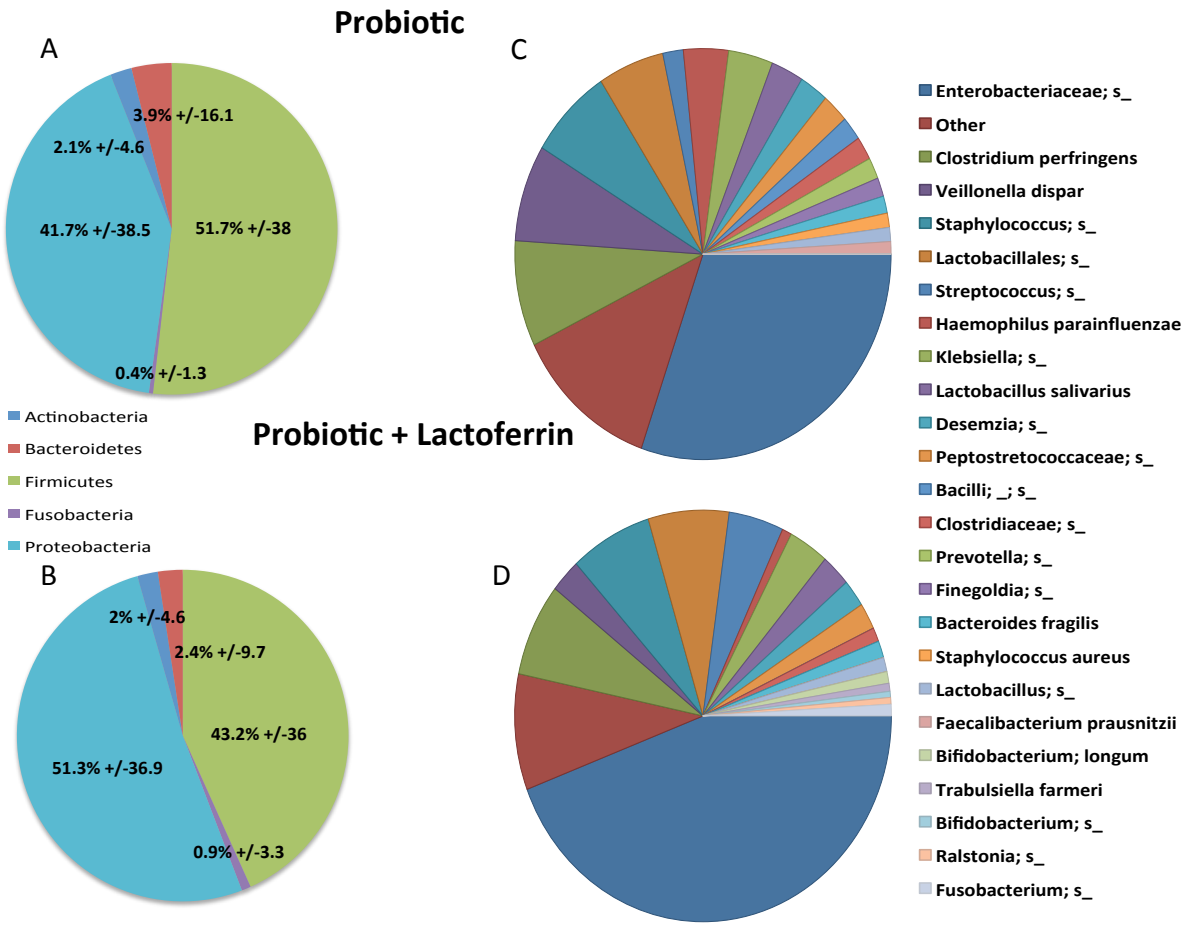

Figure 3.14: Relative Abundance of bacteria at the phylum level (A,B) and lowest possible taxa (C,D) in the probiotic and probiotic+lactoferrin treatment groups.

A

	Mean %	SD %
Enterobacteriaceae; s_	30.32	33.11
Other	12.40	15.30
Clostridium perfringens	8.32	18.39
Veillonella dispar	7.61	17.88
Staphylococcus; s_	7.23	15.82
Lactobacillales; s_	5.68	11.96
Haemophilus parainfluenzae	3.86	14.48
Klebsiella; s_	3.83	12.56
Lactobacillus salivarius	2.79	10.18
Desemzia; s_	2.48	6.14
Peptostretococcaceae; s_	2.30	7.54
Bacilli; _; s_	1.88	9.26
Clostridiaceae; s_	1.84	8.42
Streptococcus; s_	1.76	4.56
Prevotella; s_	1.67	10.85
Finegoldia; s_	1.47	10.33
Bacteroides fragilis	1.35	10.60
Staphylococcus aureus	1.13	4.54
Lactobacillus; s_	1.10	4.83
Faecalibacterium prausnitzii	0.97	5.69

B

	Mean %	SD %
Enterobacteriaceae; s_	44.21	36.11
Other	9.10	11.30
Clostridium perfringens	7.33	15.46
Staphylococcus; s_	7.06	12.99
Lactobacillales; s_	6.93	14.70
Streptococcus; s_	4.72	14.75
Klebsiella; s_	3.46	11.79
Veillonella dispar	2.63	11.02
Lactobacillus salivarius	2.54	9.55
Desemzia; s_	2.16	6.41
Peptostretococcaceae; s_	2.08	7.73
Bacteroides fragilis	1.33	9.28
Lactobacillus; s_	1.12	3.21
Clostridiaceae; s_	1.10	2.54
Bifidobacterium; longum	0.93	2.34
Fusobacterium; s_	0.91	3.34
Haemophilus parainfluenzae	0.82	5.45
Trabulsiella farmeri	0.63	5.02
Ralstonia; s_	0.53	2.12
Bifidobacterium; s_	0.48	1.38

Table 3.2: Relative abundance of bacteria
Lowest possible taxa identified in the samples from the Probiotic (A) and Probiotic+Lactoferrin (B) treatment groups. Note that "s_" refers to species unknown. All analysis was performed at the OTU level.

3.10 In the first month of life Gammaproteobacteria is consistently the most abundant class

La Rosa et al. claimed that the colonization of the intestinal microbiome of premature babies follows a fixed progression of bacterial classes (La Rosa et al., 2014). In order to investigate for a similar pattern in our infant cohort, a subset of patients for whom we had 4-5 consecutive weekly samples, were analyzed separately over time at the class level (Figure 3.15). The x-axis depicted both the day of life as well as the post-conceptual age of the patient at the time of stool collection. Days where the infants received antibiotics or were NPO were also detailed in Figure 3.15. Patient 18 was a C-section delivered male born at $29^{5/7}$ weeks of gestation who was breast-fed and randomized to the P group. Patient 19 was a female, vaginally born at $28^{5/7}$ weeks of gestation, who received both breast milk and formula and was also in the P group. Patient 22 was a male vaginally born at $25^{5/7}$ weeks of gestation, who received breast milk, and was in the PL group. Lastly patient 58 was a male vaginally born at $27^{1/7}$ weeks of gestation who received both breast milk and formula and was also randomized to the PL group. Gammaproteobacteria was consistently the bacterial class with the highest relative abundance. A trend was observed with Bacilli present early on followed by Gammaproteobacteria and then lastly Clostridia especially for patients 18 and 19.

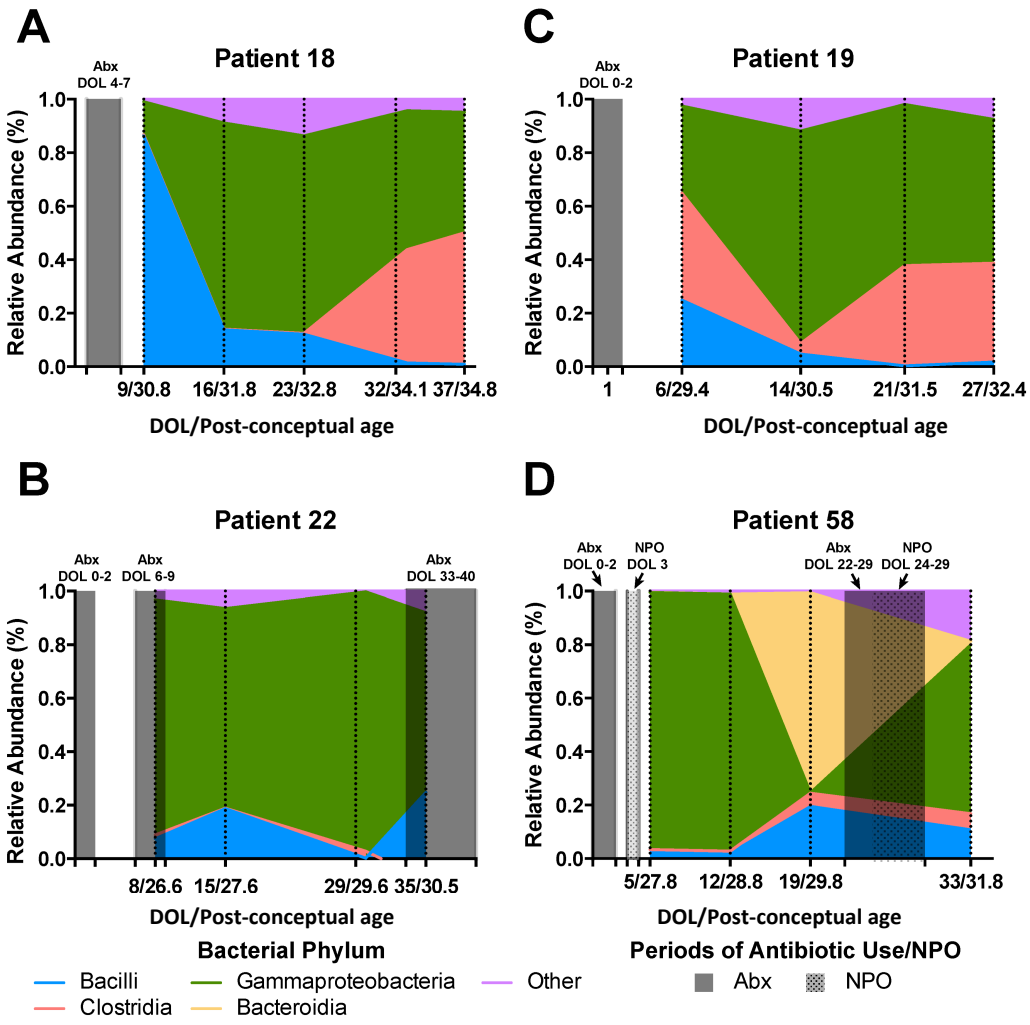

Figure 3.15: Bacterial relative abundance over time at the class taxa level

A subset of patients with 4-5 stool samples each was analyzed. X-axis represents time DOL=day of life, Abx=antibiotics, NPO=nil per os (not fed).

3.11 Clostridiales is more abundant in infants receiving probiotic+lactoferrin

To further study the effect of probiotic and lactoferrin supplementation on the microbiota composition, I sought to identify the taxa exhibiting differential relative abundance between infants receiving probiotic alone versus those receiving probiotic with the addition of lactoferrin using the LEfSe algorithm (Segata et al., 2011). LEfSe performs a non-parametric factorial Kruskal-Wallis (KW) sum-rank test and Linear Discriminant Analysis (LDA) to determine which taxa are statistically different among the treatment groups. Six taxa were found to be differentially abundant in the PL group and 2 taxa were more abundant in the P group (Figure 3.16). Of the 6 taxa, 4 belonged to the Clostridiales order with an LDA score of 2-3 (log 10 scale). Of these, 3 belonged to the *Peptostreptococcaceae* family, 2 of which belonged to the *Peptostreptococcus* genus, a known component of the human oral microbiome (Zarco et al., 2012). The treatment groups were further subdivided and analyzed by mode of delivery, gestational age, post conceptual age, prior antibiotic use, and week of stool collection, however no differences in differential relative abundance were seen (data not shown).

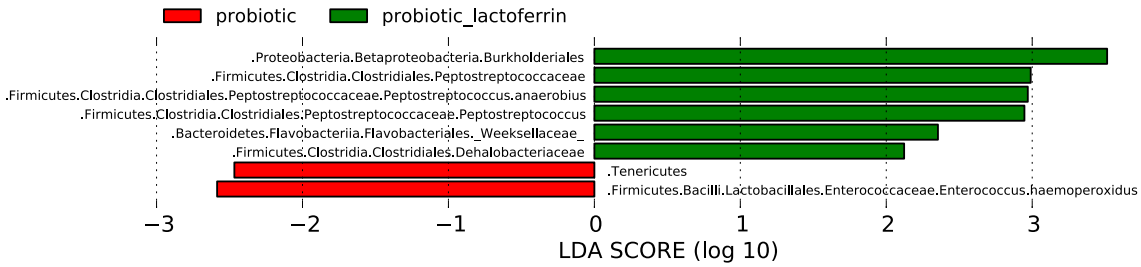

Figure 3.16: Differential abundance of taxa between treatment groups

Histogram of the linear discriminant analysis (LDA) scores are shown for taxa that are differentially abundant between the probiotic alone group (red) versus the probiotic+lactoferrin group (green).

3.12 Enterobacteriacea is more abundant in the PL group

Given the complexity of the clinical data associated with our infant cohort we thought to analyze the microbiota data using a statistical approach that controls for confounding factors - metagenomeSeq R package (Figure 3.17). The following covariates were taken into account; sequence run, gestational age bracket (<26, 26-28, >28 weeks gestation), post-conceptual age bracket (<30 weeks, 30-33weeks, >33 weeks), type of milk ingested, and mode of delivery. Results were considered significant at a fold change of ≥ 2 and a p-value <0.01. The *Enterobacteriaceae* family, represented by 3 different OTUs, was more abundant in the PL group. Note that there were occasionally more than one OTU that represented the same taxa. In contrast to the results seen using LEfSe, *Peptostreptococcus* was identified as more abundant in the P group. Furthermore many taxa belonging to the Clostridiales order such as *Ruminococcaceae* (family) and *Veillonella* (genus) were identified as more abundant in the probiotic alone group as compared to probiotic with lactoferrin. Interestingly, OTUs corresponding to Ruminococcaceae (which are represented by more than one OTU) were differentially abundant in both groups, but more OTUs were found in the P group as compared to the PL group (4 OTUs vs 1 OTU). Other significant findings were the increase of *Staphylococcus aureus, Haemophilus parainfluenzae, Fusobacterium* and *Faecalibacterium prausnitzii* in the P group.

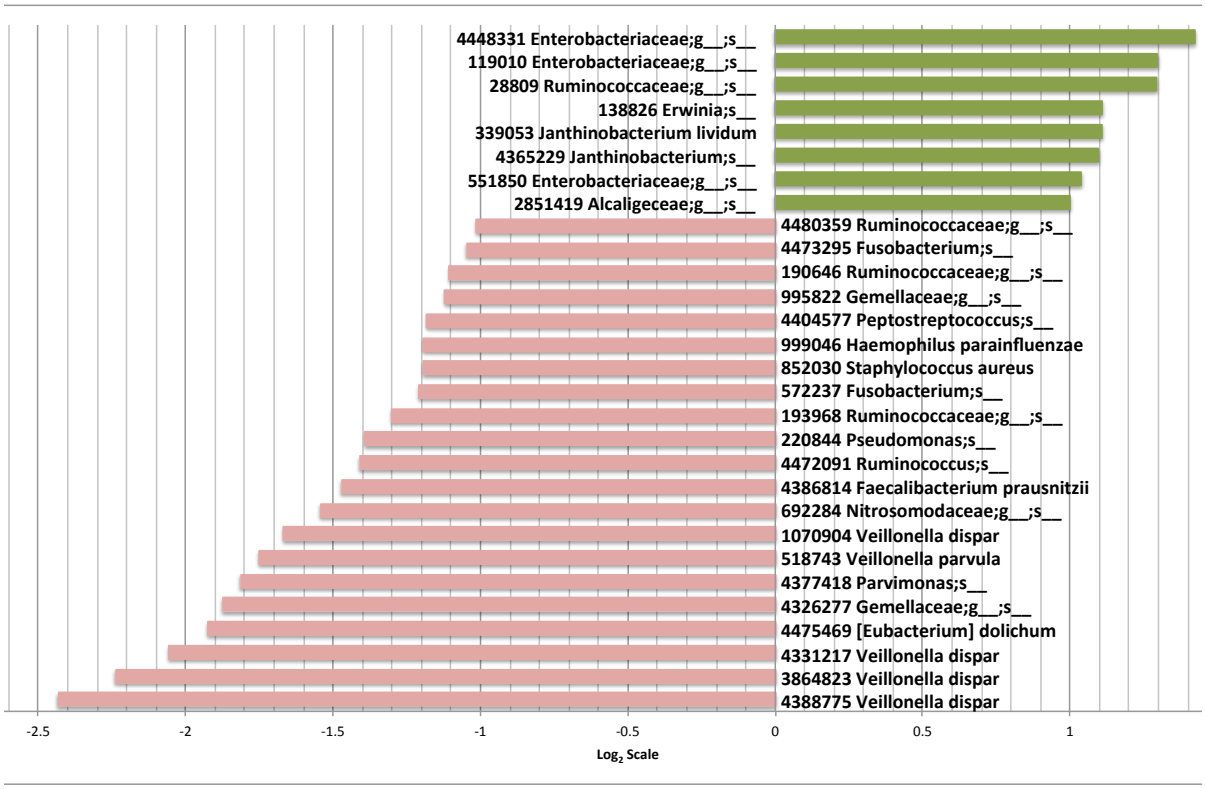

Figure 3.17: Bacteria found to be differentially abundant between treatment groups

MetagenomeSeq was used to identify differentially abundant OTUs using a $\log_2$ scale of fold change. Taxa that are more abundant in the lactoferrin+probiotic group are highlighted in green and those that are more abundant in the probiotic group alone are highlighted in red. OTUs shown had at least a 2-fold change and were significant at a p-value ≤ 0.01.

3.13 Twin stool samples cluster together despite treatment group

Studying the intestinal microbiome of premature twins and triplets may provide additional information on the importance of the host genetic versus environmental factors on the microbiota composition. Eighteen infants belonging to one of the 8 twin or triplet groups in our cohort were analyzed separately. Table 3.3 outlines the demographic information pertaining to each subject. Beta diversity testing with UniFrac distance based PCoA plots showed that twins and triplets from the same family tended to cluster together regardless of whether they received P or PL (Figure 3.18).

Patient	Family ID	Delivery Mode	Gestation (weeks)	Birth weight (g)	Sex	Chorionicity	Milk Ingested	Treatment Group	Samples Collected	Samples Analyzed
11	1	Cesarian	29	1240	M	DT	Both	Probiotic	3	2
12	1	Cesarian	29	1250	M	DT	Both	Probiotic_Lactoferrin	3	2
13	1	Cesarian	29	1195	M	DT	Both	Probiotic_Lactoferrin	4	3
16	2	Cesarian	29	1310	M	TT	Breast	Probiotic_Lactoferrin	4	3
17	2	Cesarian	29	1470	F	TT	Both	Probiotic_Lactoferrin	1	0*
18	2	Cesarian	29	1310	M	TT	Breast	Probiotic	5	5
19	3	Vaginal	28	1105	F	DD	Both	Probiotic	4	4**
20	3	Vaginal	28	1160	F	DD	Both	Probiotic_Lactoferrin	2	2**
33	4	Vaginal	30	1390	M	DD	Breast	Probiotic	4	3**
34	4	Vaginal	30	1510	M	DD	Breast	Probiotic	3	3**
52	5	Cesarian	26	920	M	MM	Breast	Probiotic_Lactoferrin	4	2
53	5	Cesarian	26	970	M	MM	Breast	Probiotic_Lactoferrin	4	3
56	6	Cesarian	30	1350	M	DD	Both	Probiotic	3	3**
57	6	Cesarian	30	1600	M	DD	Both	Probiotic_Lactoferrin	1	1**
59	7	Cesarian	30	1400	M	DD	Formula	Probiotic	4	3
60	7	Cesarian	30	1020	F	DD	Formula	Probiotic	1	0**
72	8	Cesarian	29	1440	M	DD	Breast	Probiotic_Lactoferrin	2	0
73	8	Cesarian	29	1170	M	DD	Breast	Probiotic	3	2

Table 3.3: Demographics of twin and triplet patients. A total of 18 babies separated into 8 groups of multiples are depicted. Infants belonging to the same family are grouped by color
*Infant died
** Infant transferred to another center
DT: Dichorionic triamniotic
TT: Trichorionic triamniotic
DD: Dichorionic diamniotic
MM: Monochorionic monoamniotic

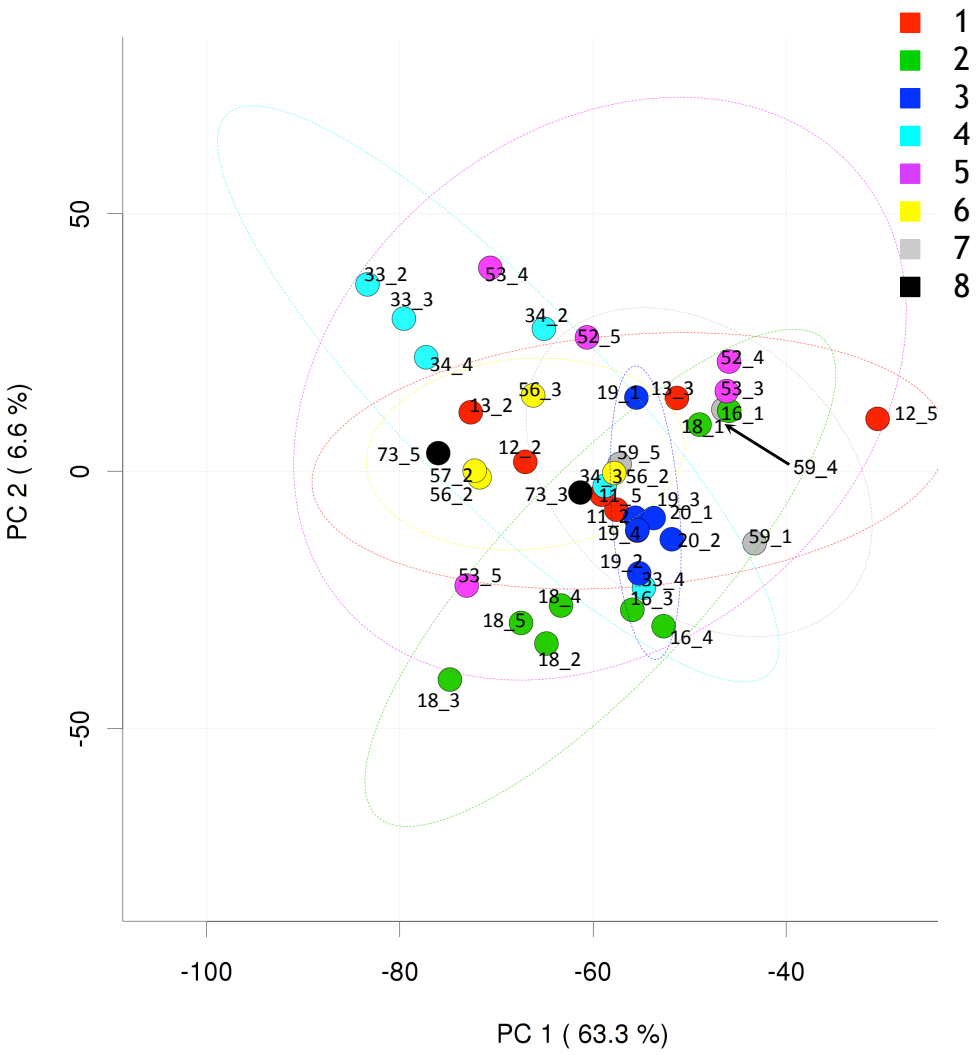

Figure 3.18: Principle Component Analysis (PCA) plots for Twins
PCA plot of 8 different twin/triplet sets colored by family performed in Metagenassist. The first digit indicates the twin/triplet patient identification number and the second number identifies the week of life at which the sample was taken for example sample18_3 refers to patient number 18 at the third week of life. Twin siblings are grouped together by color.

