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Investigating the enrichment and metabolic output of *Bifidobacterium* in the mammalian gut

By

JULES ANDREW LARKE
DISSERTATION

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Abstract

The human gut microbiota is intimately linked to our overall health by performing various functions that augment our metabolic, immunologic and physiologic characteristics. A key commensal taxon that resides within the infant gut is the genus *Bifidobacterium*. For over a century bifidobacteria have been studied in the context of the infant gut microbiota with continual discoveries describing their mutualistic relationship with the host. Diet is a major determinant of bifidobacteria colonization during infancy by which human milk feeding enriches for *Bifidobacterium* through the prebiotic effect of human milk oligosaccharides. In contrast, conventional formulas typically lack prebiotic structures consumed specifically by bifidobacteria. As such, the microbiota of human milk-fed compared with formula-fed infants assemble into distinct community states leading to disparate effects on the infant's metabolic and immunologic parameters. However, there are still knowledge gaps regarding the beneficial health effects conferred by *Bifidobacterium* species and the molecular basis of their enrichment in the gut. Through leveraging the strengths of different experimental designs centered on bifidobacteria, this body of work seeks to characterize the metabolic interactions of the host-*Bifidobacterium* relationship and better understand their role in the human gut microbiome.

The first chapter provides an overview of the relationship between bifidobacteria and the host including factors affecting early gut colonization by *Bifidobacterium* and their metabolic interactions. Cell culture of *Bifidobacterium pseudocatenulatum* (*B. pseudocatenulatum*), an infant gut isolate, grown in 2'-fucosyllactose (2'-FL) or lactose reveal that the fermentation product profiles are substrate specific. The second chapter focuses on developing a mouse model of HMO driven bifidobacteria colonization in which a synbiotic of *B. pseudocatenulatum* and 2'-FL enable persistent populations of bifidobacteria. Comparing local and systemic differences in microbial

derived metabolites in these mice provide insight into the dynamics of gut microbial perturbations that occur via persistent, rather than transient, enrichment of *Bifidobacterium*. Lastly, a prospective observational study in preterm infants supplemented with *B. longum* subsp. *infantis* (*B. infantis*) or *Lactobacillus reuteri* (*L. reuteri*) was conducted to contrast the changes in metabolite profiles in the gut from these probiotics and assess gut inflammation in relationship to these treatments.

The results of these studies have provided important insights pertaining to the modulation of host metabolism in the presence of bifidobacteria. Fermentation products excreted in cell culture are driven by the starting substrate, in this case 2'-FL, which are observed to increase in the intestine and circulation in mice colonized with the probiotic. Furthermore, absorption of 1,2-propanediol produced by *Bifidobacterium pseudocatenulatum* in the gut is enriched in tissues including liver and brain. This novel finding demonstrates that modulation of the gut microbiome has the potential to influence peripheral tissue metabolism with the possibility of altering brain biochemistry independently of the gut-brain axis. In preterm infants, metabolites in the gut lumen are consistent with a *Bifidobacterium*-enriched microbiota showing significant increases in several fermentation products produced by bifidobacteria. Additionally, gut inflammation is reduced in preterm infants provided bifidobacteria and is associated with the anti-inflammatory metabolite indole-3-lactate. Together, these studies demonstrate the potential of pre- and probiotics, i.e. 2'-FL and bifidobacteria, to enrich the gut microbiota with commensal microorganisms and impact host metabolism.

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Chapter 1: Introduction

1. Establishing the infant gut microbiome

The acquisition and development of intestinal microbiota represents a critical factor in the progression toward health or disease during early life stages. The developmental origins of health and disease (DOHaD) hypothesis states that exposures early on exert a strong influence on the health span of an individual¹⁻³. Thus, exposures during the neonatal period shape the microbial community structure that influence biochemistry within the host. Following parturition, the neonatal gut that was once free from microbial activity becomes populated with organisms present in the environment⁴. The succession of microbiota development is punctuated by exposures such as diet, antibiotics and mode of delivery⁵. Following vaginal birth, vertical transmission of maternal microbes more stably colonize the infant gut relative to bacteria acquired from the environment⁶. Moreover, the species acquired by vertical transmission are often associated with microbes known to be beneficial. Conversely, high rates of antibiotic exposure and delivery by caesarean section in preterm infants are associated with disrupted intestinal microbiota development⁷⁻⁹. Assembly of gut microbial communities is also observed as a function of gestational age at birth whereby the degree of prematurity is commensurate with the microbiota age, reflecting a delayed progression toward a stable microbial ecosystem^{10,11}.