3.14 Sepsis patients

Late onset sepsis (occurring after the third day of life) is a serious problem in premature infants with high rates of morbidity and mortality. We wondered if we could identify changes in the intestinal microbiome prior to sepsis onset, and perhaps even isolate the causative organism by a concordant increase in relative abundance in the infant's intestinal microbiome. Sixteen cases of late onset sepsis were identified within our cohort. Blood cultures at the time of sepsis for the 6 infants were positive for *E. coli* (2 cases), *Klebsiella pneumonia* (2 cases, with 1 case simultaneously positive for *Enterobacter cloacae)*, and lastly *Staphylococcus epidermidis* (2 cases) (Table 3.4). Eleven of the 16 infants had stools collected prior to the septic episode and 6 of these stools could be analyzed. Importantly, members of the family *Enterobacteriaceae* (*E. coli, Shigella* etc*.)* could not be differentiated with our experimental approach at the 97% level in the Greengenes database, so we were unable to confirm the presence of *E. coli* in patients with positive blood cultures. Nevertheless, the 2 patients (36-4 and 74-1) with *E. coli* positive blood cultures clustered close together on PCoA (Figure 3.19). Similarly the patients with *S. epidermidis* positive blood cultures (25-3 and 41-2) also clustered together. Furthermore these patients with identical blood cultures were randomized to different treatment groups (Table 3.4). Analyzing the LEfSe (Figure 3.20) results for the sepsis patients it was striking that 6 of the differential taxa in the sepsis group belong to the Proteobacterium phyllum. In addition 2 of the taxa with LDA scores greater than 3 in the sepsis group belonged to the *Veillonella* genus. For this analysis presence of sepsis was used as the class and treatment group was used as the subclass.

PatientID-week	Δ Days	Treatment Group	Blood Culture
21-3	10	Probiotic	*Klebsiella pneumoniae*
25-3	7	Probiotic+Lactoferrin	*Staphylococcus epidermidis*
36-4	1	Probiotic+Lactoferrin	*E. coli*
41-2	5	Probiotic	*Staphylococcus epidermidis*
67-3	12	Probiotic+Lactoferrin	*Klebsiella pneumoniae, Enterobacter cloacae*
74-1	0	Probiotic	*E. coli*

Table 3.4: Sepsis cases among samples that were analyzed
Δ Days refers to the number of days between stool collection and positive blood culture

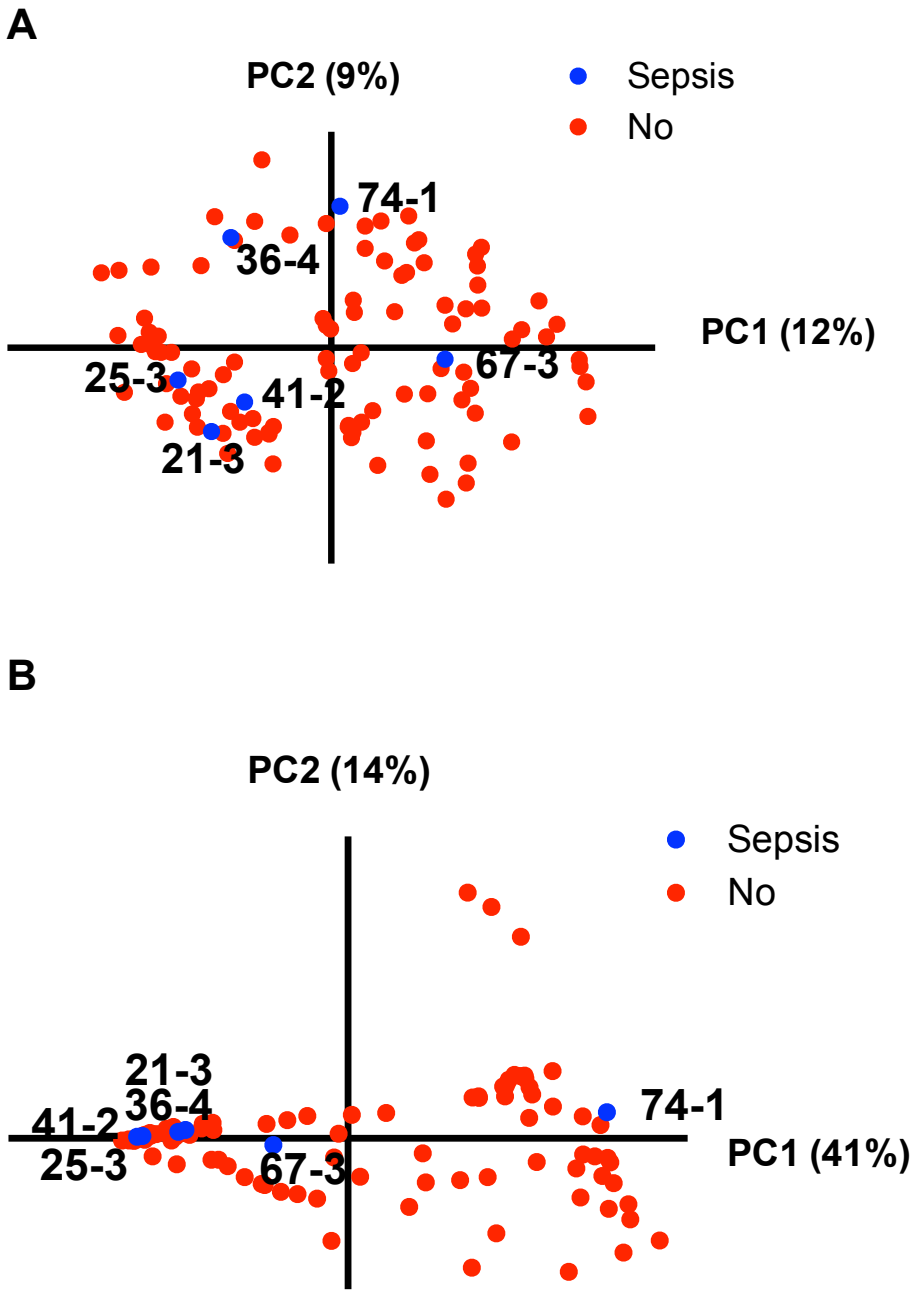

Figure 3.19: PCoA plot of stool samples with and without sepsis based on UniFrac distance. Blue dots identify sepsis cases and red dots are non-sepsis cases (a: unweighted, b: weighted UniFrac)

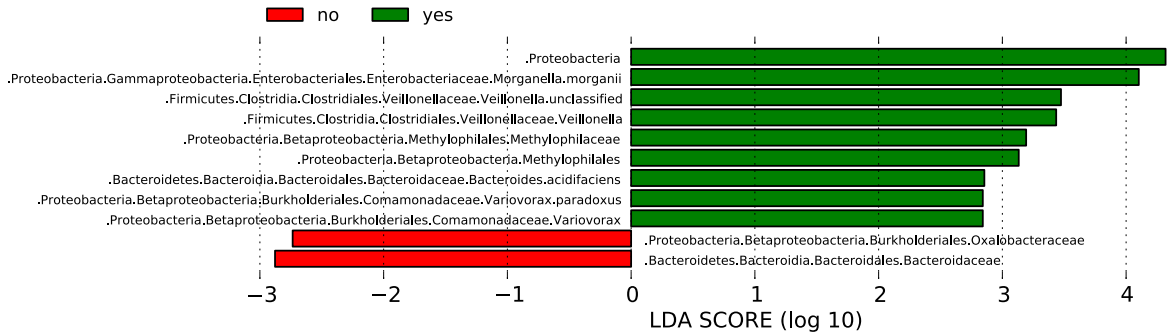

Figure 3.20: LEfSe results showing LDA score for differential abundance of bacterial taxa between patients with and without sepsis (yes/no).

CHAPTER 4

Piglet Results

In the second part of my project I replicated the intervention of probiotic with and without lactoferrin supplementation in an animal model. I used a piglet model to compensate for the lack of a true control group in our infant study (i.e. a group without probiotic or lactoferrin supplementation). Secondly, an animal model provided a controlled environment where exogenous factors seen in the infant study, such as antibiotics do not play a role. Lastly an animal model allowed us to collect not only stool samples, but also intestinal mucosal and luminal contents, which for ethical reasons would not be possible in a human study. A total of 14 piglets were included in our study (Table 4.1). Pigs were healthy at the start of the study and their daily weights can be seen in Figure 4.1. Two piglets were euthanized prematurely (one from the P group and one from the PL group) due to signs of sepsis. Please refer to section 2.2.3 for details pertaining to piglet sample collection.

4.1 Rarefaction

Similar to our infant data, the shape of the rarefaction curves (Figure 4.2) suggests that deeper sequencing would not have identified additional OTUs. As such, a threshold of 100,000 reads was once again chosen as a cut-off of sequences per sample. The mean number of reads per piglet sample was 610,308.850 with a standard deviation of 271,035.941.

Condition	Piglets (N)	Gastrointestinal tract (Luminal/Mucosal Scraping)							Stools								
		Duodenum	Jejunum	Ileum	Cecum	Ascending	Transverse	Descending	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
Control	4	4/4	4/4	4/4	4/4	4/4	4/3	4/3	4	4	4	4	4	4	4	4	4
Lactoferrin	2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2	2	2	2	2	2	2	2	2
Probiotics	4	4/4	4/4	4/4	4/4	4/4	3/4	4/4	4	4	4	4	4	3	3	3	3
PL	4	3/3	3/3	3/3	3/4	3/3	3/3	3/4	4	4	4	4	4	3	3	3	3

Condition	Piglets (N)	Gastrointestinal tract (Luminal/Mucosal Scraping)							Stools								
		Duodenum	Jejunum	Ileum	Cecum	Ascending	Transverse	Descending	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
Control	4	4/4	4/4	4/4	4/4	4/3	4/3	4/3	2	4	4	4	4	3	4	4	4
Lactoferrin	2	1/2	1/1	2/2	2/2	2/2	2/2	2/2	2	2	2	1	2	2	2	2	2
Probiotics	4	3/3	3/4	4/4	3/4	4/4	3/4	4/4	2	4	3	4	4	3	3	2	2
PL	4	2/3	1/3	3/3	3/3	3/3	3/3	3/3	3	3	4	4	4	3	3	3	3

Table 4.1: Summary of all piglet samples collected (A) and analyzed (B) during the study
107 unique stool samples were analyzed
168 unique intestinal samples were analyzed

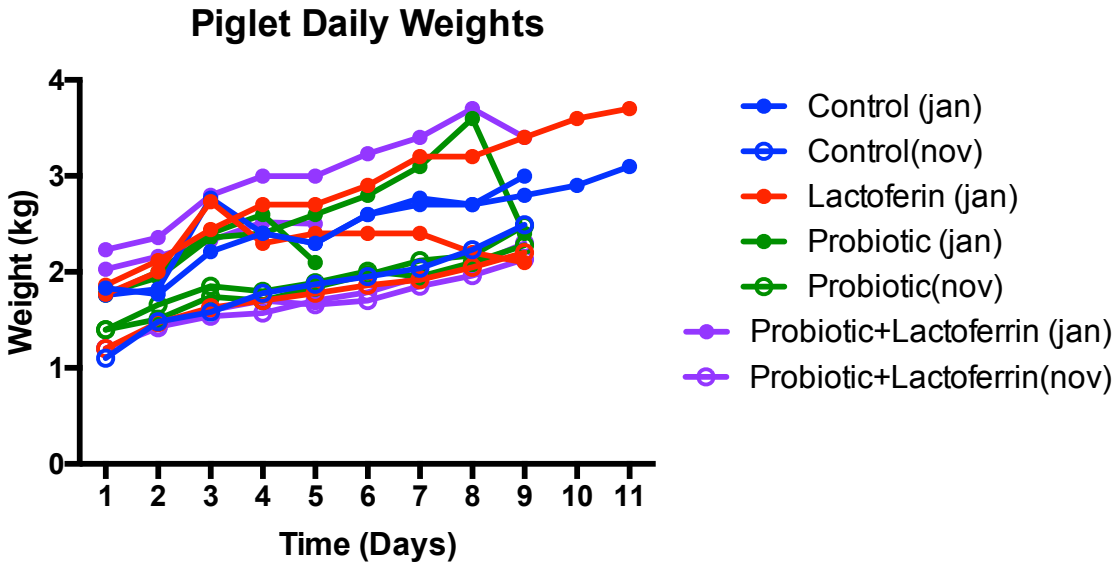

Figure 4.1: Piglet weights
Daily weights were taken over the course of the study

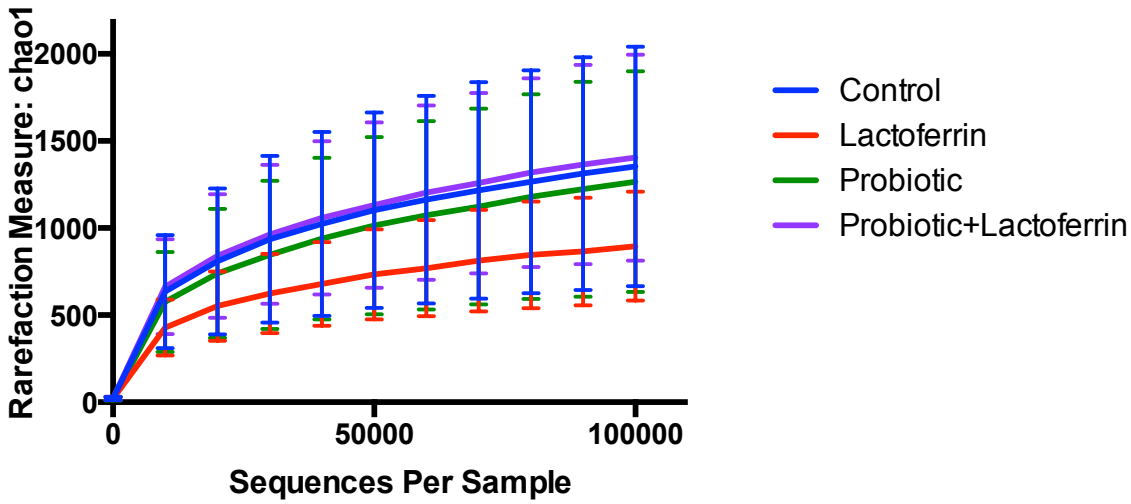

Figure 4.2: Rarefaction curves

Rarefaction curves indicating the depth of sequencing from stool samples. We chose a threshold of 100,000 sequences per sample for rarefication.

4.2 Piglet litter impacts the microbial community

Similar to the results seen in infants, the stools of piglets treated with probiotic alone, lactoferrin alone and the two combined did not lead to a noticeable separation using UniFrac distance (Figures 4.3A, 4.4A). As samples appeared to separate based on sequence run (AS07 and AS18) (Figure 4.3B). We added an additional sequence run (AS27) which incorporated piglet stool samples from both the January and November litters. In doing so it became clear that piglet litter (January versus November), as opposed to sequence run (AS07, AS18, AS27) was driving the separation. The difference was especially apparent on unweighted UniFrac analysis (Figure 4.3C), and less so for weighted UniFrac analysis (Figure 4.4C) which takes relative abundance into account.

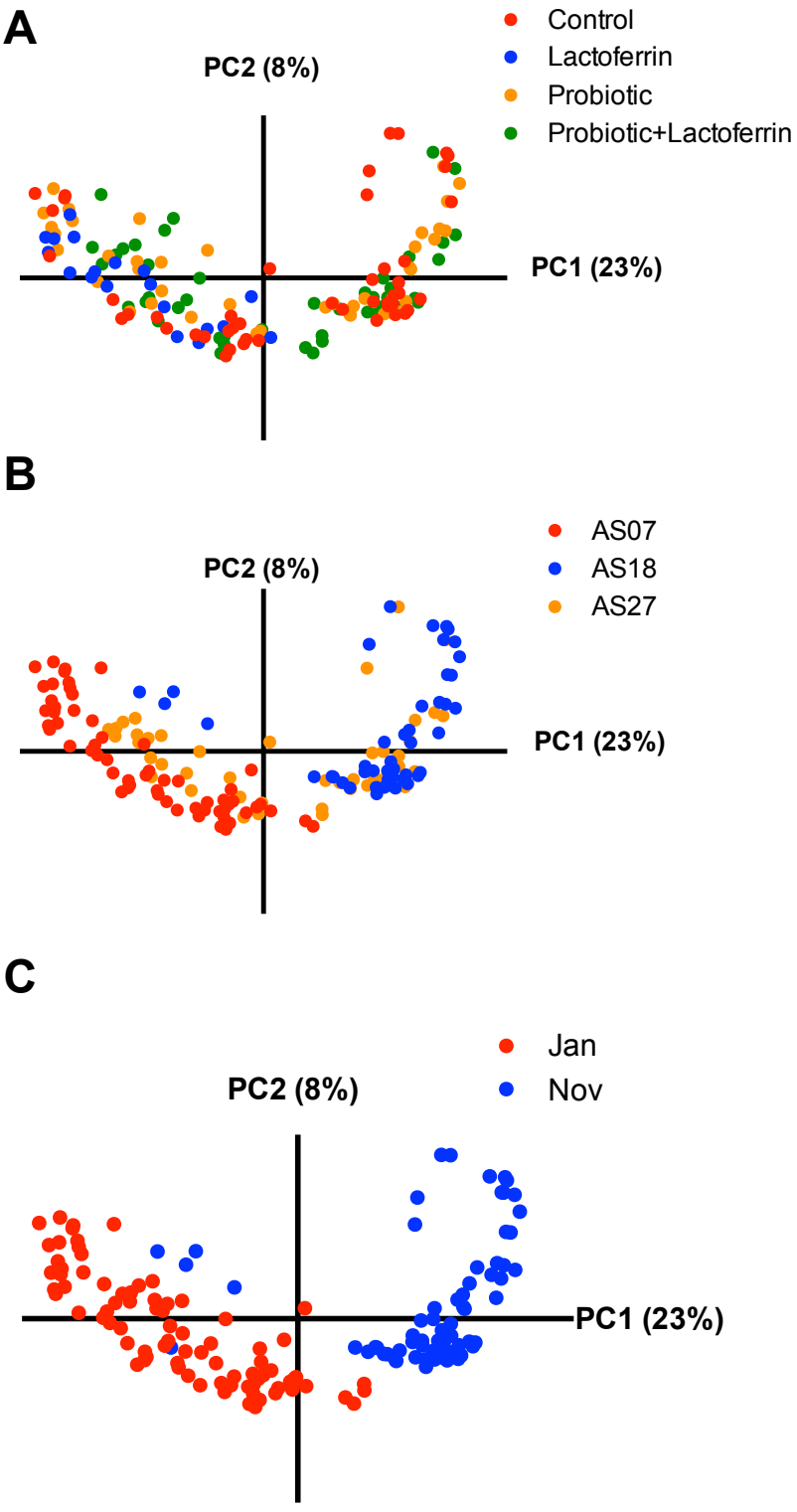

Figure 4.3: PCoA plots of stool samples based on unweighted UniFrac distance by A) treatment group, B) sequence run and C) piglet litter

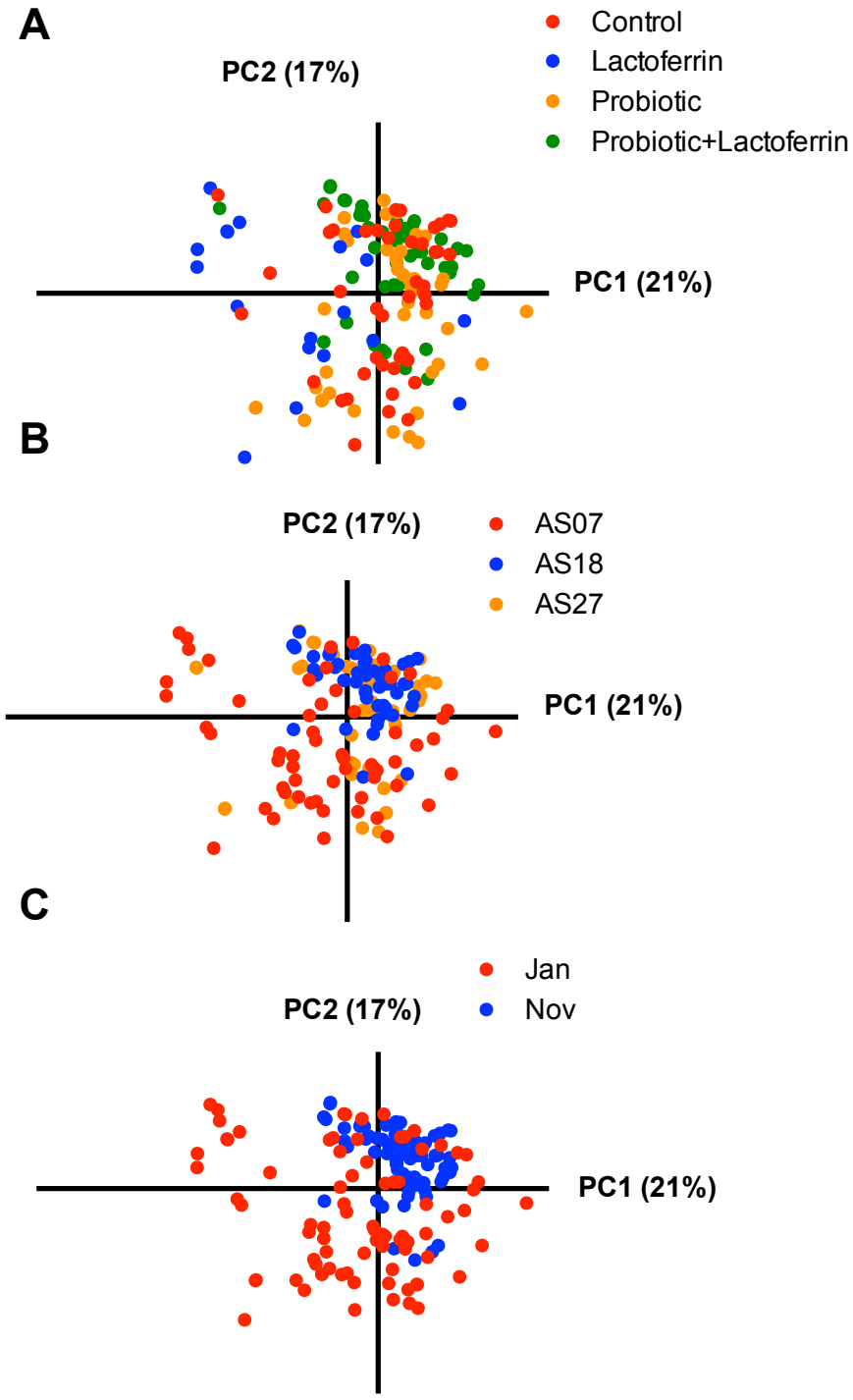

Figure 4.4: PCoA plots of stool samples based on weighted UniFrac distance by A) treatment group, B) sequence run and C) piglet litter

4.3 Alpha Diversity of stools and intestinal samples

To assess bacterial diversity in piglets receiving lactoferrin and/or probiotic supplements, we used the same methods as for the infant study using the chao 1 and the Shannon indices. We found that there was no difference in richness or bacterial diversity between treatment groups for piglet stool samples. Likewise no difference was observed for intestinal luminal and mucosal scrapings (Figures 4.5-4.7). Furthermore, when the intestinal luminal segments were grouped together into small bowel (duodenum, jejunum, ileum) and large bowel (cecum, ascending colon, transverse colon, descending colon) there was no statistically significant difference in the diversity scores between the 2 anatomical regions (Figure 4.8). Examining the diversity between each treatment group in the small bowel and large bowel separately yielded some interesting findings. Analysis of the small intestinal samples revealed that PL samples possessed a greater richness compared to controls ($p=0.03$) (Figure 4.9B). These findings were not replicated in the large bowel. In the latter I found that the controls were more diverse than the probiotic specimens ($p=0.0008$) as well as the lactoferrin samples ($p=0.0013$, $p=0.0102$) using the Shannon index (Figure 4.9D). Controls also displayed greater richness than lactoferrin alone (Figure 4.9C). In addition the PL samples were more diverse than the probiotic alone ($p=0.0387$), and more diverse and rich than the lactoferrin group ($p=0.0313$, $p=0.0012$) using both Shannon and chao1 indices (Figure 4.9C,D).

In order to compare our animal model with the infant study I supplemented the piglets with FloraBaby^{TM} and bovine lactoferrin, using the same doses received by the infants. Prior to supplementation with probiotic, lactoferrin or a combination of the two, all groups had a similar bacterial diversity (data not shown). When we compared the microbial

diversity before supplementation (days 1+2) versus at the end of the study (days 8+9) within each treatment group, we found a higher bacterial diversity and richness in stools supplemented with probiotics as shown by an significant increase in the chao 1 and Shannon indexes ($p=0.0134$ and $p=0.037$ respectively) (Figure 4.5). A trend toward increased diversity between the before and after supplementation categories was observed for the control group and the PL group, but was not statistically significant.

In order to account for the influence of each individual piglet litter on alpha diversity, we analyzed our data with a generalized linear mixed model by taking into account the piglet litter as a covariate (using metagenomeSeq). None of the various measures of alpha diversity or richness (Shannon index, chao1, observed species, PD whole tree and goods coverage) were found to be significantly affected by the lactoferrin and/or probiotics treatments, with all days of the experiment pooled together (data not shown).

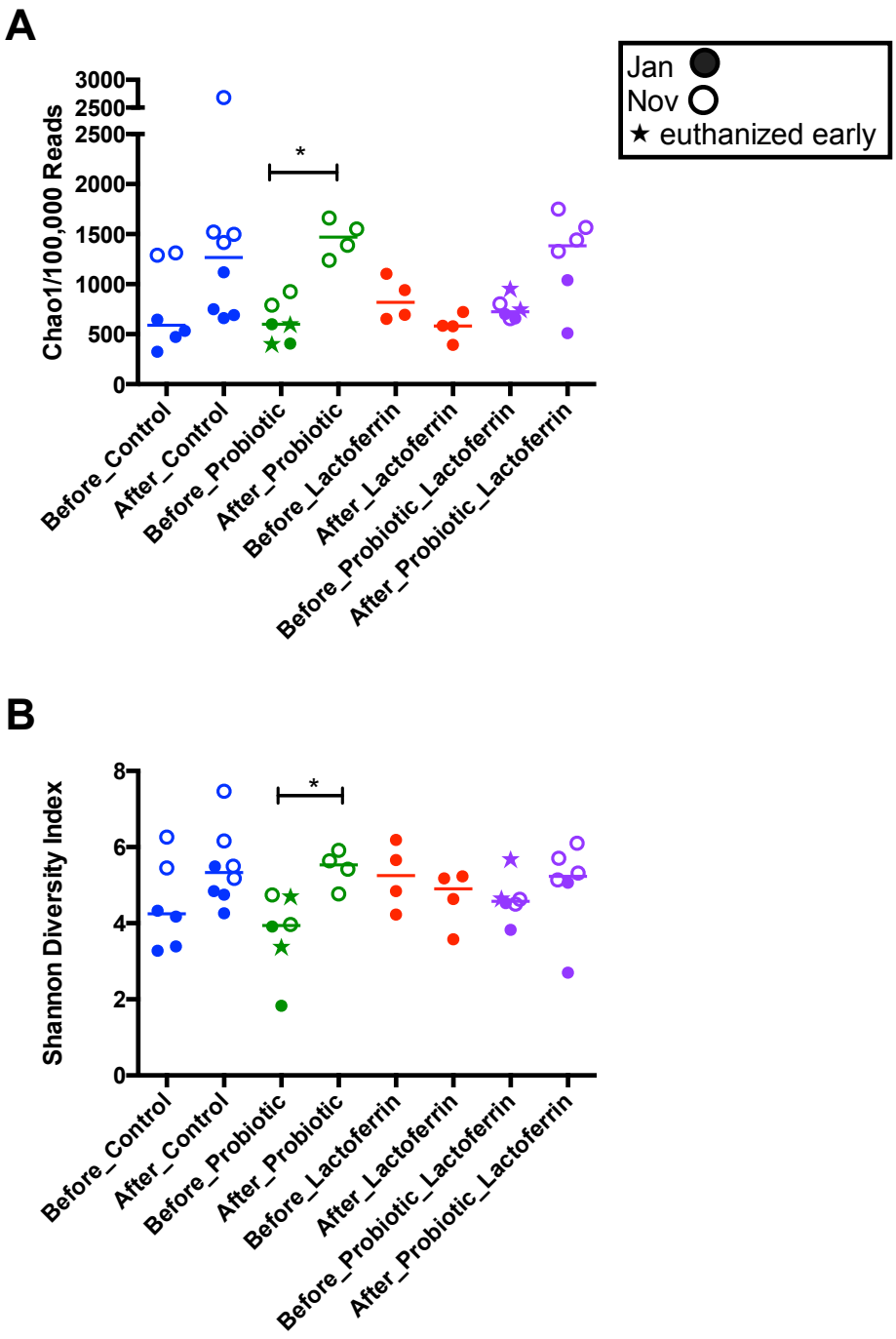

Figure 4.5: Alpha Diversity before and after supplementation with probiotics +/- lactoferrin

A: Chao 1 score before and after supplementation

B: Shannon diversity index before and after supplementation

"Before" refers to experiment days 1+2 and "after" refers to days 8+9

Samples are colored by treatment group (Blue=control group, Green=P alone, Red=Lactoferrin alone, Purple=PL)

* $p<0.05$ Kruskal Wallis

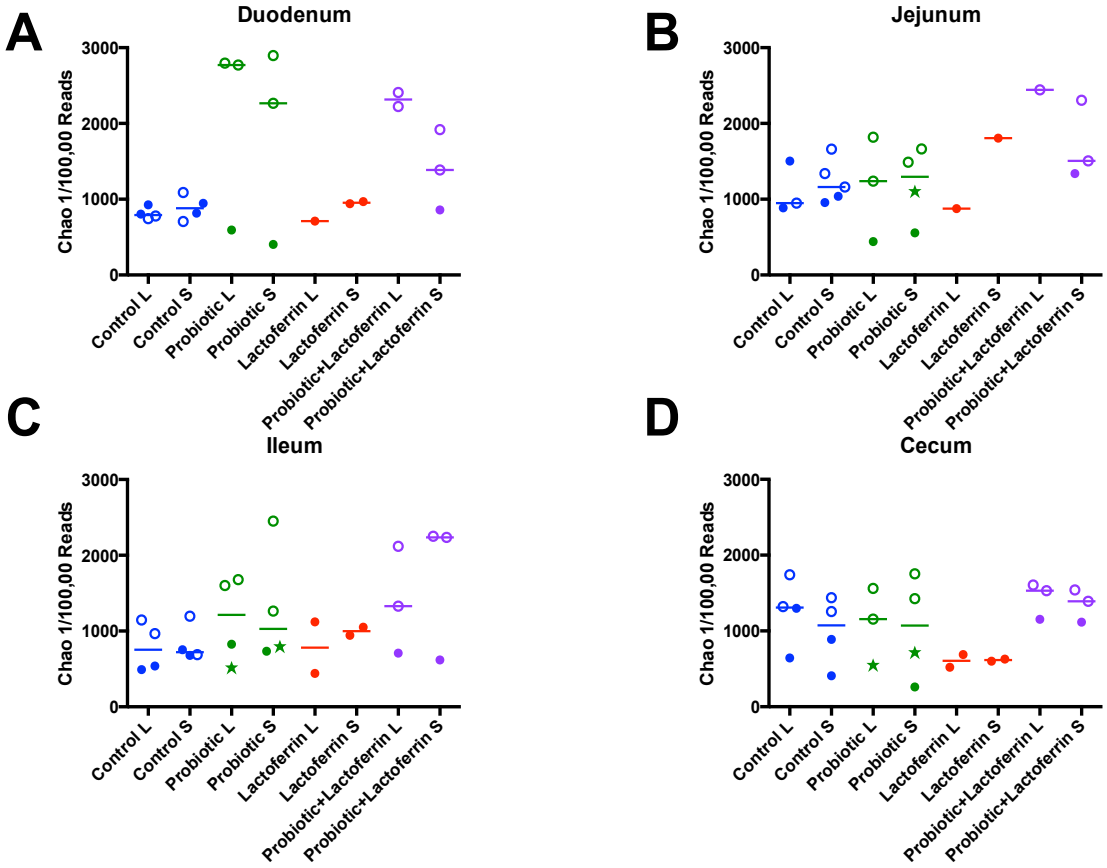

Figure 4.6: Bacterial richness observed in each intestinal segment

Piglets were sacrificed and both the intestinal luminal contents as well as the mucosal scrapings were analyzed for bacterial richness using the chao 1 and compared by treatment group. Samples are colored by treatment group (Blue=control group, Green=P alone, Red=Lactoferrin alone, Purple=PL)
A=duodenum, B=jejunum, C=ileum, D=cecum
L=luminal contents, S=mucosal scrapings

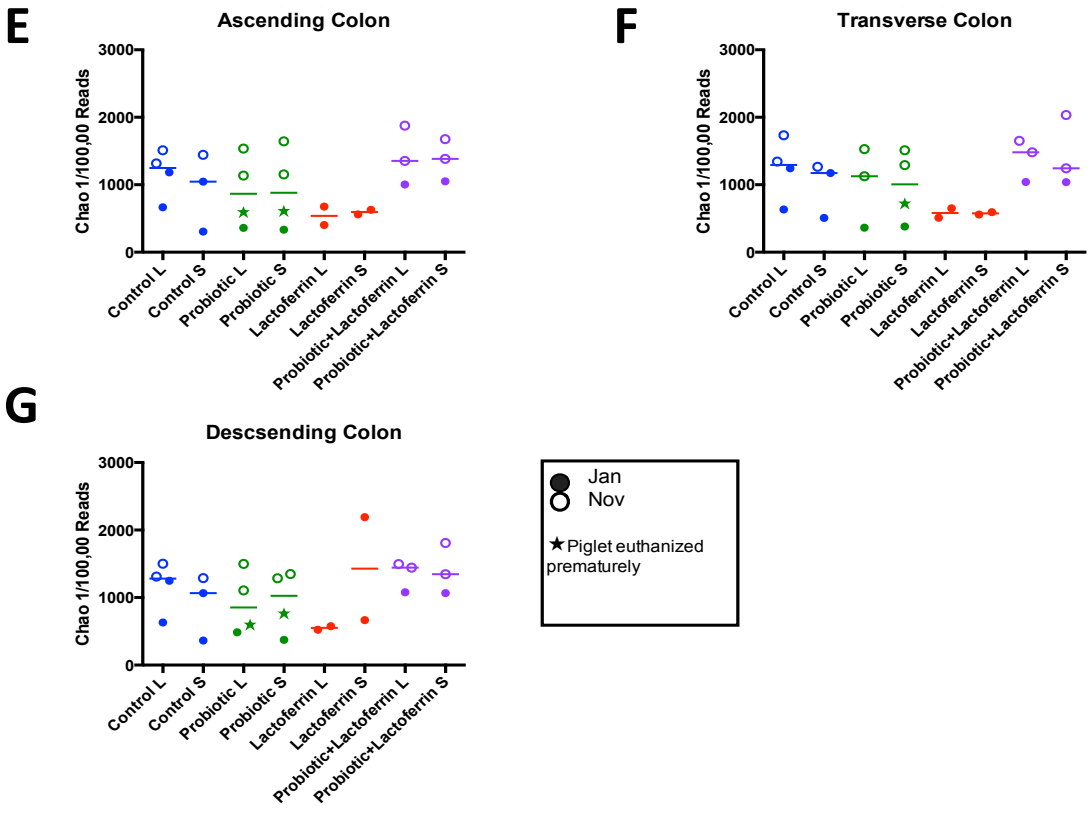

Figure 4.6: Bacterial richness observed in each intestinal segment

Piglets were sacrificed and both the intestinal luminal contents as well as the mucosal scrapings were analyzed for bacterial richness using the chao 1 and compared by treatment group. Samples are colored by treatment group (Blue=control group, Green=P alone, Red=Lactoferrin alone, Purple=PL)
E=ascending colon, F=transverse colon, G=descending colon
L=luminal contents, S=mucosal scrapings

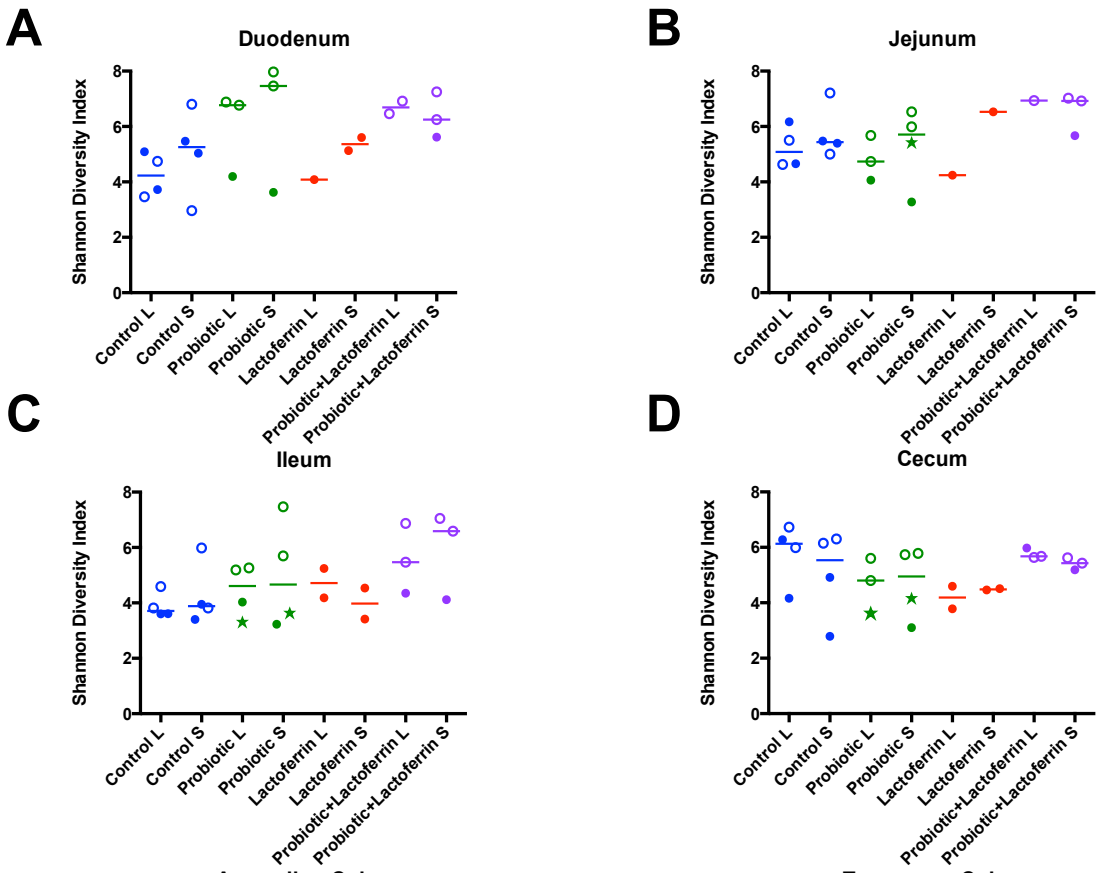

Figure 4.7: Bacterial diversity observed in intestinal segments

Piglets were sacrificed and both the intestinal luminal contents as well as the mucosal scrapings were analyzed for bacterial diversity using the Shannon diversity index and compared by treatment group. Samples are colored by treatment group (Blue=control group, Green=P alone, Red=Lactoferrin alone, Purple=PL)
A=duodenum, B=jejunum, C=ileum, D=cecum
L=luminal contents S=mucosal scrapings

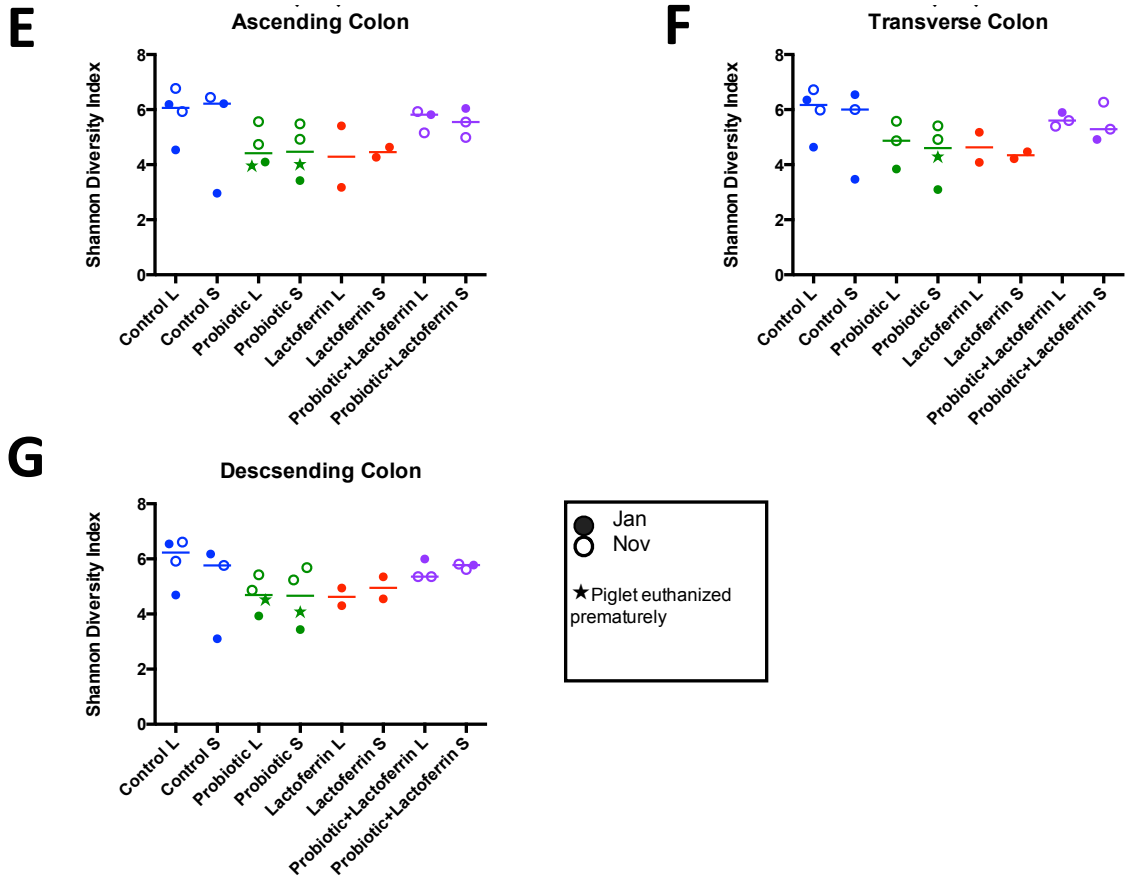

Figure 4.7: Bacterial diversity observed in intestinal segments

Piglets were sacrificed and both the intestinal luminal contents as well as the mucosal scrapings were analyzed for bacterial diversity using the Shannon diversity index and compared by treatment group. Samples are colored by treatment group (Blue=control group, Green=P alone, Red=Lactoferrin alone, Purple=PL)
E=ascending colon, F=transverse colon, G=descending colon
L=luminal contents S=mucosal scrapings

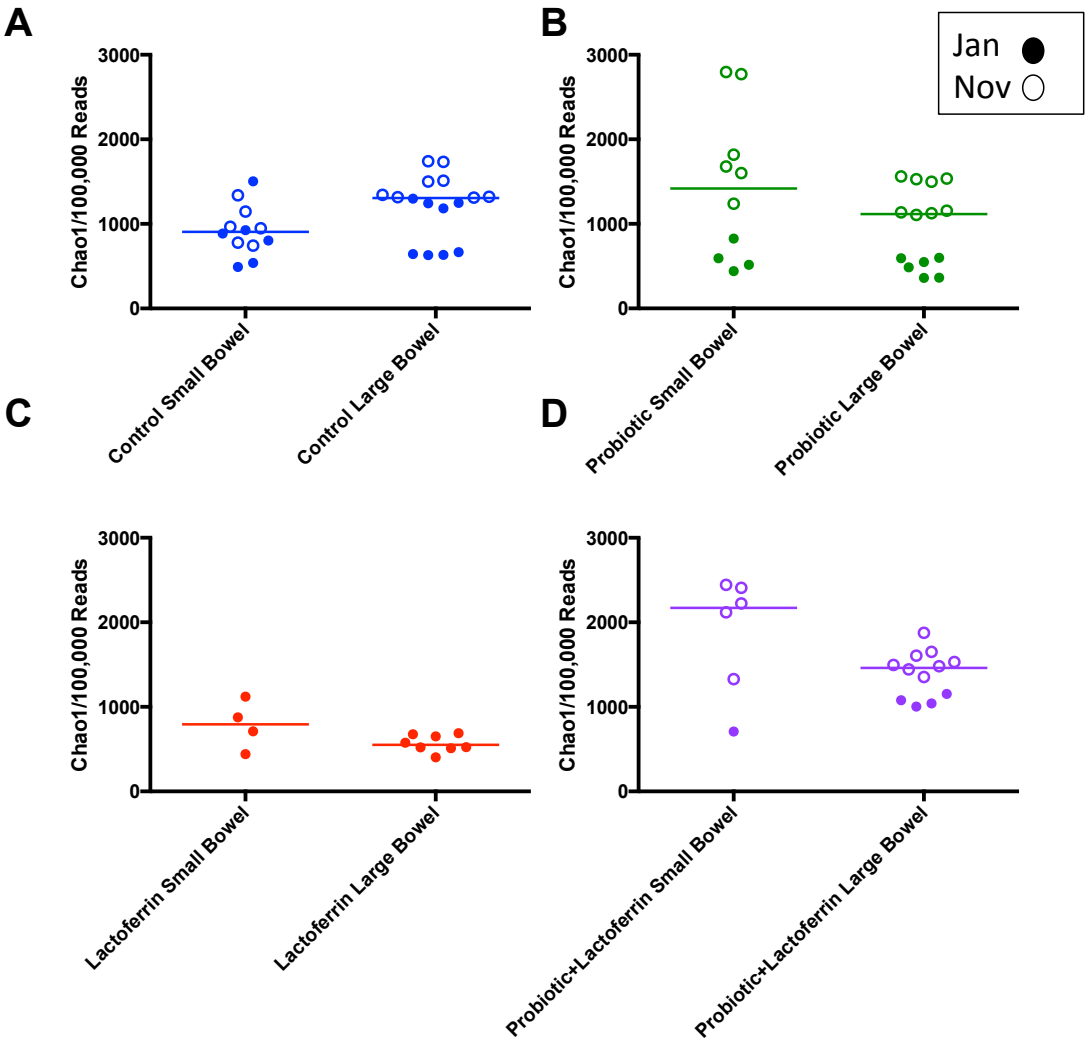

Figure 4.8: Diversity of luminal bowel samples- small bowel (duodenum, jejunum, ileum) compared to luminal large bowel specimens (cecum, ascending colon, transverse colon, descending colon) for each treatment group
A=Control group, B=P alone, C=Lactoferrin alone, D=PL

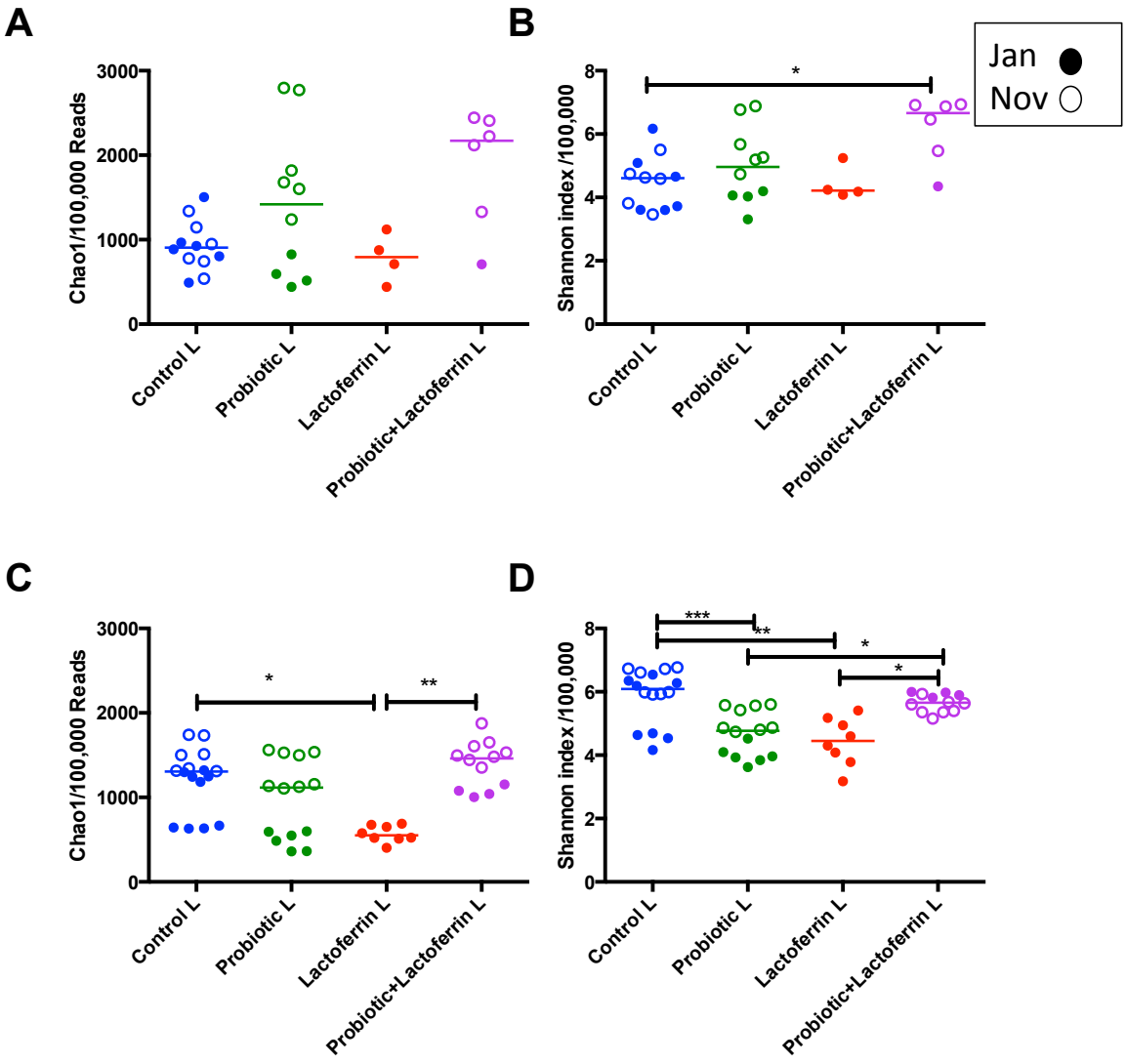

Figure 4.9: Comparison of alpha diversity measures between treatment groups

Bacterial richness and diversity were measured using the chao 1 and Shannon diversity index for the luminal contents (L) of the small bowel (duodenum, jejunum, ileum) and large bowel (cecum, ascending colon, transverse colon, descending colon)
A=Chao 1 for small bowel samples separated by treatment group
B=Shannon diversity index for small bowel samples separated by treatment group
C=Chao1 for large bowel samples separated by treatment group
D=Shannon diversity index for large bowel samples separated by treatment group
*p<0.05, **p<0.001, ***p<0.0001 Kruskal Wallis

4.4 Alpha Diversity over time

Bacterial diversity and richness is expected to increase over time in the neonatal period (Jacquot et al., 2011; Madan et al., 2012). In our piglets we observed a trend towards increased richness over time in all treatment groups except for lactoferrin alone (Figure 4.10). We also observed a trend towards a faster rate of rise in richness for the probiotic and probiotic+lactoferrin group compared to the controls. Similarly we observed a trend in increased diversity over time in the probiotic alone group (Figure 4.11B) although less pronounced than the bacterial richness results. Conversely the rate of rise in diversity for the probiotic+lactoferrin group peaked at day 6 and then decreased (Figure 4.11D).