An important taxon during establishment of the gut microbiota is the genus *Bifidobacterium*. These gram positive microbes have long been studied in association with humans, particularly in the context of the infant gut where their domination of the microbial community is not uncommon in breastfed infants. Bifidobacteria are uniquely situated to thrive within the infant gut due to adaptations in this environment, notably from the capacity to access nutrients within human milk^{12,13}. Utilization of milk glycans rapidly transforms the intestinal

lumen by reducing pH¹⁴ due to the release of short chain fatty acids and creating an anaerobic environment by the coupled metabolic actions of host and microbe¹⁵. The resulting environmental conditions are less favorable for colonization by gut pathogens, providing protection against virulent species¹⁶. Additional benefits of a bifidobacteria-dominated microbiota arise from fermentation products that enhance the development of host metabolism and immunologic function¹⁷. These early impacts on host physiology subsequently influence the individual's disposition toward or away from disease; leveraging this knowledge will help to develop new strategies of care such as microbiome-based therapies that extend the health span of future generations.

2. Bifidogenic properties of formula and milk oligosaccharides

The infant diet consists primarily of human milk and/or formula for at least the first six months of life. Diet has a major role in directing the configuration of the intestinal microbiome, and this effect is distinct when comparing exclusively breastfed (BF) and formula fed (FF) infants. This difference exists due in part to human milk oligosaccharides (HMOs) that influence the composition of the infant gut microbiota. Compositionally, HMOs are the third largest macromolecular component in human milk¹⁸. The glycosidic linkages within HMOs are configured such that they resist host enzymatic cleavage and pass through the intestine undigested. Notably, several members of the *Bifidobacterium* genus possess the glycoside hydrolases required to cleave HMOs¹⁹, permitting utilization of the sugar monomers. Many infant associated bifidobacteria have some ability to grow on HMOs in pure culture²⁰⁻²² with *B. longum* subsp. *infantis* (*B. infantis*) showing the greatest efficiency for growth on HMO substrates²³. In effect, *Bifidobacterium* are selected for and enriched by utilizing the HMOs that are largely inaccessible

to other microbes residing in the infant gut. However, differences in composition of human milk and formula have a substantial impact on gut microbiota development.

2.1 Formula prebiotics and Bifidobacterium enrichment

Similar to human milk, infant formulas are designed to provide complete nutrition until weaning. As such, formula is meant to perform as a human milk replacement by supplying all macro- and micronutrients. While this aspect of nutrition has been achieved, formulas have historically been deficient in providing non-digestible carbohydrates needed to feed the intestinal microbiota. It was only during the mid-1990s that the concept of adding prebiotics to modulate the colonic microbiota gained traction leading to the start of their addition to bovine milk based formulas²⁴. Additionally, the lack of added fiber can be attributed to the policy that guide nutritional intakes in the United States. The most current dietary reference intakes (DRIs) that indicate nutritional requirements during the first six months of life have no determined fiber intake level based on the reasoning that “human milk does not contain dietary fiber”²⁵. This statement is correct under their definition that dietary fiber is plant derived; however, the antiquated definition lacks meaning in this context and new recommendations are needed given the role of fiber in gut microbial development. A committee formed by the National Academy of Sciences has more recently acknowledged the importance of HMO related health benefits and the lack of DRIs for non-digestible carbohydrates during the first year of life²⁶. Together, the definitions dietary fibers and their level of consumption will need to be expanded to guide optimal intakes during infancy.

Beyond understanding the total amount of fiber needed to nourish the gut microbiota is the need to know the ideal types of fiber that encourage growth of commensal bacteria, specifically bifidobacteria. Many infant formulas include fructooligosaccharides (FOS) and/or galactooligosaccharides (GOS) to simulate the prebiotic effect that occurs from human milk