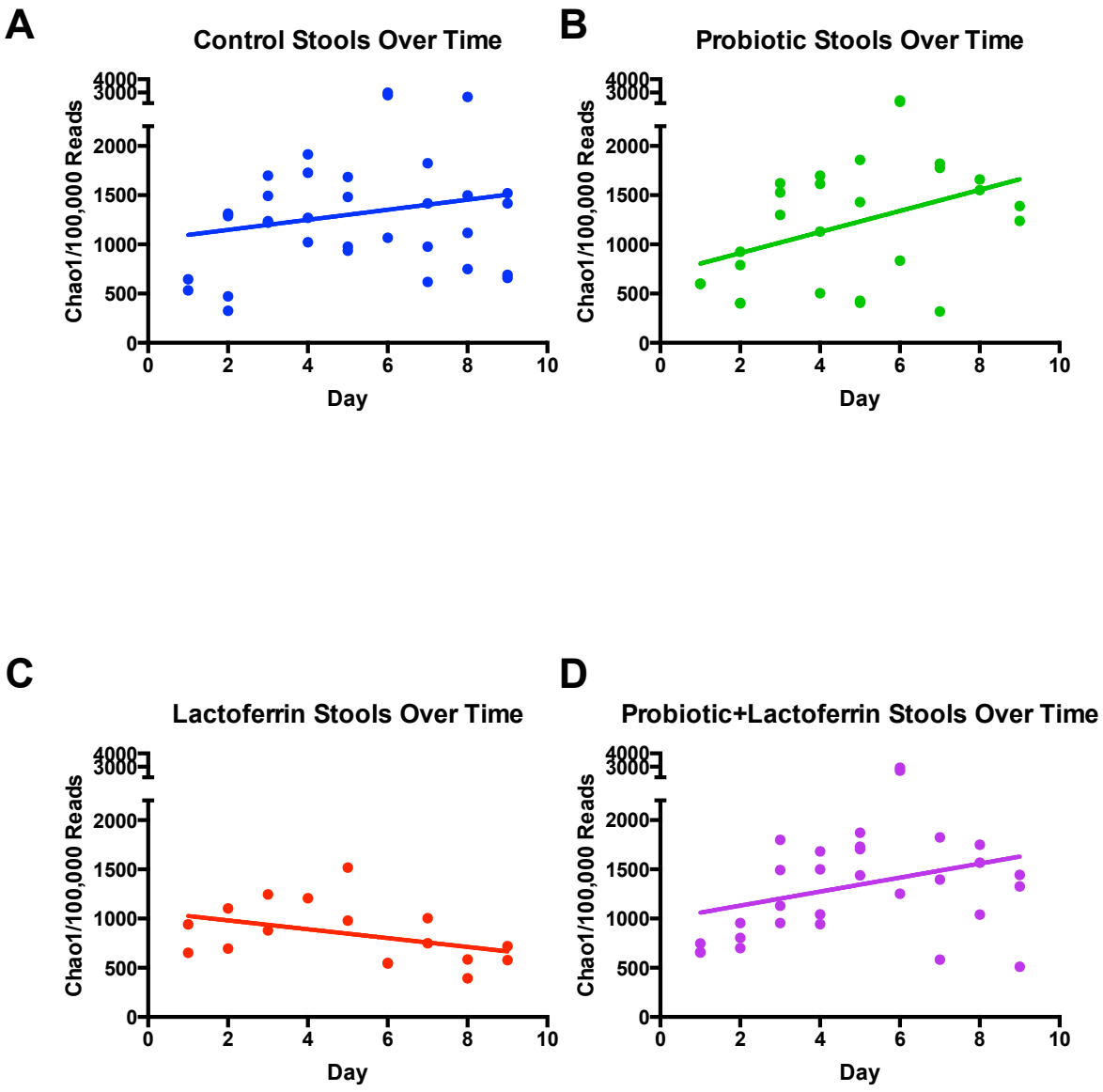

Figure 4.10: Stool sample richness over time

Chao 1 measures of richness (y-axis) over time (x-axis) for piglets in the control group (A), P alone (B), Lactoferrin alone (C) and PL (D).

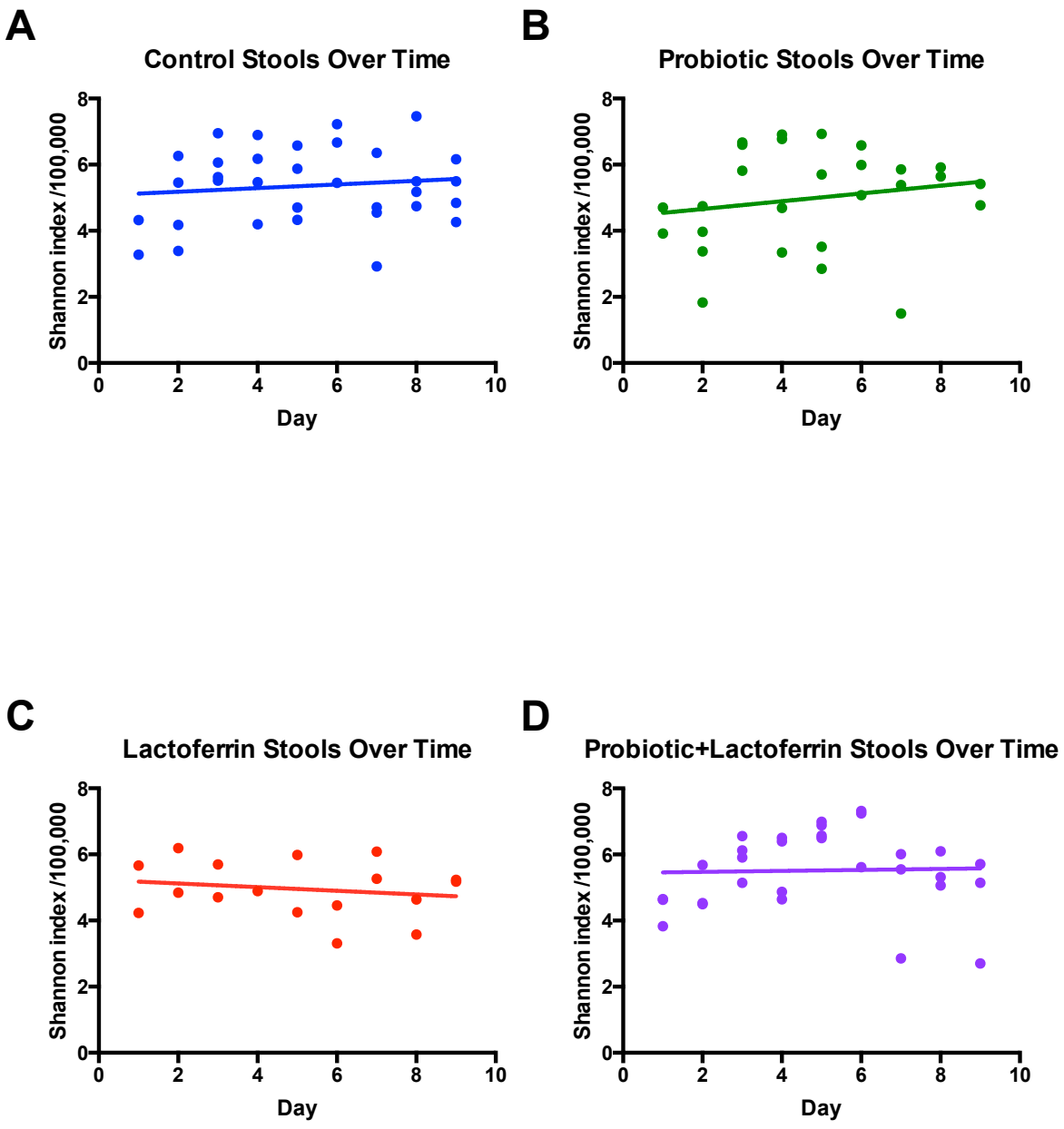

Figure 4.11: Stool sample diversity over time

The Shannon diversity index measures diversity (y-axis) over time (x-axis) for piglets in the control group (A), P alone (B), Lactoferrin alone (C) and PL (D).

4.5 **Stool samples cluster with colonic samples**

I studied two types of intestinal samples from the euthanized piglets; luminal contents which were manually squeezed out of the dissected intestinal segment and mucosal scrapings, which were obtained with a blade. Our objective was to assess whether a difference in microbial populations could be appreciated between the 2 intestinal specimens. Luminal samples should be more similar to stool, and others have shown a different microbial population between mucosal biopsies and stool (Lepage et al., 2005; Momozawa et al., 2011). We performed a PCoA using weighted and unweighted UniFrac distance on piglets stool and intestinal segments from the control group alone to test the similarity in bacterial populations between these two sample types. Once again the same separation was seen based on piglet litter as in Figures 4.3 and 4.4 (Figures 4.12A, 4.13B). Interestingly no separation was seen based on the type of specimen collected; intestinal lumen, versus mucosal scraping versus stool (Figures 4.12B, 4.13B). As expected, we observed a separation between samples located in the upper gastrointestinal tract (duodenum, jejunum, ileum) versus those in the colon (cecum, ascending, transverse, descending colon) (Figures 4.12C, 4.13C). It has previously been demonstrated that the colon contains a greater abundance and diversity of bacteria compared to the small bowel and stomach with concentrations of bacteria of 10^{12} in the colon compared to $<10^{5}$ in the duodenum and jejunum (Mowat and Agace, 2014). It is not surprising then that stools, which are produced in the colon, clustered more closely with the colonic samples as compared to small bowel samples by weighted PCoA (Figure 4.13C).

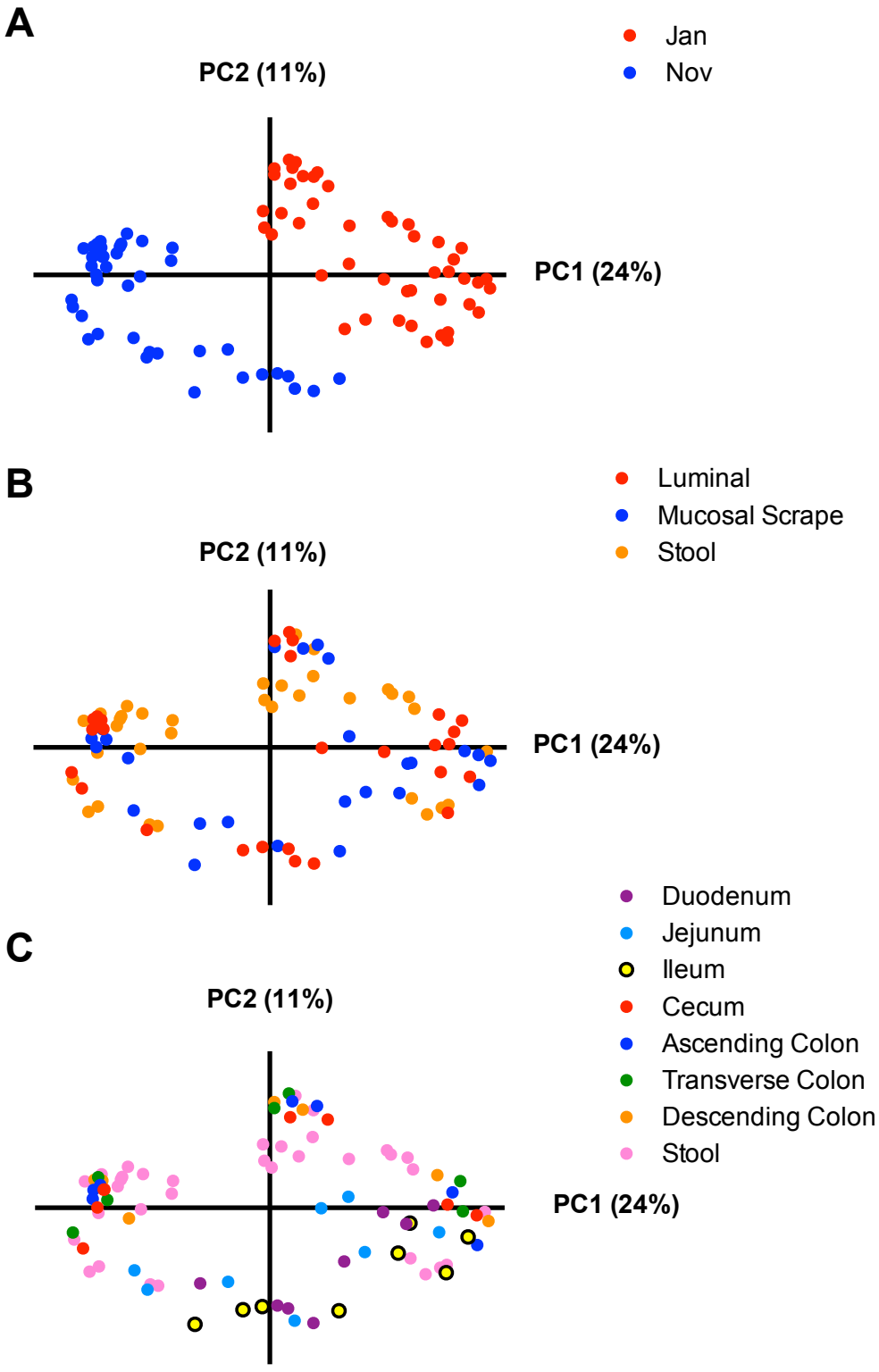

Figure 4.12: PCoA plots of control piglets based on unweighted UniFrac distance colored by piglet litter (A), sample type (B) and sample location (C)

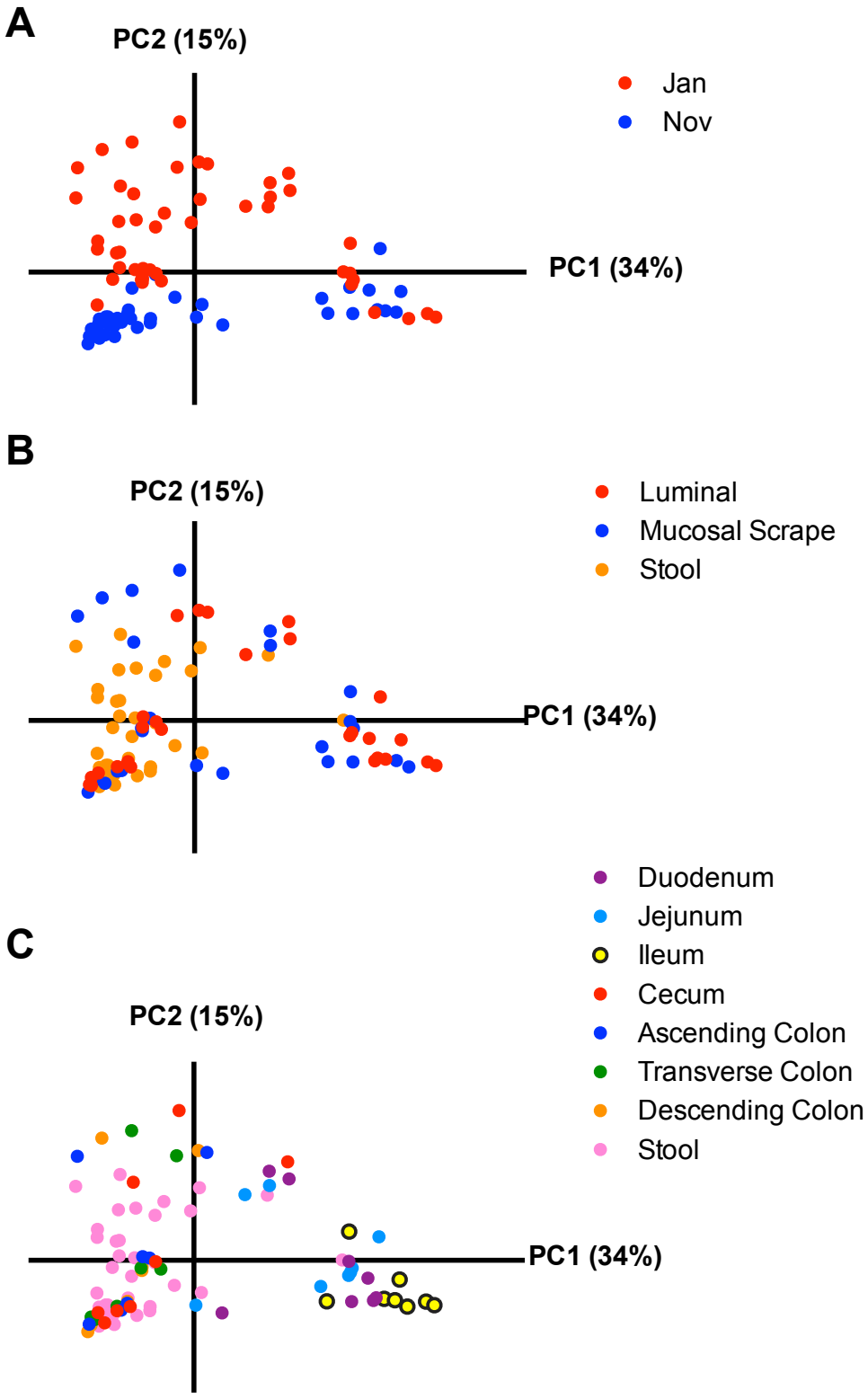

Figure 4.13: PCoA plots of control piglets based on weighted UniFrac distance colored by piglet litter (A), sample type (B) and sample location (C)

4.6 Supplementation with probiotics and lactoferrin yields higher relative abundance of probiotic species in stool samples compared to controls

Unlike the infant stools, we did not identify *Bifidobacterium bifidum* in our samples. Similar to the infant stools we were also unable to detect *Bifidobacterium infantis* from our piglet stools. Once again the *Lactobacillus; s_* OTU which we hypothesize may in fact have represented *LGG* was the most abundant of the probiotic species ranging from 2-6% in the treatment groups versus 1.27% (+/-1.45) in the control group (Figure 4.14). *Bifidobacterium breve, Bifidobacterium longum* and an unidentifiable species (*Bifidobacterium;_s*) were present however in extremely small abundance, at less than $1x\ 10^{-3}$ % relative abundance.

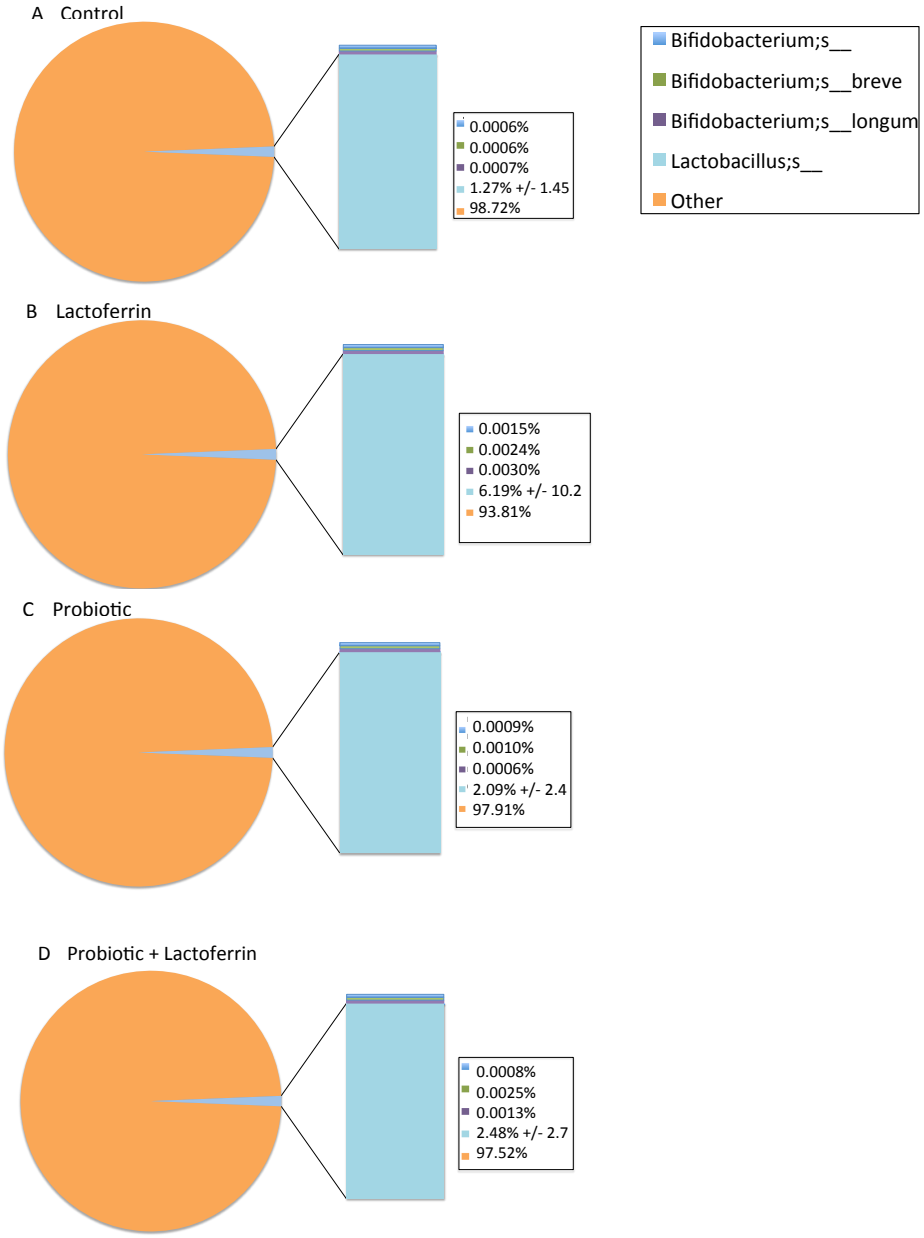

Figure 4.14: Relative abundance of probiotic strains in piglet stool samples
The relative abundance of *Bifidobacterium* and *Lactibacillus*, were calculated in the piglet stools of the control group (A), lactoferrin alone group (B), probiotic alone group (C) and probiotic + lactoferrin group (D). Each bacterial species is identified by a different color; blue: *Bifidobacterium* (species unknown), green: *Bifidobacterium* breve, purple: *Bifidobacterium longum*, pale blue: *Lactobacillus* (species unknown), and orange: other.

4.7 Firmicutes dominates the intestinal microbiota of piglets

Looking at the relative abundance of taxa at the phylum level in piglet stools, it is clear that Firmicutes was a major member of the microbiota representing approximately 40-50% of the total microbial community (Figure 4.15). Of note, piglet groups that received lactoferrin, either alone or in addition to probiotics (Figure 4.15 C,D) showed a trend towards increased relative abundance of the Firmicute phylum (however it was not statisticially significant). Interestingly, although the percent relative abundances of each phylum varied, the order of each phylum from highest to lowest relative abundance was consistant in each piglet group. Bacteroidetes was second highest in all groups, but noticeably lower in the lactoferrin alone group. Fusobacterium and Proteobacterium were next, followed by a very low relative abundance of Actinobacteria (which includes the *Bifidobacterium* genus) in all groups. When comparing these results to those found in our infant study (Figure 3.14) it is evident that the microbiota of infants were characterized by a greater abundance of Proteobacteria as compared to the piglets as well as a much lower abundance of Bacteroidetes and Fusobacteria.

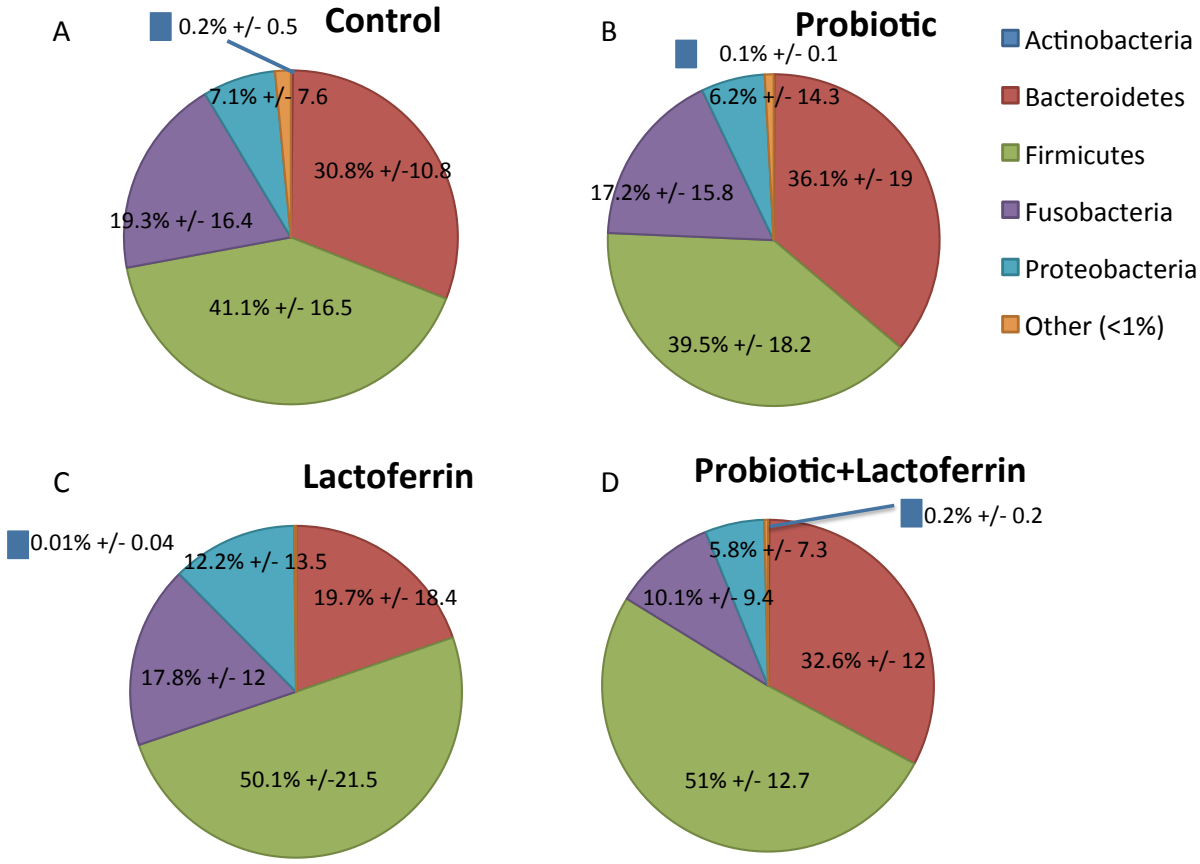

Figure 4.15: Relative Abundance of taxa at the phyla level for piglet stools by treatment groups, Controls (A), P alone (B), Lactoferrin alone (C) and PL (D)

4.8 Relative abundance over time at the phyla and class level

In order to compare piglet results to the infant data, I analyzed the abundance of bacterial taxa at the phylum and class level over the entire duration of the experiment. Over the 9-day course of the experiment it is clear that the Firmicutes and Bacteroidetes phyla dominated throughout in all treatment groups (Figure 4.16). Small increases in the relative abundance of Fusobacteria and Proteobacteria were visible especially after day 3 in the Lactoferrin group. In both groups containing Probiotics (B and D) Proteobacteria was present early on and then tapered off. In contrast the relative abundance of Proteobacteria fluctuated over time in the Lactoferrin group (C).

At the class level Clostridia and Bacteroidia predominated (Figure 4.17). Clostridium was also seen in high abundance for 2 of 4 infants shown in figure 3.15. Gammaproteobacteria (phylum Proteobacteria) once again was present early on in the 2 groups receiving Probiotics (B and D), but decreased over time. While it was the class present in highest abundance for the infants (Figure 3.15), such is not the case in the piglet stools. Similarly the infant study showed high abundance of the Bacilli class while this did not hold true for the piglets.

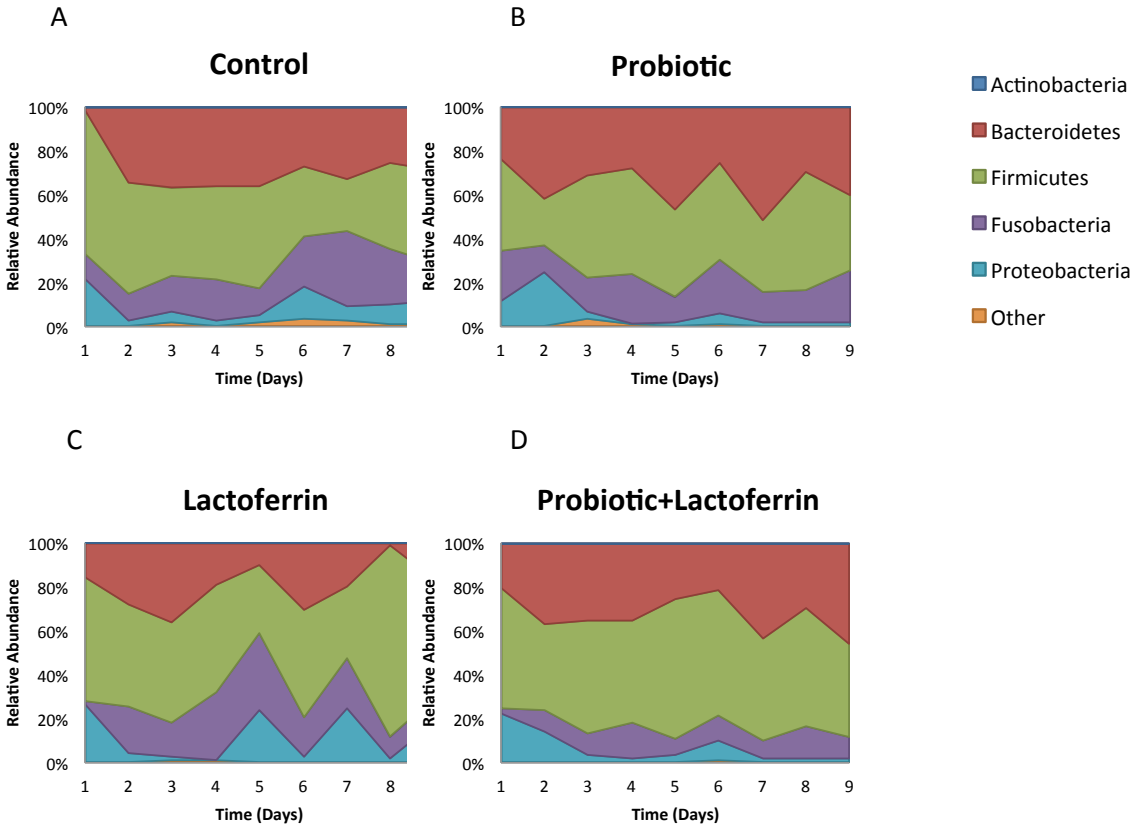

Figure 4.16 Relative abundance over time at the phyla level of piglet stools
The mean relative abundance of each phylum (y-axis) is expressed over time (x-axis) Phyla are represented in different colors. Control piglets (A), P alone (B), Lactoferrin alone (C), PL (D).

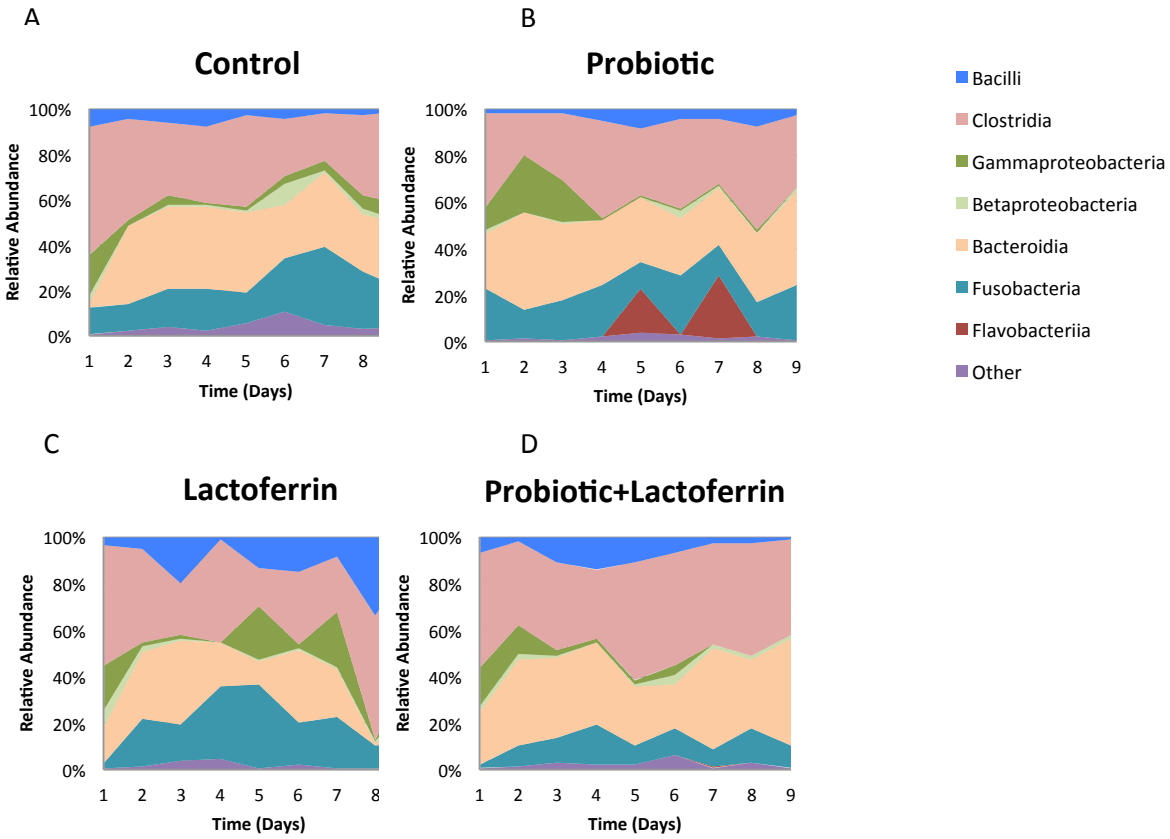

Figure 4.17: Relative abundance over time at the class level of piglet stools
The mean relative abundance of each class (y-axis) is expressed over time (x-axis) Class levels are represented by different colors. Control piglets (A), P alone (B), Lactoferrin alone (C), PL (D).

4.9 Differentially abundant taxa between piglets receiving probiotics and lactoferrin using LEfSe

LEfSe analysis was repeated in the piglet study to facilitate comparisons with the differentially abundant taxa from the infant study. LEfSe results showed a larger than 2 (log_{10}) LDA score for the *Succinivibrionaceae* family for the lactoferrin alone group compared to probiotic alone (Figure 4.18). The probiotic alone group displayed 3 taxa from the Alcaligenaceae family (Burkholderiales order) with a LDA score of 2.5-3.5 (log_{10}). Lastly *Bacteroides coprophilus* was also found to be more abundant in the probiotic alone group. Note that due to the effect of piglet litters on the composition of the microbiote as shown by UniFrac based PCoA plots (Figures 4.3 and 4.4), we used piglet litter as a subclass in the LEfSe analysis. Interestingly when comparing these results to those seen in the infant study Burkholderiales also displayed a greater than 3 log_{10} LDA score for the probiotic+lactoferrin group.

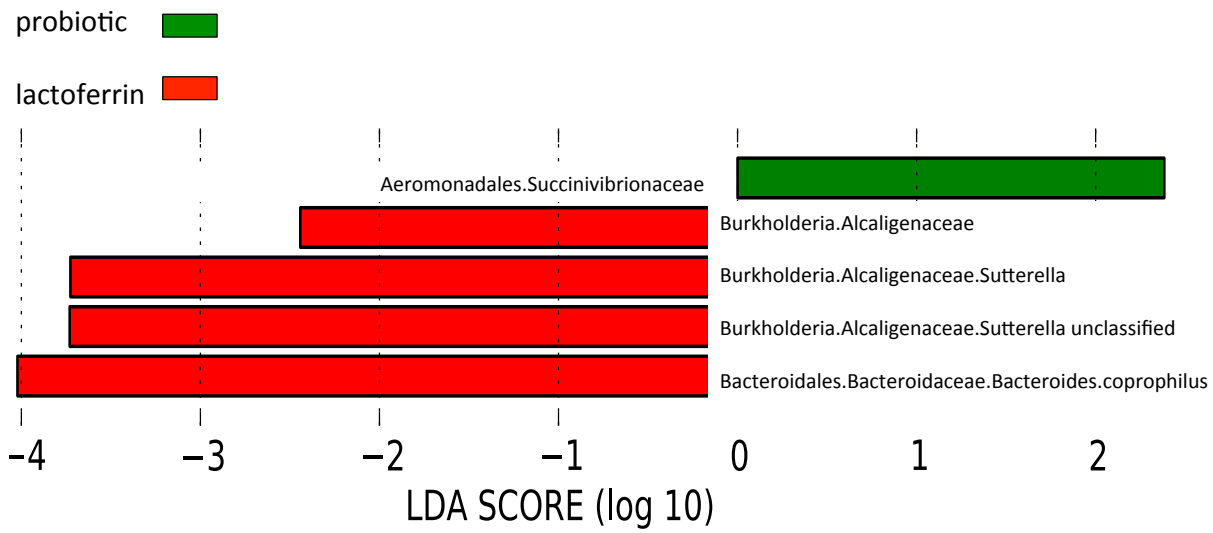

Figure 4.18: LEfSe results for piglet stools
Histogram of the linear discriminant analysis (LDA) scores are shown for taxa that are differentially abundant between the probiotic alone group (green) versus the lactoferrin group (red)

4.10 Identification of differentially abundant taxa between treatment groups by using metagenomeSeq

MetagenomeSeq was used to identify differentially abundant taxa between the 3 treatment groups (probiotic alone, lactoferrin alone, and probiotic+lactoferrin) as compared to the piglet control group. Due to the previously noted differences on PCoA plots seen between the 2 piglet litters, we included it as a covariate. Often multiple OTUs are shown representing the same bacterial taxa. The lowest possible taxonomy level was used for analysis. The results outlined below, show bacterial taxa with a greater than or equal to 4-fold change in differential abundance, and are statistically significant with a p-value less than or equal to 0.01. Over 100 OTUs were found to be differentially abundant in each group (119 OTUs in the probiotic alone, 137 OTUs in the lactoferrin alone, and 107 in the probiotic+lactoferrin group) (Figures 4.19-4.221 for selected taxa and Appendix IV-VI for a complete list of differential taxa). The *Lactobacillus* genus was more abundant in all treatment groups compared to controls, with 1 OTU represented in each of the probiotic and probiotic+lactoferrin groups, however the lactoferrin alone group contained 17 lactobacillus OTUs that were more abundant compared to controls (Figure 4.20). Other interesting findings included the greater relative abundance of *Faecalibacterium prausnitzii* (1-4 OTUs) and *Fusobacterium* (1-3 OTUS) in the control groups as compared to lactoferrin alone and PL. Except when comparing probiotic to controls, Fusobacterium was in fact more abundant in the probiotic group. *Prevotella* genus and more specifically *Prevotella stercorea* was also more abundant in all treatment groups as compared to controls. Comparing these results to the LEfSe analysis, *Bacteroides coprophilus* was more abundant in the lactoferrin group based on both the LDA and metagenomeSeq analyses (2 OTUs, $>4\log_2$ fold change).

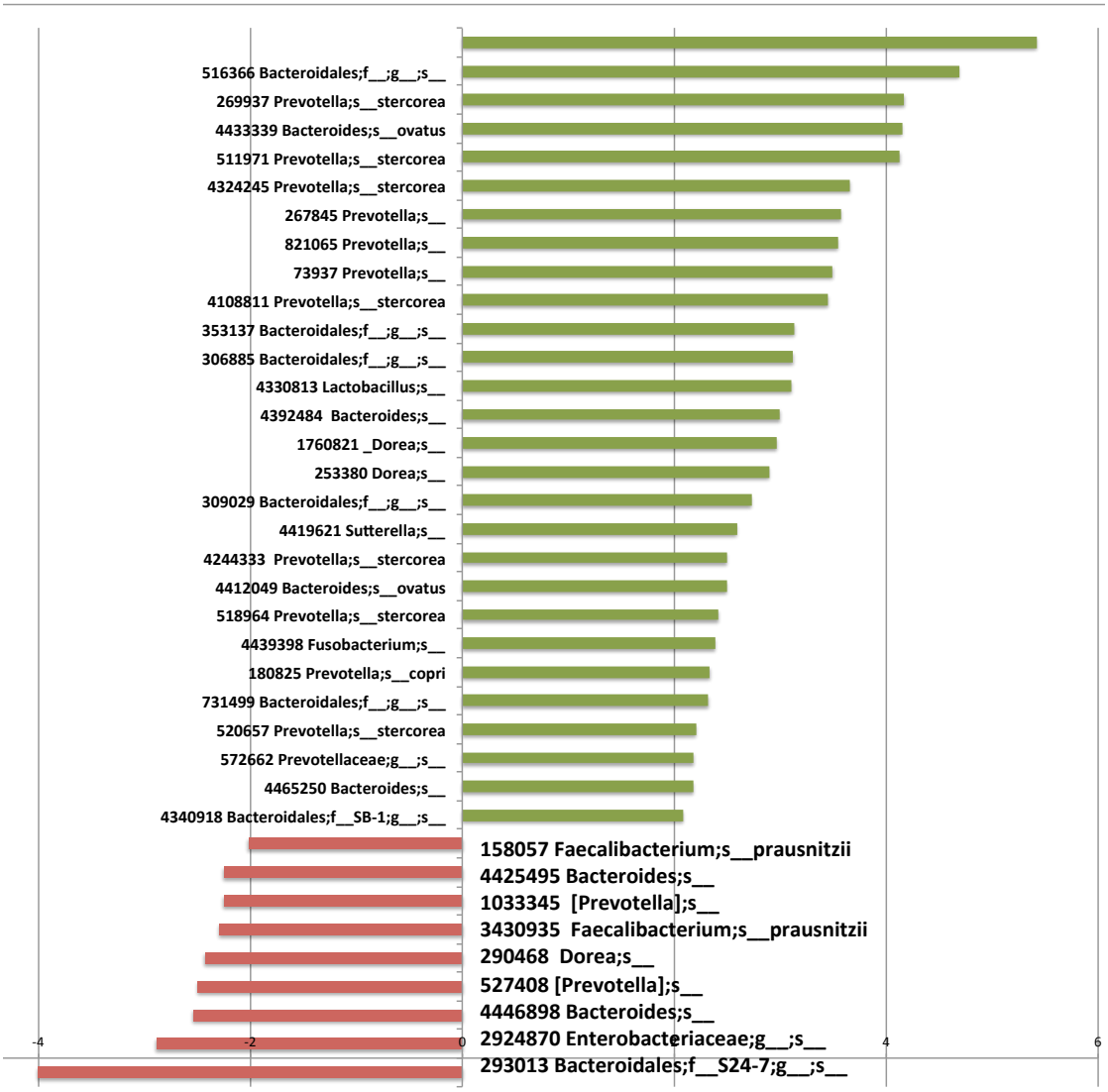

Figure 4.19: Differentially abundant taxa of piglet treatment groups: probiotic vs control

The figure depicts selected taxa, P alone in green, control group in red that were differentially abundant using metagenomeSeq. The x-axis displays the fold change using a $\log_2$ scale

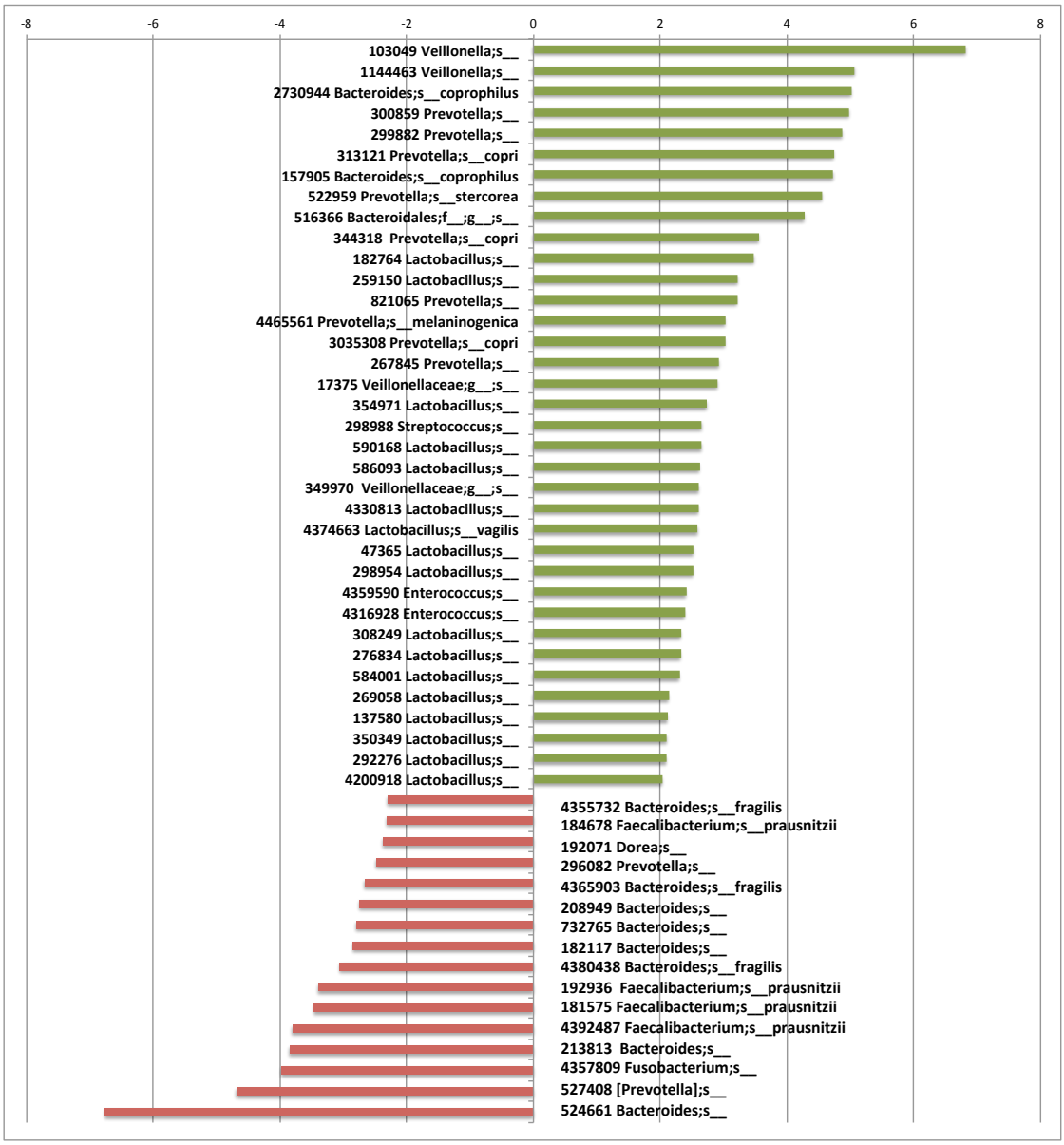

Figure 4.20: Differentially abundant taxa of piglet treatment groups: lactoferrin vs. control