consumption. GOS produced from transglycosylation of lactose yields structures with a degree of polymerization (DP) between 2 and 8 units consisting of β -1,3, β -1,4 and β -1,6 linkages between galactose and glucose²⁷. Fructooligosaccharides have a DP of 5 to 60 units containing β -1,2 linked fructose with a terminal glucose residue²⁸. The bifidogenic effect of GOS and FOS has led to many studies aimed at identifying their efficacy in infant formulas. Previous studies using infant formula containing GOS have found an increase in bifidobacteria relative to formula containing no prebiotic²⁹⁻³¹. However, several past studies supplementing GOS/FOS lack species level resolution that can provide important ecological insights³²⁻³⁶. Understanding the growth characteristics of different *Bifidobacterium* species on various substrates can be used to tailor the prebiotic supplement to optimize intestinal colonization. Commonly observed *Bifidobacterium* species in the infant gut include *B. longum* subsp. *longum* (*B. longum*), *B. infantis* subsp. *infantis* (*B. infantis*), *B. breve*, *B. bifidum*, *B. catenulatum* and *B. pseudocatenulatum*³⁷⁻⁴¹. When grown in pure culture, *B. longum*, *B. infantis*, *B. breve* and *B. bifidum* each show strain specific growth responses to GOS or FOS⁴². Moreover, when strains of *B. longum*, *B. infantis* and *B. breve* are grown together under continuous co-culture, *B. breve* significantly outgrows either *B. longum* subspecies. Despite having the same capacity to use GOS and FOS, *B. breve* outperforms *B. infantis* during growth on the GOS/FOS media (0.2% wt/vol) with a doubling time for *B. infantis* nearly twice as long compared to *B. breve*. The rationale for this observation provided by the author was the increased utilization rate of GOS by *B. breve* relative to *B. infantis* in their pure culture growth assays. Interrogation of these strains at the gene level may help to identify the genetic determinates of GOS utilization and growth as has been shown with fucosyllactose transport and growth kinetics⁴³.

2.2 Human milk oligosaccharides and *Bifidobacterium*

Two main routes for HMO assimilation have been observed in species of *Bifidobacterium*; one in which the entire HMO structure is internalized and subsequently digested intracellularly, and the second where digestion of the glycosidic linkages occur externally with import of the sugar monomers. Bifidobacteria using the strategy of intact HMO internalization are also referred to “inside-eaters”, with examples including *B. infantis* and *B. breve*⁴⁴. Conversely, the “outside-eating” method of cleaving HMOs extracellularly has been observed in *B. bifidum*^{45,46} as well as some *B. longum*^{47,48} strains. The consequence of external HMO digestion is that it may foster cross-feeding in which liberated monosaccharides are captured and metabolized by other microbial community members. An example of this has been demonstrated *in vitro* by examining metabolism and growth of *B. bifidum* and *B. breve* in the presence of 2'-FL⁴⁹. In this experiment, cultures of *B. bifidum* were able to grow on 2'-FL, but not fucose alone, whereas strains of *B. breve* were able to grow on free fucose, but not on 2'-FL. Co-culture of the two species with 2'-FL revealed a disappearance of fucose indicating a syntrophic effect in which the fucose released by *B. bifidum* was used by *B. breve*. Strikingly, in continuous co-culture, the consumption of the 2'-FL hydrolysis products (lactose and fucose) by *B. breve* resulted in a growth advantage over *B. bifidum*. This study illustrates there are important ecological interactions across bifidobacteria and HMO metabolism that may translate to the developing infant microbiome. Interestingly, a study of breastfed infants showed that when *B. bifidum* was detected at greater than 10 percent of the total proportion of *Bifidobacteriaceae*, a significantly greater relative abundance of bifidobacteria was present relative to infants with less than 10% of *B. bifidum* as *Bifidobacteriaceae*⁵⁰. These findings may reflect a similar syntrophic interaction *in vivo*, where extracellular digestion of HMOs by *B. bifidum* enables cross-feeding of species that would otherwise be unable to consume those substrates. In summary, the routes of HMO transport vary across infant associated

bifidobacteria and together may promote syntrophic interactions that shape the presence and diversity of *Bifidobacterium* in the infant gut.