The figure depicts selected taxa, lactoferrin alone in green, control group in red that were differentially abundant using metagenomeSeq. The x-axis displays the fold change using a $\log_2$ scale

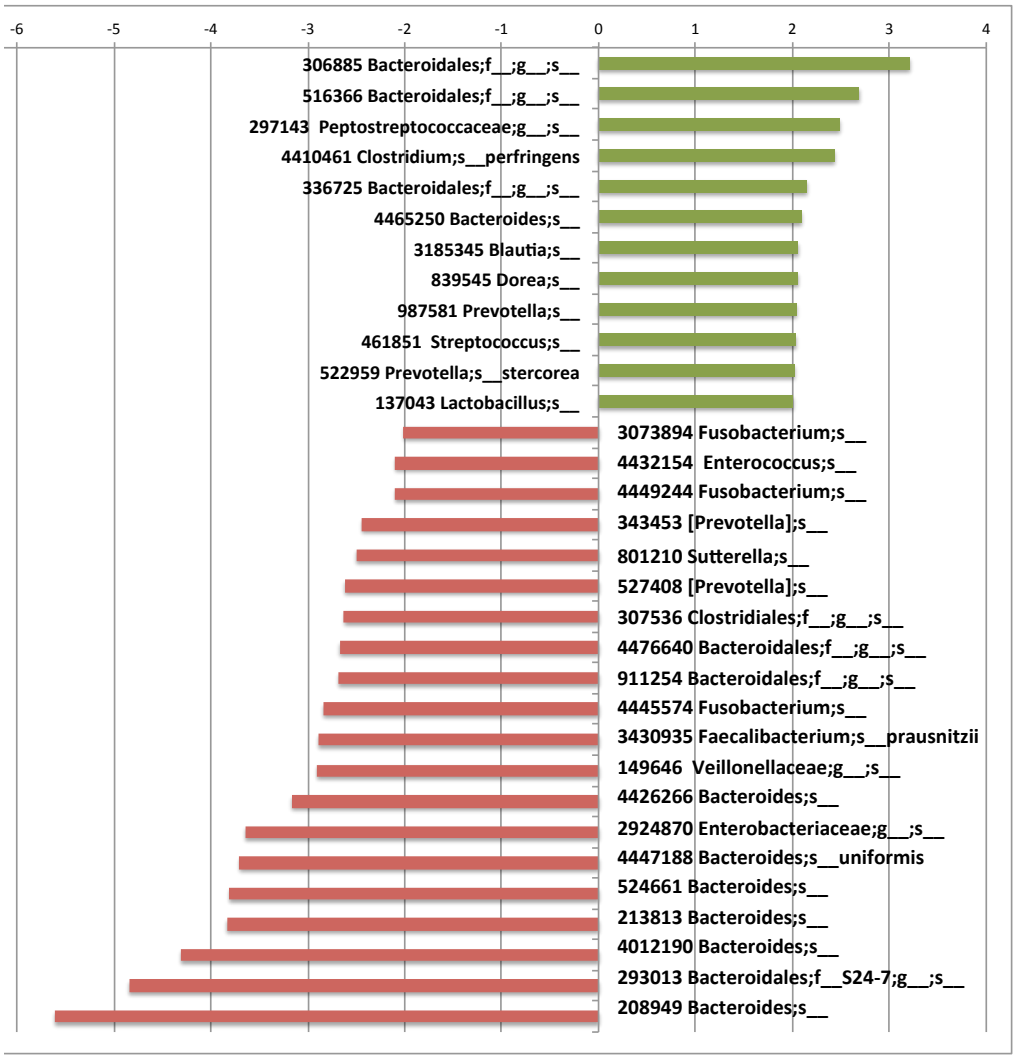

Figure 4.21: Differentially abundant taxa of piglet treatment groups: Probiotic+lactoferrin vs control.

The figure depicts selected taxa, PL in green, control group in red that were differentially abundant using metagenomeSeq. The x-axis displays the fold change using a $\log_2$ scale

CHAPTER 5

Discussion

Probiotics are routinely being used in many NICUs with the goal of reducing rates of NEC and late onset sepsis (Janvier et al., 2014). Lactoferrin, which can be classified as a prebiotic, has also been shown to reduce rates of NEC and sepsis in preterm infants, in a recent Cochrane review (Pammi and Abrams, 2015). Apart from animal models, published studies using probiotics and lactoferrin have focused on clinical outcomes. Our study remains the first to perform a prospective human and animal study exploring the effects of probiotic and lactoferrin supplementation on the intestinal microbiome of premature infants and piglets using molecular-based techniques and next generation sequencing. We conclude that probiotics have a beneficial effect on the microbiome as hypothesized, by speeding up the acquisition of a diverse microbiome as well as potentially protecting from the loss of diversity seen with antibiotics. However, with our current findings, we cannot conclude that lactoferrin confers an additional benefit at the level of the microbiome. As of this writing, there are only two similar studies. The first is a project in the Netherlands investigating the effects of lactoferrin and prebiotics supplemented in formula on the intestinal microbiota of premature infants for 6 weeks (ISRCTN71737811) (Pandita 2015). Their study differs from ours, in that they began supplementation only after attaining full enteral feeds (which can take days or even weeks) while our subjects were included in the study within the first 48 hours of life and were recruited within 12 hours of their first enteral feed. Additionally, we used a fixed amount of Lactoferrin i.e. 100 mg once per day during a single feed, while their infants received 1 mg/100 ml of infant formula. As such, the total amount of lactoferrin received by each infant per day fluctuated and depended on the enteral intake of the infant.

Many NICUs have a feeding protocol, which dictates the volume of milk an infant receives per day. For instance at the CHU Sainte Justine premature infants receive only 1-2 ml of milk every 2-3 hours during the first few days of life. It can take 2-3 weeks to reach full feeds, which is defined as 160 ml/kg of body weight per day. For example an infant weighing 1.5 kg would receive a total of 240 ml of milk per day, or approximately 2.4 mg of lactoferrin per day. In a recent study the microbiome of infants receiving recombinant lactoferrin at a higher dose of 150mg/kg of body weight twice daily, versus placebo was compared (Sherman et al., 2016). While their study drew conclusions about the presence of particular bacterial species in each group i.e. less *Enterobacter*, *Klebsiella* and *Staphylococcus* in the lactoferrin treatment group, no diversity analysis was performed. Furthermore only a small subset of infants enrolled had microbiome analysis (23/120).

We experienced the same limitations as other researchers who study the intestinal microbiome of human neonates. Due to ethical considerations we used fecal samples as a proxy for the distal intestinal microbiome. That said we know from the work done on the intestinal microbiome in healthy and IBD patients, that stool samples are not the perfect proxy for mucosal biopsies (Momozawa et al., 2011). One study showed that the rectal mucosal-associated microbiome was a predictor of Crohn's disease severity, but this was not true for fecal samples (Gevers et al., 2014). In our animal study we did note however, that stool samples clustered with colonic specimens taken at piglet necropsy on UniFrac PCoA plots, suggesting that fecal samples are indeed an acceptable proxy for what is occurring at the level of the intestinal mucosa for preterm infants.

We analyzed our piglet colonic samples even further by dividing them into mucosal scrapings and luminal contents, and no difference was noted on UniFrac PCoA plots. This is

contradictory to what others have shown (Lepage et al., 2005). The dominant fecal microbiota was found to differ from the mucosal associated microbiota in patients with inflammatory bowel disease and healthy controls (Lepage et al., 2005). A possible explanation for our results is a contamination of the luminal samples with mucosal scrapings; our scraping technique may have included luminal contents. Perhaps a more standardized method would have yielded the expected differences. Alternatively, perhaps mucosal and luminal samples in young infants have not had enough time to develop the distinct microbial profiles that are apparent in adult studies. In addition perhaps a greater number of piglet samples would have yielded different results.

Overall piglet intestines have been shown to be a good proxy for the human gut microbiome (Guilloteau et al., 2010). That said we found that the Firmicutes phyllum predominated in the piglet microbiota whereas Enterobacteria prevailed in the preterm infants. Therefore, full term piglets may be an excellent surrogate for full term infants, but less so for premature infants, which was a limitation in our study.

5.1 Microbiota composition in the early life of preterm infants

Our findings are in keeping with the recent literature regarding the composition of the neonatal microbiome of premature infants (Arboleya et al., 2015; Jacquot et al., 2011; La Rosa et al., 2014). At the lowest possible taxa level we observed that the *Enterobacteriaceae* family comprised between 30-45% of the relative abundance in our samples. These results are quite similar to other studies of premature infants, one of which reported a greater than 50% relative abundance of *Enterobacteriaceae* as of 10 days old which persisted through day 90 (Arboleya et al., 2015). We again showed similar findings to the published literature at the class level. Gammaproteobacteria was a taxa that displayed high relative abundance in

our infants and Arboleya *et al*. also reported this taxa to be greater in premature infants when compared to their full term counterparts. At the lowest taxa level our third highest taxa in the probiotic with lactoferrin group and fourth highest taxa in the probiotic alone group, identified at approximately 7% relative abundance in both treatment groups was *Staphylococcus*. The presence of increased *Staphylococcus* is consistent with other studies of premature babies (Jacquot et al., 2011; Madan et al., 2012). *Clostridium perfringens, Klebsiella* and *Veillonella* were found to be in our top 7 for relative abundance, and all three of these genera have previously been reported as predominant in premature infants (Brooks et al., 2014; Madan et al., 2012; Penders et al., 2006). Interestingly, Madan et al.'s study of 6 preterm infants showed that the latter 3 genera were highest in the healthy preemies compared to preemies with sepsis suggesting that the presence of certain bacteria in preemies may be protective against sepsis.

Overall our results are consistent with the currently published literature on the intestinal microbiome of premature infants and most comparable to Arboleya et al. (Arboleya et al., 2015). Our sequencing techniques were similar with the use of the *16S rRNA* gene to assess the evolution of the intestinal microbiome of premature infants. Baseline demographics were also comparable since the majority of their preterm infants were exposed to antibiotics in the perinatal period (14/27 of their preterm infants). We too did not show a difference in diversity based on mode of delivery. Arboleya et al. reported only minor differences in the bacterial profile of premature babies born via C-section, namely higher *Bacteroides* in vaginally delivered babies. We differ in that they followed the progression of the microbiota for the first three months of life, and compared their findings to a control group of full term vaginally delivered infants.

Important differences in the microbiome of premature infants versus full term infants have been published. Firstly, full term infants have a more diverse microbiome as compared to their preterm counterparts (Schwiertz et al., 2003). Bifidobacterium is predominant in the microbiota of full term infants, and is present as early as the first week of life (Palmer et al., 2007). Lastly, potentially pathogenic bacteria such as *Staphylococcus*, *E. coli*, and *Klebsiella* have been identified in higher abundance in the microbiota of preterm infants (Jacquot et al., 2011; Schwiertz et al., 2003)

5.2 NEC, sepsis and variables that affect diversity

NEC and LOS are two of the most severe and frequent morbidities that occur in premature infants (Neu and Walker, 2011; Vergnano et al., 2011). Much research is focused on prophylactic interventions to reduce these serious complications. Clinically speaking, lactoferrin with and without probiotic supplementation has proven to be safe and effective in the reduction of late onset sepsis and NEC based on a recent Cochrane review that encompassed 4 RCTs (Pammi and Abrams, 2015). For the administration of lactoferrin alone, the relative risk was 0.49 (95% CI 0.32-0.73) for the decrease in LOS and 0.30 (95% CI 0.12-0.76) for the prevention of NEC (stage II or greater). From a clinical perspective, an important statistical measure is the number needed to treat (NNT), simply stated, the average number of infants needed to be treated with lactoferrin in order to prevent an undesirable outcome. In this case, for every 8 and 20 infants treated with lactoferrin we could potentially prevent 1 case of sepsis and 1 case of NEC (stage II or greater) respectively. These are low numbers for the prevention of two frequent and morbid complications. Given that the Sainte Justine NICU receives more than 1100 admissions per year, if lactoferrin administration becomes the standard of care, the future clinical implications would be impressive. We could

potentially prevent greater than 130 cases of sepsis and greater than 50 cases of NEC by routinely administering lactoferrin.

In our study no decrease in Health Care Associated Infections (HCAIs) was observed with the use of lactoferrin in addition to probiotics unlike what has previously been reported in the literature (Pammi and Abrams, 2015). HCAIs were defined as a positive culture of a normally sterile body fluid (blood, cerebrospinal fluid, peritoneal fluid), occurring after 3 days of age, thereby incorporating sepsis (Janvier et al., 2014). This negative finding was likely due to the fact that this was a small pilot study. Rouge et al. have suggested that probiotics may only be beneficial at preventing sepsis caused by bacteria that translocate from the gut (Rouge et al., 2009). Probiotic strains may exert their benefit by competitively binding epithelial binding sites and preventing the entry of potentially pathogenic bacteria, and, or by modulation of the immune system (Sherman, 2010). In clinical practice sepsis can occur both from bacteria that translocate from the gut (Hill et al., 1974) as well as from the skin into the bloodstream. Premature babies are more at risk of bacterial skin translocation compared to their full term counterparts due to the presence of central catheters that puncture the skin. Interestingly, in our group of infants, those with sepsis due to the same bacterial strain (identified with a blood culture) tended to cluster together on PCoA plots using UniFrac distance, indicating a similar microbiome profile. This finding suggests that the bacteria identified by blood culture, may have originated from the intestines. This further suggests that probiotics did not prevent bacterial translocation from the gut of these infants.

Unfortunately the LACUNA study (the branch of our study looking at clinical outcomes associated with probiotic and lactoferrin use from which we analyzed the microbiome of a subset of the enrolled infants (70/79 patients included)) was not powered to

study NEC. However, in keeping with the NEC and probiotic literature, a prior and larger study in the same CHU Sainte Justine NICU showed a decrease in NEC (odds ratio of 0.51) with the use of the identical probiotic as our current study (Janvier et al., 2014). It is reassuring that lactoferrin was found to be safe in the CHU Sainte Justine NICU study. To date there have been no published cases of bacterial or fungal contamination of lactoferrin, unlike what has been reported with probiotics (Vallabhaneni et al., 2015).

Since the precise mechanism of NEC has yet to be elucidated, it is even more difficult to understand the mechanisms by which probiotics and lactoferrin lead to decreased rates of NEC (AlFaleh and Anabrees, 2014; Deshpande et al., 2010; Pammi and Abrams, 2015). Experts in this area of research believe that modulation of the gut bacteria is playing a role in the prevention of NEC (Neu and Walker, 2011). A healthy newborn will begin with a predominance of *Enterobacteriaceae* as well as other facultative anaerobes, and around one month of age *Bifidobacteriaceae* will become predominant (Palmer et al., 2007). The bacterial colonization of the newborn gastrointestinal tract influences both the anatomical development of the intestine as well as the immune system (Arrieta et al., 2014). Previous studies have shown that increased bacterial diversity has a beneficial effect on humans (2012). As we strive to attain the ideal microbiome-which no one has clearly defined yet, we can hypothesize that imitating the microbiome of healthy, full term, breast fed, vaginally delivered, no antibiotic exposure infants is the gold standard. Future research may one day provide us with the gold standard newborn gut 16S sequence database with which we can compare premature infants. That said, one of our strengths was that babies and piglets were recruited from a single center and farm thereby limiting the effect of environment.

Unfortunately we had no true control group in our infant study (without probiotic and

without lactoferrin supplementation) since probiotic supplementation has become the standard of care in the CHU Sainte Justine NICU. McFarland recently published an extensive review of probiotic and microbiome studies for the prevention or treatment of numerous medical conditions (McFarland, 2014). Furthermore they categorized studies based on whether they a) changed the microbiome of healthy patients, b) restored the microbiome of healthy patients after a significant event (e.g. antibiotics, infection), or c) improved the microbiome of patients with a chronic medical condition and a baseline altered microbiome. Of the 29 studies performed on healthy volunteers 4 were shown to alter the microbiome. We expected an increased diversity in those receiving lactoferrin in addition to probiotics, since probiotics alone have been shown to increase bacterial diversity in the colon of healthy adult patients with early stages of atherosclerosis (Karlsson et al., 2010). In contrast, we were unable to demonstrate an increased diversity in the microbiome of infants who received lactoferrin along with probiotics. The most likely explanation is that the addition of lactoferrin to probiotics does not confer an additional increase in bacterial diversity. Another plausible explanation is the effect of many confounding clinical factors, and especially the very high prevalence of antibiotics in our cohort that could have impacted the microbiome masking differences in bacterial diversity. Furthermore our methodology had limits as well that could potentially have masked differences in diversity. For instance a minimal amount of bacteria, in our case 50 ng of DNA was necessary for PCR and subsequent sequencing. Therefore samples containing low amounts of bacteria would not have been adequately represented. In addition using a known database, such as Greengenes (DeSantis et al., 2006) could introduce bias since not all bacteria will be equally represented in the database, and bacteria from animals, such as piglets may be underrepresented. The microbiota of our two infants treatment groups resembled each other on PCoA plots. The P

alone and PL infant groups separated on unweighted, but clustered together on weighted UniFrac analysis as well as on the Bray-Curtis dissimilarity measure. This implies that any differences between the 2 bacterial communities can be attributed to rare taxa with low relative abundance. In addition analyzing the infant results on two different sequencing runs did not introduce bias since once again little separation was observed on weighted UniFrac.

Unlike what has been previously reported in the literature (Azad et al., 2013; Bezirtzoglou et al., 2011; Biasucci et al., 2008) our diversity scores did not differ based on clinical factors such as type of milk ingested, or delivery method. This could imply that supplementation with probiotics standardizes the number of bacterial taxa in the microbiota. Alternatively, these environmental variables may only play a role in full term infants, and may not influence preterm infants to the same degree. The latter however, is not in keeping with the findings of Jacquot et al. since their preterm infants born via C-section had higher diversity scores (Jacquot et al., 2011). In our cohort, gestational age was the only variable that impacted diversity. Infants born at a more advanced gestational age (i.e. those that were the least premature) exhibiting higher bacterial diversity scores. Furthermore, post conceptual age which takes the postnatal age into account along with the gestational age at birth did not affect our diversity scores, unlike what has previously been published (La Rosa et al., 2014). It may be that young gestational age, which is a prenatal factor, genetically predisposes infants to a less diverse microbiota. We did however, note similarities with the La Rosa paper, in that in a small subset of our patients that had consecutive sampling, we also observed that Gammaproteobacteria was the most relatively abundant bacterial class. In addition 2 of our subjects displayed the pattern of bacterial composition described by La Rosa et al. namely early appearance of Bacilli followed by Gammaproteobacteria, and

subsequently presence of Clostridia. This finding supports the argument that gestational age, a prenatal variable, pre-programs the microbiome. Another clue that genetics may influence the preterm infant microbiome more than post-natal environmental factors was our finding that twin and triplet infants clustered together with their siblings on PCA irrespective of their treatment group (P versus PL).

Our demographic results showed that we had comparable rates of antibiotic use in those receiving probiotics alone versus probiotics with the addition of lactoferrin. We know from multiple studies that antibiotics can affect bacterial diversity for months and perhaps years after administration (Dardas et al., 2014; Fouhy et al., 2012; Greenwood et al., 2014; Jacquot et al., 2011; Tanaka et al., 2009). An additional important finding in our cohort, with important clinical implications, was that infants exposed to antibiotics did not have lower diversity or richness scores compared to those who had never been exposed to antibiotics. This suggests that the use of probiotics and lactoferrin prevents the reduction in diversity normally associated with antibiotics. Although we would have expected to see increasing diversity and richness over time as the infants grew further away from their antibiotic exposure this was not the case. This may be due to the fact that our study only took into account how many days had passed since the infant had last been exposed to antibiotics. It was impossible to factor in the number of antibiotic courses per patient as well as the duration of each course of antibiotics into a meaningful analysis. A more uniform study with a standardized antibiotic approach where all infants receive the same type, duration and number of antibiotic courses would perhaps have yielded different results, yet for ethical reasons this is not feasible.

Our piglet model, which provided a more ideal experiment without the confounding

variables encountered in a clinical human study, did however show increased diversity after 1 week of probiotic supplementation compared to a control group. Furthermore looking at diversity over time we observed that the addition of probiotic as well as probiotics with lactoferrin had a steeper slope suggesting that even though all pigs will attain an increased diversity with time, supplementation with probiotics and lactoferrin in the newborn period speeds up the process, a desirable outcome from a clinical perspective. In contrast, the lactoferrin alone group did not display a trend towards increased bacterial richness over time. A logical explanation is the fact that lactoferrin has a bacteriostatic effect (Embleton et al., 2013), thereby inhibiting the replication of bacteria, yielding a less rich bacterial community. The addition of probiotic in addition to lactoferrin may inhibit the bacteriostatic effect of lactoferrin alone. This line of reasoning is similar to our conclusion that probiotic supplementation in the infant group prevented the usual decrease in alpha diversity observed when infants receive antibiotics.

5.3 Possible explanations for the beneficial effect of lactoferrin

A recent study in piglets supplemented with bovine lactoferrin found that this dietary supplement affected the mesenteric lymph nodes and spleen immune cells response to stimulation with lipopolysaccharide and showed a protective immune response (Comstock et al., 2014). More specifically, the spleen cells from supplemented pigs produced 2 times more interleukin-10 and tumor necrosis factor alpha and the mesenteric lymph nodes produced 40% more interleukin-10 and interleukin-6 compared to control piglets. Furthermore serum immunoglobulin G levels were increased in the piglets supplemented with a high dose of lactoferrin. Comstock et al. also looked at the effect of dietary bovine

lactoferrin on the intestinal growth and maturation of piglets (Reznikov et al., 2014). They observed a 60% increase in crypt proliferation in the piglet group supplemented with bovine lactoferrin. These are interesting findings and while we would like to extrapolate their findings to our piglet group there are some important differences in methodology. Unlike our animals, their piglets were colostrum deprived, since colostrum contains very high level of lactoferrin, and were instead given pregnant sow serum by oro-gastric gavage after birth to ensure the piglets received some passive immunity. Their dose of lactoferrin was also much higher at 367 mg and 1300 mg of lactoferrin per kg of birth weight per day for the low and high dose lactoferrin groups respectively. In contrast, our piglets received 100 mg per day or approximately 62.6 mg/kg of admission weight per day, based on the average weight of our piglets on arrival to our animal care facility. The authors explain that they attempted to imitate the daily dose (low dose group) and five times the daily dose (high dose group) of lactoferrin that an exclusively breast-fed infant would receive. These results are important since they show a link between lactoferrin and cytokine response. What remains to be determined is the precise mechanism. The authors postulate that lactoferrin might exert its effect by absorption from the gastrointestinal tract and direct binding of lactoferrin to lactoferrin receptors found on immune cells (T cells, B cells, NK cells, and dendritic cells). Alternatively lactoferrin may act locally on gut immune cells that then travel to the lymph nodes and spleen. Our study shows that lactoferrin significantly affects the microbiota composition of preterm infants and piglets suggesting that lactoferrin might be exerting its effect on the immune system via changes to the microbiota. However, our results are descriptive and focus on which bacterial taxa are present in the intestine of piglets receiving lactoferrin, additional studies are clearly needed to explore the modulation of the microbiome in relation to the immune system.