Understanding the ontology of bifidobacteria enriched infant gut microbiota necessitates further discussion of interspecies differences with respect to HMO utilization. *In silico* analysis of HMO utilization genes for 12 major HMOs using metagenome data for 91 *B. breve* and 21 *B. infantis* genomes revealed these genes are highly conserved in *B. infantis* (>75% prevalence)⁴⁴. In comparison, very few *B. breve* genomes had genes encoding fucosyllactose transport. Of the analyzed *B. breve* genomes, only 4% possessed FL transporter-1, and 8% with FL transporter-2 despite a common occurrence of intracellular α -1,2 fucosidase. The reduced capacity to catabolize HMOs has been described in previous work showing a select few *B. breve* strains that express fucosidase activity necessary to yield ample growth on 2'-FL⁴⁰. Beside fucosylated HMOs, each of the 24 strains in this study displayed a high growth capacity on the non-fucosylated HMOs LNT and LNnT, which is consistent with other reports on their degradation of these tetrasaccharides^{51,52}. Altogether, *B. infantis* is more specialized for overall HMO utilization through catabolism of a greater range of HMO structures compared to *B. breve* and other bifidobacteria. However, this does not lessen the importance of *B. breve* as an infant gut commensal. In the context of an infant fed human milk containing a variety of fucosylated and sialylated HMOs, *B. infantis* is more favored to proliferate. Conversely, infants fed formula supplemented with GOS or non-fucosylated HMO containing LNT or LNnT should yield a relatively equal competition between *B. infantis* over *B. breve*.

Subtle, yet discriminating genomic differences across strains of bifidobacteria can have notable effects on its growth via differences in substrate utilization. *B. infantis* is well-known for efficiently degrading HMOs; however, this efficiency is strain dependent and based on the compliment of

HMO utilization genes. *In vitro* culture of *B. infantis* EVC 001 exhibited significantly increased growth on both LNT and LNnT compared to 10 other strains of *B. infantis*, although no difference in growth was detected when 2'-FL was the sole carbon source⁵³. Comparing the bacterial genomes of these *B. infantis* strains revealed that only strain EVC 001 had an ABC-type transporter devoted to HMO binding, conferring a growth advantage. However, contrary to growth on single HMO structures, when growth was assessed using a combination of LNT, LNnT, and 2'-FL, no difference was observed between strains EVC 001 and NLS as a result of greater LNT, LNnT utilization in EVC 001 and higher 2'-FL consumption by NLS. The importance of substrate availability and bacterial strain cannot be overstated when assessing fitness and efficacy in probiotic organisms. With the more recent addition of HMOs to infant formulas there is a greater emphasis to consider these traits in conjunction with probiotic supplementation. A recent randomized double-blinded controlled trial examined the addition of 2'-fucosyllactose (2'-FL) and lacto-N-neotetraose (LNnT) to infant formula compared to the same formula without added HMOs beginning from the first two weeks of life to observe change in the microbiota composition at 3 months of age⁵⁴. Exclusively breastfed infants served as a reference group. The results showed that the HMO formula increased the total proportion of *Bifidobacterium* relative to control formula; however, there was no significant difference in the relative abundances of *B. longum* or *B. infantis*. Compared to breastfed infants the proportion of *Bifidobacterium* was not statistically different. However, a caveat to this study is the amount of HMO used in their formula is considerably lower than that found in mature human milk. The HMO content for this formula totaled 1.5 g/L (1.0 g/L of 2'-FL and 0.5 g/L of LNnT) which is an order of magnitude lower compared to milk collected during early lactation from secretor mothers at approximately 19 g/L⁵⁵. As infant formulas continually improve in an effort to more closely reflect the composition of human milk, attention

should be given to the types and concentrations of HMOs or other fibers and their impact on gut microbial development. Tailoring formula prebiotics will help to inform how to optimize infant gut microbiota and their related clinical outcomes as the microbiome assembles and progresses to its stable adult configuration.

2.3 Maternal factors affecting HMO composition

Human milk is the gold standard of infant nutrition, supporting optimal growth and development while encouraging colonization of commensal microbes in the infant gut microbiome. The oligosaccharide fraction of milk is incredibly diverse in its structural composition⁵⁶. Moreover, the structural diversity and concentration of HMOs varies based on maternal genotype⁵⁷⁻⁵⁹ and stage of lactation^{18,55,60}. Together, these factors mediate the prebiotic potential of milk that has a direct impact on the infant intestinal microbiome. Herein, variables that affect the composition of HMOs are explored and related to the influence of bifidobacterial colonization in the infant gut.

Maternal differences in genotype responsible for HMO composition includes a set of fucosyltransferase (FUT) enzymes which catalyze the linkage of fucose to the terminal glucose and galactose and N-acetylglucosamine residues in various tissues and biofluids⁵⁷. These FUT enzymes are widely studied due to their participation in a large proportion of glycosidic linkages in the milk glycome. Regarding the FUT family enzymes influencing HMO composition, FUT2 and FUT3 link fucose via respective α 1,2 and α 1, 3/4 linkages to the oligosaccharide backbone⁶¹. Additional terminology is often used to describe the activity of FUT2 and FUT3, which includes secretor status, with Se⁺ denoting a functional FUT2 enzyme, and Lewis group, with Le⁺ indicating a functioning FUT3 phenotype⁶². Phenotypically, secretor positive individuals show differences in the concentration of several HMOs with α 1,2 linkages, notably 2'-FL and lacto-N-

fucopentaose I (LNFP I) with smaller variations in other HMO structures^{56,59}. However, non-secretor mothers have a higher concentrations of lacto-N-tetraose (LNT), 3-fucosyllactose (3-FL) and LNFP II relative to those with secretor status^{63,64}. Differences in the infant microbiota have been observed due to maternal secretor status, with infants of secretor mothers reported to have higher levels of fecal *Bifidobacterium*⁶⁵. This observation may be a result of multiple factors including structural differences in HMOs from Se+ and Se- mothers as well as higher total HMO concentrations in secretor positive compared to secretor negatives⁶⁶.

Lactation stage has a prominent effect on the HMO content in human milk. Colostrum, the milk expressed in the first week of life has the highest concentration of HMOs ranging from 20-25 g/L, but as lactation progresses the quantity decreases over time to 5-15 g/L^{63,67-69}. Interestingly, there is an inverse temporal pattern in which bifidobacteria colonize the gut, illustrated by a lag period in which colonization starts within the first week and increases in breastfed infants up to around 6 months of age followed by a decline upon the introduction of complimentary foods^{39,41,70,71}. The initial high milk HMO content may be part of an evolutionary strategy to maximize colonization potential of *Bifidobacterium* and limit that of pathogenic microbes early on.

Mothers delivering preterm have different HMO profiles compared to those giving birth at term. Preterm delivery is associated with more variable concentrations of total or fucosylated HMOs compared to a term delivery^{64,69}, whereas other studies have found lower levels of fucosylated HMOs⁷² or greater sialylated HMOs⁷³ in milk from moms giving birth preterm. These results have important implications for the preterm infant microbiota. Many studies have found gestational age at birth to be a strong determinant of microbiome assembly with preterm infants exhibiting delayed maturation relative to term infants^{10,74-76}. While there are several factors that

contribute to this, it is reasonable to infer that an aberrant, or immature, HMO profile would contribute to this effect.

2.4 Donor milk

In the absence of a mother's own milk (MOM), donor human milk can be available for ill, preterm, or at-risk infants. Donor milk is considered second best to MOM as it retains some of the properties of breastmilk that confers immunologic and other benefits to the infant⁷⁷. The safety of donor milk has been well established when proper measures for handling and pasteurization are used^{78,79}, but it is reasonable to consider how the HMO composition differs in donor milk compared with MOM. The first consideration would be the effect on the milk matrix due to processing; pasteurization and storage^{80,81}. Fortunately, HMOs are stable during pasteurization⁸² unlike other milk bioactives⁸³. Secondly, donor milk is pooled from multiple individuals representing a mixture of milks spanning lactation stages and host genotypes resulting in a heterogeneous final product. One study revealed a greater total HMO content of expressed milk from 26 mothers compared to 31 different batches of donor milk⁸⁴. It is important to note that the milk collected from each group in the study had different donor characteristics in that the MOM group had mostly delivered preterm (76.9%) compared to the PDHM group (21.5%), and the race/ethnicities differed between groups. Additional studies are needed to generalize these results across demographics. With these differences in mind, it brings to attention what, if any, differential effect on the microbiota could result from feeding MOM compared with PDHM. Previous observational studies comparing microbiotas of preterm infants consuming MOM or PDHM found that MOM fed infants have increased levels of *Bifidobacterium*^{85,86}. However, further studies are needed to attribute HMO composition to differences in gut microbiotas. Additionally, the other bioactive components of human milk (including the milk microbiota) that are degraded during

pasteurization contribute a constellation of effects on the neonatal microbiota⁷⁷. Future research is needed to understand how adding human milk bioactives lost during processing of PDHM might result in desirable health outcomes including microbiota development.

3. *Bifidobacterium*-host interactions

Our microbial inhabitants are major determinates affecting the balance of health versus disease. This balance, or homeostasis, is guided in part by the interactions that occur between the molecular stimuli exerted by the microbiota. Theoretically, a gut microbiome with high diversity provides ample functionality that contributes to our own fitness through enabling enzymatic processes not encoded by our own genome. Therefore, assessing the functional impact of a specific microbial community member can be used to build an understanding of how inclusion of that member will affect the host phenotype. **Figure 1** depicts the metabolism of 2'-FL by *Bifidobacterium* in the gut lumen followed by general host interactions. In the following sections, *Bifidobacterium* species and their contribution to the host immune and metabolic systems are explored in more detail by describing several major metabolites and their interactions with the host.