5.4 The inflammatory microbiome profile

Dysbiosis has been defined by Petersen et al. (Petersen and Round, 2014) as "any change to the composition of resident commensal communities relative to the community found in healthy individuals". The authors further classify dysbiosis into three different subtypes; firstly, the loss of beneficial bacteria, secondly, an increased abundance of potentially pathogenic bacteria, and lastly a decrease in bacterial diversity. Interestingly, premature infants similar to Crohn's disease (CD) patients, display a gut microbiota with decreased presence of commensal bacteria compared to their healthy peers (Gevers et al., 2014). In the same way that no single pathogen induces CD, no single bacterial species is responsible for the dysbiosis present in premature infants. One of the theories behind dysbiosis in CD patients is that oxygen levels in the luminal gut increase in the presence of inflammation, and as such, a shift in the microbiota towards more aerobic bacteria can be observed as an adaptive measure to oxidative stress (Mimouna et al., 2011). Likewise our premature infants displayed a high relative abundance of facultative anaerobes like *Enterobacteriacea* and *Staphylococcus*.

Some bacteria may be classified as pro-inflammatory, for example, those that produce hydrogen disulfide (H_2S), since H_2S is detrimental to colonocytes due to its inhibition of butyrate oxidation (Pitcher and Cummings, 1996), the food source for colonocytes (Cummings, 1981). In fact, increased hydrogen disulfide production has been proposed in the pathogenesis of ulcerative colitis (Roediger et al., 1993). *Desulfovibrio* is one such example of a sulfate reducing bacteria that has been found in the gut of ulcerative colitis patients (Gibson et al., 1991). In contrast commensal bacteria may be exhibiting their beneficial effect to the host by reducing inflammation. One such example is

Faecalibacterium prausnitzii, which was found to block NF-kB activation and IL-8 production both in vitro and in mouse models of ulcerative colitis (Sokol et al., 2008).

In our infants although we only had 2 groups (P versus PL) and lacked a true control group, our results, suggested that the addition of lactoferrin to probiotics decreased some of the IBD associated pro-inflammatory bacteria. We used metagenomeSeq to identify taxa with at least a 2-fold change in relative abundance between treatment groups. We know from the work of Gevers et al. that the intestinal mucosal microbiome of pediatric CD patients is richer in specific bacteria such as the *Enterobacteriacea*, *Pasteurellaceae*, *Veillonellaceae*, *Neisseriaceae*, *Gemellaceae*, and *Fusobacteriaceae* families and depleted in *Bacteroidales, Clostridiales* (specifically *Faecalibaceterium prausnitzii*)*, Erysipelotrichaceae*, and *Bifidobacteriaceae* (Gevers et al., 2014). Moreover, the presence of antibiotics amplified this dysbiosis. A different adult study also found increased *Fusobacterium* in the inflamed mucosa of IBD patients (Strauss et al., 2011). In infants receiving PL as compared to P alone we observed a decreased relative abundance of *Fusobacterium*, *Gemellaceae*, *Hemophilus parainflenza*, and *Veillonella*. In contrast we observed an increase in *Enterobacteriacea* in the PL versus P group, which suggests that the addition of lactoferrin to probiotics also promotes the growth of pro-inflammatory bacteria. The *Enterobacteriacea* family contains many potentially pathogenic bacteria, and we were expecting to observe a decrease in their relative abundance to explain the clinical results of decreased incidence of sepsis in low birth weight infants receiving lactoferrin (Manzoni et al., 2009). These results suggest that an increase in the relative abundance of *Enterobacteriaceae* is not a predictor of sepsis in preterm infants. *Enterobacteriaceae* may thrive in the presence of lactoferrin due to the presence of siderophores, an advantage that not all bacteria possess. Lactoferrin is an iron

chelator, thereby preventing pathogenic bacteria from obtaining iron, which in part explains its bacteriostatic effect. However *Enterobacteriaceae* produce siderophores (Searle et al., 2015), which can bind free iron as well as extract iron from other binding proteins (Parrow et al., 2013), thereby competing with lactoferrin.

It is not surprising that premature infants show a higher preponderance of pro-inflammatory bacteria. NEC, an often-devastating gastrointestinal emergency predominantly affects preterm infants. The precise mechanism of NEC is unknown but it is associated with inflammation and results in ischemic necrosis of the bowel wall (Neu and Walker, 2011). Furthermore sepsis is also much more common among preemies compared to full term infants, and many of the pathogens identified on blood culture originate from the gut (Vergnano et al., 2011).

With regards to our piglet results, overall for the potential pro-inflammatory bacteria we observed a decrease in *Enterobacteriaceae* in the P and PL group compared to controls, as well as a decrease in *Fusobacterium* in the L and PL group (but enriched in P group) compared to controls. With regards to what have been proposed as beneficial bacteria, we observed an increase in the relative abundance of the *Prevotella* genus (Bacteroidales order) in all supplemented pigs (P, L and PL), and an increase in the relative abundance of *Erysipelotrichaceae* in the P group, (but OTUs both increased/deceased for L and PL) as compared to controls. *Prevotella* is a bacterium commensal to the vaginal microbiota and is predominant in the gut of vaginally delivered infants (Dominguez-Bello et al., 2010). It has also been suggested that *Prevotella* exerts a beneficial effect on the host by converting dietary plant lignans into antioxidants (Schogor et al., 2014). Additionally our L, P and PL groups had a far greater relative abundance of the *Lactobacillus* genus for all 3 supplemented

groups (P, L and PL), as compared to control piglets who were not supplemented. *Lactobacillus rhamnosus* has been shown to be beneficial via its effect on tight junctions in the maintenance of the intestinal barrier (Laval et al., 2015). Probiotic and lactoferrin supplements may, therefore, be demonstrating their clinical benefit (decreased NEC and sepsis) by suppressing an inflammatory-like microbiome and promoting beneficial microbes.

5.5 Can probiotic strains colonize the gastrointestinal tract?

Molecular based sequencing methods utilize DNA as a means of identifying the bacteria present in the microbiome. In doing so it is not possible to differentiate live from dead bacteria, and therefore it is unclear whether probiotic strains are actually colonizing the intestinal tract. One adult study demonstrated a fecal probiotic recovery rate of approximately 20% compared to the amount that was orally ingested (Rochet et al., 2008). There is some evidence to support colonization of the gastrointestinal tract with probiotic strains in preterm infants. A study using *LGG* and *Bifidobacteriun longum* supplementation was able to show that their probiotic strains did indeed colonize premature infants (Rouge et al., 2009). At the genus level *Bifidobacterium* and *Lactobacillus* colonized a higher percentage of infants who received the probiotic compared to those that received a placebo using culture-PCR methods. Rouge et al. also observed differences in clinical outcomes, such as time required to achieve >50% enteral feeds based on infant weight. Those with a birth weight greater than 1000g had a shortened time to reach full enteral feeds. Rouge et al. used similar strains to our Florababy^{TM} and also studied premature infants, which suggests that our subjects may also have been colonized by the probiotic strains. Ideally we would have identified the "functional human gut microbiota," (Gosalbes et al., 2011) or the active bacteria in our samples, using a metatranscriptomic approach, and this remains another

limitation of our study.

In our study, *Bifidobacterium* was present at very low relative abundance. Despite administering probiotics with high levels of *Bifidobacterium*, this genus represented less than 1% of the total relative abundance in our fecal samples. Others have reported low levels of *Bifidobacterium* in preemies as well (Jacquot et al., 2011). Although it is possible that *Bifidobacterium* was absent in our subject's fecal microbiota, this is unlikely. Our choice of primer and/or DNA extraction technique likely introduced a bias. This is the most plausible explanation for the discrepancy between the low relative abundance of *Bifidobacterium* in our samples compared to the large amounts of administered probiotic. With regards to DNA extraction it has been shown that the addition of bead-beating for mechanical cell lysis greatly increases the abundance of *Bifidobacterium* identified from infant specimens (Walker et al., 2015). We incorporated this step in our DNA extraction method. Another important finding by Walker et al. is that universal primers do not amplify all bacteria equally. Using a broader universal forward primer led to the identification of 30% more *Bifidobacterium* compared to results obtained with the usual single universal primer (Walker et al., 2015). The PCR amplification step is a crucial step in identifying bacterial taxa through *16S rRNA* gene sequencing and the choice of primers can greatly influence the results obtained. Our primers were found to be inefficient at amplifying *Bifidobacterium* (data not shown). This result in all probability explains our findings.

5.6 Future directions

We plan on continuing to follow the microbiome of our cohort over time. We are fortunate that most NICU graduates are monitored closely, and as such we have begun collecting stool samples at the 18 month corrected age neurodevelopmental visit. In addition

parents were given a questionnaire to complete at the time of the scheduled clinic visit with questions related to their child's general health and more specifically presence of atopic disease or food allergies. We would eventually like to compare our cohort to a group of age-matched children who did not receive perinatal probiotic and lactoferrin supplementation.

We have also been approached by the manufacturers of FloraBABY^{TM}, who would like to explore other formulations of their probiotic such as single use sachets. The current product is supplied in powder form in a large container that is open and closed numerous times. Single serving formulations would even further reduce the risk of contamination with bacteria and fungi, which is a rare, but real concern. Recently a large supplier of probiotic (Solgar Inc, Leonia, NJ) recalled their ABC Dophilus®**,** a mixture of *Bifidobacterium lactis* and *LGG*, due to fungal contamination causing a fatal case of intestinal mucormycosis in a preterm infant (Vallabhaneni et al., 2015).

Many questions remain unanswered such as; which combination of probiotics is most advantageous, what is the ideal dosing of lactoferrin and which type (bovine versus human recombinant) would be most effective. It is evident that future research is required to help answer these questions.

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Contributions of Collaborators

Guillaume Romain and Jennifer Li: Guillaume (Stintzi lab, University of Ottawa) and Jennifer (Stintzi lab, University of Ottawa), helped with DNA extraction and 16S rRNA sample library preparation for sequencing. In addition Jennifer helped with LEfSe analysis.

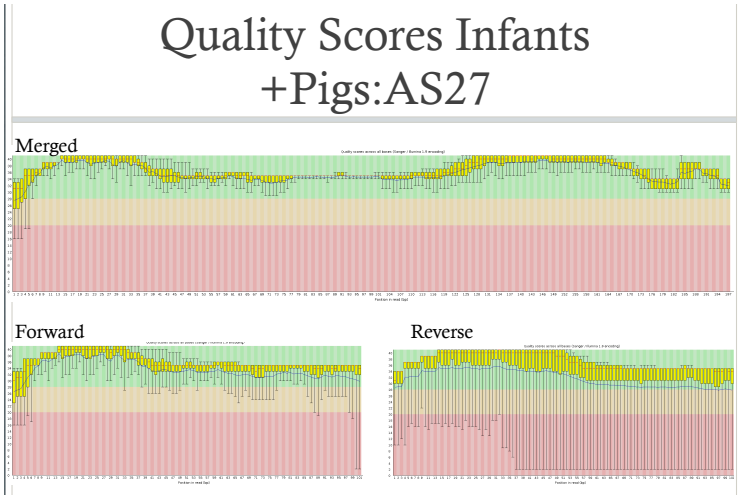
Dr. James Butcher and Dr. Annika Flint: Dr. James Butcher (Stintzi lab, University of Ottawa), contributed to the preparation of figures 2.2 and 2.3, and both he and Dr. Annika Flint (Stintzi lab, University of Ottawa) assisted in the maintenance and necropsy of neonatal piglets. Furthermore Dr. Butcher assisted with data analysis specifically by writing automated scripts for use in QIIME as well as MetagenomeSeq.

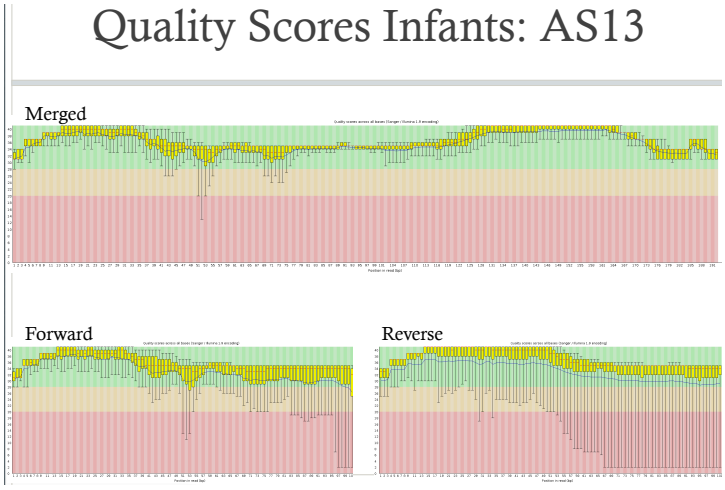
Dr. Alain Stintzi: Dr. Alain Stintzi (Department of Biochemistry, Microbiology and Immunology, University of Ottawa), contributed both as a supervisor to the project as a whole, and assisted with the maintenance, euthanasia, and necropsies for all experiments involving neonatal piglets.

Appendix I.
List of primers used for PCR reactions
F: forward, R: reverse

PCRFWD1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
PCRRVS1	CAAGCAGAAGACGCCATACGAGATCGGTCTCGGCATTCCGTCTGAACCGCTCTTCCGATCT
MF1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATAGCGAAACTCAAAKGAATTGACGG
MF2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGGGTAAACTCAAAKGAATTGACGG
MF3	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTCATAAACTCAAAKGAATTGACGG
MF4	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGATCGTAAACTCAAAKGAATTGACGG
MF5	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCCCGTAAACTCAAAKGAATTGACGG
MF6	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTGTCAAACTCAAAKGAATTGACGG
MF7	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCACGTAAACTCAAAKGAATTGACGG
MF8	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGTACGAAACTCAAAKGAATTGACGG
MF9	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGACAAACTCAAAKGAATTGACGG
MF10	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTAGAAAACTCAAAKGAATTGACGG
MF11	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCATAAACTCAAAKGAATTGACGG
MF12	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACTTAAACTCAAAKGAATTGACGG
MR1	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTATAGCGACGAGCTGACGACARCCATG
MR2	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTAGGGTACGAGCTGACGACARCCATG
MR3	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTCATACGAGCTGACGACARCCATG
MR4	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTGATCGTACGAGCTGACGACARCCATG
MR5	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTGCCCGTACGAGCTGACGACARCCATG
MR6	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTCTGTCACGAGCTGACGACARCCATG
MR7	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTCACGTACGAGCTGACGACARCCATG
MR8	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTCGTACGACGAGCTGACGACARCCATG
MR9	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTGGACACGAGCTGACGACARCCATG
MR10	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTTAGAACGAGCTGACGACARCCATG
MR11	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTTCATACGAGCTGACGACARCCATG
MR12	CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTACTTACGAGCTGACGACARCCATG

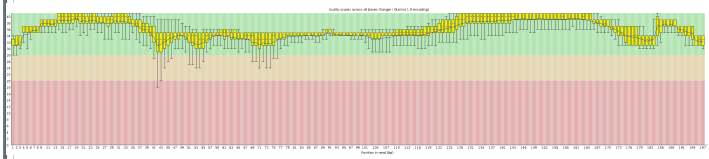
Appendix II.
Quality scores for the merged reads as well as forward and reverse reads received from the sequencing facility. Results are represented by sequence run (AS_ _). Samples were analyzed from 4 different sequence runs.



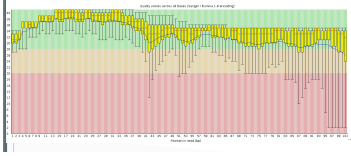


Quality Scores Piglets: AS07

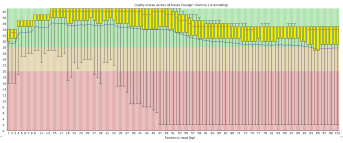
Merged



Forward

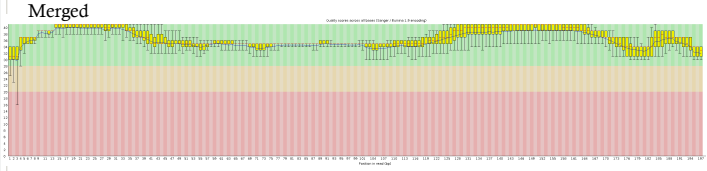


Reverse

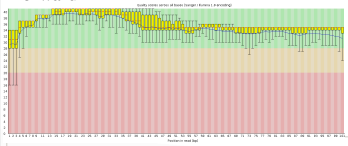


Quality Score Piglets: AS18

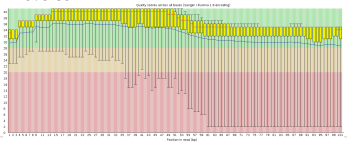
Merged



Forward



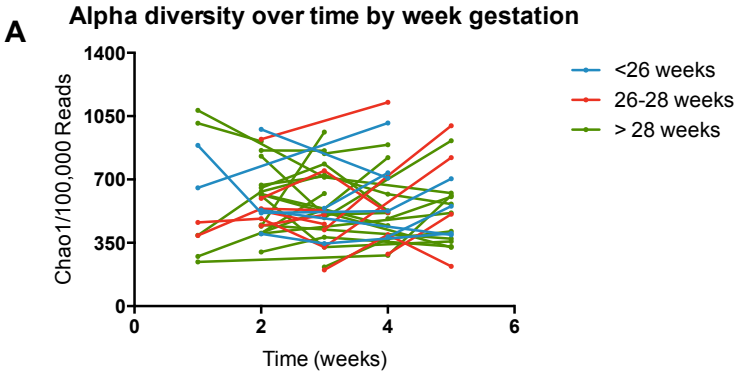
Reverse



Appendix III.

Alpha Diversity over time by week gestation

Each line represents a distinct patient and is color coordinated based on gestational age bracket at birth. Note that infants with only 1 sample were removed from the analysis.



Appendix IV. Differential relative abundance- probiotic group

Taxa	Probiotic	Taxa	Probiotic
988375 Butyricimonas;s__	5.740710075	341657 Anaerovibrio;s__	-2.011709002
175535 Bacteroides;s__	5.418478863	4267520 Ruminococcaceae;g__;s__	-2.014908775
208479 Butyricimonas;s__	5.41155383	158057 Faecalibacterium;s__prausnitzii	-2.016270702
516366 Bacteroidales;f__;g__;s__	4.684236957	185001 Lachnospiraceae;g__;s__	-2.041043235
269937 Prevotella;s__stercorea	4.171787604	512250 Blautia;s__	-2.115154993
4433339 Bacteroides;s__ovatus	4.148887767	4397737 Lachnospiraceae;g__;s__	-2.137989784
511971 Prevotella;s__stercorea	4.130887621	2415201 Clostridiales;f__;g__;s__	-2.142284143
4324245 Prevotella;s__stercorea	3.659607824	190453 Ruminococcaceae;g__;s__	-2.176106091
267845 Prevotella;s__	3.574258207	4425495 Bacteroides;s__	-2.248697512
821065 Prevotella;s__	3.543225084	1033345 [Prevotella];s__	-2.249454329
73937 Prevotella;s__	3.497277039	196604 Catenibacterium;s__	-2.277270079
4108811 Prevotella;s__stercorea	3.452062142	3430935 Faecalibacterium;s__prausnitzii	-2.292497698
360238 Erysipelotrichaceae;g__;s__	3.299325461	4333513 Ruminococcaceae;g__;s__	-2.322615348
522582 Parabacteroides;s__	3.262164348	373928 Clostridiales;f__;g__;s__	-2.339375818
921813 Parabacteroides;s__	3.151747863	70067 Blautia;s__producta	-2.395348681
353137 Bacteroidales;f__;g__;s__	3.13609848	295861 Clostridiales;f__;g__;s__	-2.418483222
306885 Bacteroidales;f__;g__;s__	3.124072516	712142 Ruminococcaceae;g__;s__	-2.428170252
4330813 Lactobacillus;s__	3.10755631	290468 Dorea;s__	-2.429457418
2176015 Mogibacterium;s__	3.026646668	4460680 Ruminococcaceae;g__;s__	-2.464366465
4392484 Bacteroides;s__	2.999980686	72926 Anaerovibrio;s__	-2.464587003
3479430 [Tissierellaceae];g__;s__	2.979628139	185158 Lachnospiraceae;g__;s__	-2.470403279
1760821 _Dorea;s__	2.968809919	527408 [Prevotella];s__	-2.497129575
287898 Clostridiales;f__;g__;s__	2.968198108	292167 Ruminococcaceae;g__;s__	-2.536104143
253380 Dorea;s__	2.89656732	4446898 Bacteroides;s__	-2.536569466
4307330 Ruminococcaceae;g__;s__	2.847919475	4296217 Blautia;s__	-2.565275116
316378 Clostridiaceae;g__;s__	2.731112896	2924870 Enterobacteriaceae;g__;s__	-2.88769925
309029 Bacteroidales;f__;g__;s__	2.727772339	308912 Christensenellaceae;g__;s__	-2.909759047
918187 Parabacteroides;s__	2.710852174	178342 Ruminococcaceae;g__;s__	-2.964576427
66188 Oscillospira;s__	2.701583483	875643 [Paraprevotellaceae];g__CF231;s__	-3.006915137
4356697 Ruminococcaceae;g__;s__	2.617247042	676472 Clostridiales;f__;g__;s__	-3.015402767
3220120 Clostridiaceae;g__;s__	2.613093096	364527 Ruminococcus;s__	-3.039589171
186233 Parabacteroides;s__distasonis	2.600607328	287539 Blautia;s__	-3.064248464
4419621 Sutterella;s__	2.590023476	184991 Clostridiales;f__;g__;s__	-3.123384282
1849383 Clostridia ;f__;g__;s__	2.584208287	105501 Clostridiales;f__;g__;s__	-3.131954311
110458 Christensenellaceae;g__;s__	2.580607802	34413 Lachnospiraceae;g__;s__	-3.202780314
4417947 [Ruminococcus];s__torques	2.573832795	23625 Deltaproteobacteria GMD14H09;f__;g__;s__	-3.207226502
4244333 Prevotella;s__stercorea	2.498119733	183684 Lachnospiraceae;g__;s__	-3.234569671
4412049 Bacteroides;s__ovatus	2.495799645	4335229 Ruminococcaceae;g__;s__	-3.432721876
3117556 Butyricimonas;s__	2.453867183	49461 [Paraprevotellaceae];g__;s__	-3.494530231
145856 [Mogibacteriaceae];g__;s__	2.451960649	331659 Ruminococcaceae;g__;s__	-3.525724944
516752 Phascolarctobacterium;s__	2.450869162	176765 Ruminococcaceae;g__;s__	-3.535304691
584137 Lachnospiraceae;g__;s__	2.443067528	3768338 Blautia;s__	-3.718065551
528141 S24-7;g__;s__	2.423391945	293013 Bacteroidales;f__S24-7;g__;s__	-4.008620338
518964 Prevotella;s__stercorea	2.418985943	50499 Lachnospiraceae;g__;s__	-4.213044988
288224 Lachnospiraceae;g__;s__	2.397043362	195840 Ruminococcaceae;g__;s__	-4.221018436
4439398 Fusobacterium;s__	2.387798655	351067 Blautia;s__	-4.343210443
293285 Ruminococcaceae;g__;s__	2.362921657	137353 Coprococcus;s__	-4.398237846
180825 Prevotella;s__copri	2.339346944	188001 Ruminococcus;s__	-4.650617272
731499 Bacteroidales;f__;g__;s__	2.324466452	288760 Clostridiales;f__;g__;s__	-5.212122538
4452633 Clostridiaceae;g__;s__	2.321698777		
1094991 Ruminococcaceae;g__;s__	2.286638841		
4303850 Ruminococcaceae;g__;s__	2.271278913		
2670715 Clostridium;s__	2.245915037		
578588 Clostridiaceae;g__;s__	2.243348561		
316539 Ruminococcaceae;g__;s__	2.226147596		
520657 Prevotella;s__stercorea	2.20654005		
184760 Ruminococcaceae;g__;s__	2.196747193		
572662 Prevotellaceae;g__;s__	2.185132236		
4465250 Bacteroides;s__	2.181725107		
262554 Ruminococcaceae;g__;s__	2.16364948		
52166 Veillonellaceae;g__Megasphaera;s__	2.125693155		
304106 Clostridiales;f__;g__;s__	2.111449633		
185597 Ruminococcaceae;g__;s__	2.104621246		
528752 Ruminococcaceae;g__;s__	2.099602315		
308495 Ruminococcaceae;g__Oscillospira;s__	2.09605677		
183137 Lachnospiraceae;g__;s__	2.088071375		
4340918 Bacteroidales;f__SB-1;g__;s__	2.086004168		
4338161 Clostridiales;f__;g__;s__	2.085665345		
4458576 Lachnospiraceae;g__;s__	2.038245629		
254846 Lachnospiraceae;g__;s__	2.01564212		

Table of all taxa showing differential relative abundance in metagenomeSeq of piglets receiving probiotics versus control piglets. Green: increased in probiotic group, Red: decreased in probiotic group

Appendix V. Differential relative abundance- lactoferrin group

Taxa	Lactoferrin	Taxa	Lactoferrin
103049 Veillonella;s__	6.8134075	3568684 Oscillospira;s__	-2.0071568
1144463 Veillonella;s__	5.066850877	536682 Ruminococcaceae;g__;s__	-2.0478854
2730944 Bacteroides;s__coprophilus	5.011074867	363735 Blautia;s__	-2.1631599
300859 Prevotella;s__	4.977577895	579239 Oceanospirillales;f__SUP05;g__;s__	-2.1636561
817140 Megasphaera;s__	4.914242402	195052 Clostridiales;f__;g__;s__	-2.2012273
299882 Prevotella;s__	4.869751743	4442523 Phascolarctobacterium;s__	-2.2809303
313121 Prevotella;s__copri	4.733944215	4297831 Clostridiales;f__;g__;s__	-2.2891199
157905 Bacteroides;s__coprophilus	4.728704002	247557 Oscillospira;s__	-2.2912051
522959 Prevotella;s__stercorea	4.552723934	4355732 Bacteroides;s__fragilis	-2.2939014
4480090 Megasphaera;s__	4.501990302	184678 Faecalibacterium;s__prausnitzii	-2.3080646
9498 Pasteurellaceae;g__;s__	4.299333795	811453 Oscillospira;s__	-2.3289539
516366 Bacteroidales;f__;g__;s__	4.27016148	2855173 Ruminococcaceae;g__;s__	-2.3679442
587570 Aerobiospirillum;s__	4.211065074	192071 Dorea;s__	-2.3761504
301984 Ruminococcaceae;g__;s__	4.096125607	319798 Oscillospira;s__	-2.3835709
52166 Megasphaera;s__	3.941434091	1126373 Ruminococcaceae;g__;s__	-2.386991
4452633 Clostridiaceae;g__;s__	3.757303638	145856 [Mogibacteriaceae];g__;s__	-2.4005204
266210 Megasphaera;s__	3.745467872	216710 Ruminococcaceae;g__;s__	-2.4077907
4338116 Clostridiales;f__;g__;s__	3.637743234	178849 Clostridiales;f__;g__;s__	-2.4709155
3220120 Clostridiaceae;g__;s__	3.61128466	296082 Prevotella;s__	-2.480594
344318 Prevotella;s__copri	3.555869371	300619 Oscillospira;s__	-2.5064581
182764 Lactobacillus;s__	3.481929523	192111 Bacteroides;s__eggerthii	-2.5340819
181717 Clostridiales;f__;g__;s__	3.474325021	297038 Ruminococcus;s__	-2.5379284
849723 Erysipelotrichaceae;g__;s__	3.424626189	4355086 Lachnospiraceae;g__;s__	-2.5481484
1023075 Veillonella;s__	3.249618474	538256 [Paraprevotellaceae];g__YRC22;s__	-2.5506271
259150 Lactobacillus;s__	3.224559172	4365903 Bacteroides;s__fragilis	-2.6613373
4371295 Ruminococcaceae;g__;s__	3.220536101	354461 Ruminococcaceae;g__;s__	-2.661799
821065 Prevotella;s__	3.210638134	1766666 Lachnospiraceae;g__;s__	-2.7413293
4465561 Prevotella;s__melaninogenica	3.026558119	208949 Bacteroides;s__	-2.7486485
3035308 Prevotella;s__copri	3.023339793	375106 Ruminococcaceae;g__;s__	-2.7532089
581201 Ruminococcaceae;g__;s__	3.022587505	4460356 Desulfosporosinus;s__meridiei	-2.7562564
267845 Prevotella;s__	2.929881775	289449 Clostridiales;f__;g__;s__	-2.7772766
17375 Veillonellaceae;g__;s__	2.906764282	732765 Bacteroides;s__	-2.7967372
1849383 Clostridia;o__;f__;g__;s__	2.835181842	307536 Clostridiales;f__;g__;s__	-2.8156089
1121926 Clostridiales;f__;g__;s__	2.78397985	230933 Ruminococcaceae;g__;s__	-2.8350431
354971 Lactobacillus;s__	2.739231678	182377 Lachnospiraceae;g__;s__	-2.8367623
4369901 [Paraprevotellaceae];g__CF231;s__	2.714788367	182117 Bacteroides;s__	-2.8490311
3185345 Blautia;s__	2.70519002	305997 Blautia;s__	-2.8590149
298050 Megasphaera;s__	2.689360806	1869566 [Ruminococcus];s__gvus	-2.8933086
254846 Lachnospiraceae;g__;s__	2.666663872	356760 Erysipelotrichaceae;g__;s__	-2.916021
298988 Streptococcus;s__	2.659433872	4444529 Rikenellaceae;g__;s__	-3.0333377
590168 Lactobacillus;s__	2.655718955	4380438 Bacteroides;s__fragilis	-3.0592776
586093 Lactobacillus;s__	2.619930364	4333513 Ruminococcaceae;g__;s__	-3.1438998
349970 Veillonellaceae;g__;s__	2.611399589	519763 Oscillospira;s__	-3.1568843
4330813 Lactobacillus;s__	2.604079116	190676 Oscillospira;s__	-3.2117758
4374663 Lactobacillus;s__vagilis	2.594721565	584951 Ruminococcaceae;g__;s__	-3.249823
149335 Mitsuokella;s__	2.593392057	847228 Parabacteroides;s__	-3.2504637
4371786 [Barnesiellaceae];g__;s__	2.53689638	4194837 Coprococcus;s__	-3.2513165
3430775 [Tissierellaceae];g__;s__	2.5303871	192936 Faecalibacterium;s__prausnitzii	-3.3885058
47365 Lactobacillus;s__	2.527604799	36611 Lachnospiraceae;g__;s__	-3.4260982
298954 Lactobacillus;s__	2.523622203	4387469 Ruminococcaceae;g__;s__	-3.4325439
3818299 Lachnospiraceae;g__;s__	2.50380168	181575 Faecalibacterium;s__prausnitzii	-3.4627562
4359590 Enterococcus;s__	2.409313521	175761 Ruminococcaceae;g__;s__	-3.5068507
4316928 Enterococcus;s__	2.387406759	185542 Ruminococcaceae;g__;s__	-3.5202059
3479430 [Tissierellaceae];g__;s__	2.358148878	352733 Lachnospiraceae;g__;s__	-3.54614
308249 Lactobacillus;s__	2.328198081	519398 Lachnospiraceae;g__;s__	-3.5821333
276834 Lactobacillus;s__	2.323575945	712142 Ruminococcaceae;g__;s__	-3.6080207
584001 Lactobacillus;s__	2.303646644	4392487 Faecalibacterium;s__prausnitzii	-3.8029564
315256 Clostridiales;f__;g__;s__	2.291242041	213813 Bacteroides;s__	-3.8433563
3715618 Clostridiales;f__;g__;s__	2.265994472	232975 Oscillospira;s__	-3.9231506
248492 Ruminococcaceae;g__;s__	2.254084975	4357809 Fusobacterium;s__	-3.976038
187000 Ruminococcaceae;g__;s__	2.231431449	579541 Ruminococcaceae;g__;s__	-3.9888281
269058 Lactobacillus;s__	2.133701875	323024 RF39;f__;g__;s__	-4.1472246
137580 Lactobacillus;s__	2.116659234	295861 Clostridiales;f__;g__;s__	-4.4234706
350349 Lactobacillus;s__	2.108560081	2415201 Clostridiales;f__;g__;s__	-4.6190005
292276 Lactobacillus;s__	2.100811966	527408 [Prevotella];s__	-4.6882301
4349517 Clostridiaceae;g__;s__	2.086251417	4309137 Clostridium;s__	-4.8540619
194472 Lachnospiraceae;g__;s__	2.066453489	288760 Clostridiales;f__;g__;s__	-4.9602062
4200918 Lactobacillus;s__	2.03848135	531353 Parabacteroides;s__	-5.4813903
		524661 Bacteroides;s__	-6.7634007

Table of all taxa showing differential relative abundance in metagenomeSeq of piglets receiving lactoferrin versus control piglets. Green: increased in lactoferrin group, Red: decreased in lactoferrin group

Appendix VI. Differential relative abundance- probiotic+lactoferrin group

Taxa	Probiotic+Lactoferrin	Taxa	Probiotic+Lactoferrin
360238 Erysipelotrichaceae;g__;s__	4.067447563	190646 Ruminococcaceae;g__;s__	-2.000320983
3117556 Butyricimonas;s__	3.270418243	1084643 Mogibacterium;s__	-2.009317494
306885 Bacteroidales;f__;g__;s__	3.207597771	3073894 Fusobacterium;s__	-2.012432179
17518 Bacillales;f__;g__;s__	3.164659826	175761 Ruminococcaceae;g__;s__	-2.093983474
3818299 Lachnospiraceae;g__;s__	3.050608011	4432154 Enterococcus;s__	-2.101875755
2442706 Christensenellaceae;g__;s__	3.046257423	4449244 Fusobacterium;s__	-2.102014131
4338116 Clostridiales;f__;g__;s__	3.042308215	323024 Mollicutes RF39;f__;g__;s__	-2.114781719
4458700 [Ruminococcus];s__	2.980591631	323001 Ruminococcaceae;g__;s__	-2.194306531
4371786 [Barnesiellaceae];g__;s__	2.925438911	185001 Lachnospiraceae;g__;s__	-2.207929154
16512 Clostridiales;f__;g__;s__	2.858149137	1813887 Ruminococcaceae;g__Ruminococcus;s__	-2.270342598
349246 Ruminococcaceae;g__;s__	2.846836961	185542 Ruminococcaceae;g__;s__	-2.357996567
3220120 Clostridiaceae;g__;s__	2.815158268	4267520 Ruminococcaceae;g__;s__	-2.392021135
4307122 Odoribacter;s__	2.807850843	509383 Lachnospiraceae;g__;s__	-2.439492435
1121926 Clostridiales;f__;g__;s__	2.722558845	343453 [Prevotella];s__	-2.444286696
516366 Bacteroidales;f__;g__;s__	2.690546813	3308041 Clostridiales;f__;g__;s__	-2.499165588
817140 Megasphaera;s__	2.68560766	190453 Ruminococcaceae;g__;s__	-2.499340354
312364 Clostridiales;f__;g__;s__	2.674834828	801210 Sutterella;s__	-2.50247148
590085 Clostridiales;f__;g__;s__	2.558076819	291709 Clostridiales;f__;g__;s__	-2.519928981
522582 _Parabacteroides;s__	2.546736513	875643 [Paraprevotellaceae];g__CF231;s__	-2.522534973
4464787 Clostridiales;f__;g__;s__	2.525414782	23625 Deltaproteobacteria GMD14H09;f__;g__;s__	-2.531078105
4380971 Clostridium;s__	2.514373266	178342 _Ruminococcaceae;g__;s__	-2.587979479
1026524 Oscillospira;s__	2.498648912	356760 Erysipelotrichaceae;g__;s__	-2.614676406
297143 Peptostreptococcaceae;g__;s__	2.488769577	527408 [Prevotella];s__	-2.62137916
4410461 Clostridium;s__perfringens	2.435041899	307536 Clostridiales;f__;g__;s__	-2.624017015
4396688 [Ruminococcus];s__	2.387055061	4476640 Bacteroidales;f__;g__;s__	-2.673312364
301984 Ruminococcaceae;g__;s__	2.340584779	911254 Bacteroidales;f__;g__;s__	-2.685347619
606927 Peptostreptococcaceae;g__;s__	2.328379218	184991 Clostridiales;f__;g__;s__	-2.744581894
316378 Clostridiaceae;g__;s__	2.318534626	183684 Lachnospiraceae;g__;s__	-2.769079601
4446120 Oscillospira;s__	2.273995106	4445574 Fusobacterium;s__	-2.841747669
52166 Megasphaera;s__	2.273279513	3430935 Faecalibacterium;s__prausnitzii	-2.891616056
297963 Clostridiales;f__;g__;s__	2.255328189	693641 Pirellulaceae;g__;s__	-2.899565678
4429608 Lachnospiraceae;g__;s__	2.234705041	149646 Veillonellaceae;g__;s__	-2.906898358
201491 Rhodocyclaceae;g__TS34;s__	2.232613335	300853 [Paraprevotellaceae];g__CF231;s__	-2.915360158
317749 Ruminococcaceae;g__;s__	2.215835442	341657 Anaerovibrio;s__	-3.012835805
4315789 Ruminococcaceae;g__;s__	2.187059893	185158 Lachnospiraceae;g__;s__	-3.156765392
304106 Clostridiales;f__;g__;s__	2.165008486	4426266 Bacteroides;s__	-3.167054557
336725 Bacteroidales;f__;g__;s__	2.148428581	72926 Anaerovibrio;s__	-3.289750696
508875 Lachnospiraceae;g__;s__	2.128314663	4452400 Lachnospiraceae;g__;s__	-3.32046146
569872 Clostridiales;f__;g__;s__	2.12630082	515074 [Paraprevotellaceae];g__[Prevotella];s__	-3.478226653
334529 Clostridiales;f__;g__;s__	2.122998361	1033345 [Paraprevotellaceae];g__[Prevotella];s__	-3.522325483
4465250 Bacteroides;s__	2.098950711	34413 Lachnospiraceae;g__;s__	-3.572274651
4366524 Clostridiales;f__;g__;s__	2.074079348	2924870 Enterobacteriaceae;g__;s__	-3.644182383
196986 Lachnospiraceae;g__;s__	2.071326196	176765 Ruminococcaceae;g__;s__	-3.688196577
3185345 Blautia;s__	2.056280867	4447188 Bacteroides;s__uniformis	-3.702398715
839545 Dorea;s__	2.0549014	524661 Bacteroides;s__	-3.807509106
321825 Oscillospira;s__	2.047992256	213813 Bacteroides;s__	-3.835548945
2006243 Lachnospiraceae;g__;s__	2.04215522	512250 Lachnospiraceae;g__Blautia;s__	-4.094657791
987581 Prevotella;s__	2.041509488	49461 [Paraprevotellaceae];g__;s__	-4.147466926
461851 Streptococcus;s__	2.035724285	288760 Clostridiales;f__;g__;s__	-4.203531743
4476936 Epulopiscium;s__	2.030344878	4012190 Bacteroides;s__	-4.306051153
191273 [Ruminococcus];s__	2.029202287	293013 Bacteroidales;f__S24-7;g__;s__	-4.841055069
522959 Prevotella;s__stercorea	2.024941173	195840 Ruminococcaceae;g__;s__	-5.034734416
697548 Ruminococcaceae;g__;s__	2.01873692	208949 Bacteroides;s__	-5.603318822
137043 Lactobacillus;s__	2.004303445		

Table of all taxa showing differential relative abundance in metagenomeSeq of piglets receiving probiotic+ lactoferrin versus control piglets. Green: increased in probiotic+lactoferrin group, Red: decreased in probiotic+lactoferrin group

Kelly Grzywacz MD FRCPC

Curriculum vitae

Née: 23 février 1981

Citoyenne: Canada

Langues parlées: Anglais, Français (intermédiaire: Hébreu)

Gastroentérologue pédiatrique CHU Sainte Justine
Professeure adjointe de clinique du Département de pédiatrie de la Faculté de médecine de l'université de Montréal

Formation clinique

2000/08 à 2000/12	Semestre en Biologie à l'Université de Cornell, Ithaca, NY, États-Unis
2001/01 à 2003/06	BSc. Physiologie, Université de McGill, Montréal, (Québec) Canada
2003/08 à 2007/06	MD, Université de Montréal, Montréal, (Québec) Canada
2007/07 à 2010/06	Résidente en Pédiatrie, A.I. DuPont Hospital for Children, Wilmington, DE, Jefferson Medical College, Philadelphie, PA, États-Unis
2010/07 à 2012/06	Fellow en Gastroentérologie pédiatrique, CHU Sainte Justine, Université de Montréal, Montréal, (Québec) Canada

Formation complémentaire ou postdoctorale

2012/7à 2016	Maitrise en Biochimie, Université d'Ottawa, Ottawa, (ON), Canada Projet de Recherche : L'influence de la lactoferrine sur le microbiome des bébés prématurés recevant des probiotiques. Étude prospective en collaboration avec le CHU Sainte Justine. Analyse des données et rédaction de thèse en cours Superviseur : Alain Stintzi PhD

Diplômes et certificats

2005/07/14	United States Medical Licensing Examination (USMLE) Step 1
2007/04/10	USMLE Step 2 CS
2007/05/09	USMLE Step 2 CK

2008/05/05	USMLE Step 3
2008/10	Licencié du conseil médical du Canada (LCMC)
2010/10/18	Fellow of the American Academy of Pediatrics (FAAP)
2011/06/30	Fellow of the Royal College of Physicans of Canada (FRCPC) Pediatrics
2012/10/04	FRCPC Gastroenterology

Maintien de la compétence

Assistance à des congrès et colloques

2010/10/21 à 24	Participation au cours post gradué et congrès annuel du North American Society for Pediatric Gastroenterology Hepatology and Nutrition (NASPGHAN) New Orleans, LA, États-Unis
2011/01/13 à 16	Assistance au congrès des fellows de première année de NASPGHAN Fort Lauderdale, FL, États-Unis
2011/02/23 à 25 2011/02/26 à 03/01	Participation au Gastroenterology Residents-in-Training (GRIT) course Participation au congrès Canadian Digestive Diseases Week (CDDW) Vancouver, BC, Canada
2012/02/21 à 23 2012/02/24 à 27	Participation au cours de GRIT Participation au congrès CDDW, Montréal, Qc, Canada
2012/04/03	Participation au congrès conjoint de la recherche des résidents de pédiatrie UdeM-McGill , Hôpital de Montréal pour enfants, Montréal, Qc, Canada
2012/11/01 à 04	Assistance au 6^{e} congrès Canadian des fellows en gastroentérologie Programme de maladies inflammatoires de l'intestin Canada Future Directions in IBD (CCFC) Toronto, ON, Canada
2013/02/7 à 10	Participation au 32^{e} congrès annuel de recherche Mead Johnson de NASPGHAN Forum pour gastroentérologues pédiatriques Scottsdale, Arizona, États-Unis
2013/04/09	Participation au congrès conjoint de la recherche des résidents de pédiatrie UdeM-McGill, CHU Sainte Justine, Montréal, Qc, Canada

2013/04/19 à 20	Assistance au congrès de NASPGHAN Nutrition University Chicago, IL, États Unis
2013/05/19	Participation à la journée de poster de Biochimie Microbiologie, Immunologie (BMI) de l'Université d'Ottawa, Ottawa, ON, Canada
2013/06/11 à 12	Participation au congrès : Ottawa Institute of Systems Biology (OISB) Symposium Mont Tremblant, Qc, Canada
2013/10/9 à 12	Participation au cours pour post gradués et congrès annuel de NASPGHAN, Chicago, IL, États-Unis
2013/12/12 à 14	Assistance au congrès de Crohn's and Colitis Foundation of America (CCFA) Advances in Inflammatory Bowel Disease, Crohn's and Colitis Foundation's Clinical and Research Conference Hollywood, Florida, États-Unis
2014/10/22	Participation au congrès annuel de recherche de CHEO Ottawa, ON, Canada
2014/10/23 à 26	Participation au cours pour post gradués et congrès annuel de NASPGHAN Atlanta, Géorgie, États-Unis
2015/10/8	Participation au cours pour post gradués et congrès annuel de NASPGHAN Washington DC, État-Unis

Expérience et emplois antérieurs

2002/05 à 07	Projet de recherche sur la leucémie lymphocytaire chronique et l'imatinib L'Institut Lady Davis, Hôpital Général Juif Superviseur : Lawrence Panasci MD
2012-2014	Gastroentérologue au CHU Sainte Justine

Bourses de formation obtenues

2012-2014	Bourse en jumelage de fonds et bourse académique du CHU Sainte Justine
2012-2014	Bourse d'excellence aux études supérieures, Université d'Ottawa, Ottawa, ON, Canada

Prix obtenus pendant la formation

2000	Liste du Doyen, Université Cornell, Ithaca, NY, États-Unis
2002	Rhona and Irving Levitt Family Foundation Scholarship for Undergraduate Science
2003	Université de McGill, Graduated with Great Distinction
2009	Regan Fellowship Award, Operation Smile
2010/06	Philadelphia Pediatric Society Award
2013/10	Poster de distinction -Congrès annuel de NASPGHAN The effects of lactoferrin supplementation on the intestinal microbiota of premature infants receiving probiotics

PUBLICATIONS

Publications de recherche ou d'érudition dans des revues dotées de Comités de pairs

1. Aloyz R, **Grzywacz K**, Xu ZY, Loignon M, Alaoui-Jamali MA, Panasci L. Imatinib sensitizes CLL Lymphocytes to Chlorambucil. Leukemia 2004; 18(3) : 409-414

2. Bruchim I, Gotlieb WH, Mahmud S, Tunitsky E, **Grzywacz K**, Ferenczy A. HPV-related vulvar intraepithelial neoplasia: outcome of different management modalities. Int J Gynaecol Obstet. 2007; 99(1): 23-7

3. **Grzywacz K**, Brochu P, Beaunoyer M, Lallier M, Alvarez F. Neonatal Peliosis with maternal ingestion of pesticides. J Pediatr Gastroenterol Nutr. 2014 Feb; 58(2):e14-7

Abrégés publiés

1. **Grzywacz K**, Kahn S. Atopy patch testing is a strong complementary test to skin prick test in eosinophilic esophagitis. In: Annual meeting of the North American Society for Pediatric Gastroenterology Hepatology and Nutrition (NASPGHAN); 2010 Oct 21-23 New Orleans, LA. JPGN Volume 51 Suppl 2 2010 p E 41. Abstract nr 100.

2. Chapuy L, **Grzywacz K**, Pomerleau M, Perrrault P, Faure C. Lack of Efficacy of Topical Mitomycin C application in refractory esophageal strictures after surgical repair of esophageal atresia. In : Annual meeting of the North American Society for Pediatric Gastroenterology Hepatology and Nutrition (NASPGHAN); 2011 Oct 20-23; Orlando, Fl. JPGN Volume 53, Suppl 1 2011 p E 78. Abstract nr 284.

3. **Grzywacz K**, Herzog D, Faure C, Dal Soglio D. Duodenal ulcers with hemorrhage or

perforation as a first manifestation of eosinophilic gastroenteropathy in pediatric patients [abstract]. In : Annual meeting of the Canadian Association of Gastroenterology (CAG) Canadian Digestive Diseases Week (CDDW); 2011 Feb 26-Mar 1; Vancouver, BC. Abstract nr 082.

4. **Grzywacz K**, Rebeuh J, Turpin S, Faure C. Does PET scan measure up to colonoscopy? [abstract]. In : Annual meeting of the Canadian Association of Gastroenterology (CAG) Canadian Digestive Diseases Week (CDDW); 2012 Feb 24-27; Montreal, Qc. Abstract nr 76.

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Services à la collectivité

2009/11 Operation Smile Caire : Mission humanitaire, 10 jours en Égypte pour réparation des fentes labio-palantine. Responsable pour le dépistage pré-op et soins post-op

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