3.1 Bifidobacterial metabolism

Early studies examining the taxonomy of bifidobacteria discovered a unique metabolic pathway later to be termed the “bifid shunt” in which hexose sugars are metabolized via fructose-6-phosphoketolase rather than the conventional glucose-6-phosphate dehydrogenase and aldolase found in other bacterial groups such as *Lactobacillus*⁸⁷. By channeling hexose sugars through the bifid shunt, bifidobacteria are able to obtain additional energy in the form of ATP⁸⁸. Notably, not all monosaccharides are fed into the bifid shunt; the metabolism of fucose occurs through a separate pathway (known as the propanediol pathway) yielding 1,2-propanediol and pyruvate of

which the latter is the substrate for many secreted end products (**Figure 2A**). These features along with a pangenome encoding a repertoire of GHs as well as sugar binding and transport proteins clearly signifies bifidobacteria as specialists in carbohydrate utilization and are therefore well-adapted to the human gastrointestinal tract⁸⁹. In the anaerobic environment of the gut, fermentation is the means by which ATP is synthesized; however, due to an absence of oxygen, the TCA cycle is not fully operational and partially oxidized intermediates are excreted. Carbohydrate metabolism in bifidobacteria yields two major secreted end products; acetate and lactate with a ratio of 1.5:1, although other fermentation products include ethanol, formate and 1,2-propanediol (1,2-PD) with the ratio depending on the carbon source⁹⁰. Similar to the other secreted end products of sugar metabolism, the ratio of formate to other metabolites depends on the starting substrate. Compared with lactose, growth on 2'-FL produces additional formate, ethanol and 1,2-PD at the expense of acetate and lactate (**Figure 2B**). Moreover, the same pattern of increased formate and ethanol relative to acetate and lactate is observed when bifidobacteria is grown on oligofructose compared to fructose in monomeric form⁹¹. The metabolic change responsible for this shift can be attributed in part to upregulation of pyruvate formate-lyase (PFL) which hydrolyzes pyruvate into formate and acetyl-coenzyme A (acetyl-CoA) where acetyl-CoA is then converted into ethanol or acetate⁹². Previous reports suggest the increased carbon flux through PFL is favored as a means to generate additional ATP by conserving NADH that would be used to reduce pyruvate to lactate^{93,94}. Furthermore, the rate of sugar catabolism affects the end product ratio. In the initial growth phase, acetate and lactate are predominately excreted from the cell despite the complexity of the carbon source, i.e. fructose, oligofructose, lactose or 2'-FL; however, as growth proceeds, formate production increases^{91,95}. A putative explanation for this is that bifidobacteria initially use the most efficient strategy with the lowest energy cost by preferentially metabolizing simple sugars; mono-

and disaccharides, only transcribing a subset of the genes needed. This is evidenced by the preference for fructose monomers and fructose with a low DP⁹⁶. Additionally, as growth proceeds and transcription of gene clusters for complex substrates begins, the additional energy expenditure prompts the switch to increase formate production for an increase in net ATP. However, this does not explain the fermentation product composition when fucose is the sole carbon source. Similar to 2'-FL, bifidobacteria grown on monomeric fucose produce increased formate, ethanol and 1,2-PD compared to lactose despite that fucose is a monosaccharide⁹⁰. One potential explanation is the difference in oxidation state of fucose relative to glucose. 1,2-PD is produced exclusively as a product of deoxyhexose (fucose or rhamnose) catabolism. From a metabolic perspective, the production of 1,2-PD arises out of need to balance redox status with a deoxyhexose sugar such as fucose. As a more reduced sugar, fucose theoretically increases NADH production and prompts the cell to adjust by excreting a reduced product, i.e. 1,2-PD, to recycle NAD⁺. In this way redox balance is achieved whilst deriving additional ATP through PFL. By placing reducing equivalents on 1,2-PD for excretion, additional regeneration of NAD⁺ is possible and allows for efficient fermentation on varied sugar substrates. Another important consideration is the effect of environmental pH on fermentation product excretion. As pH decreases over time, the ratio of secreted metabolites will change accordingly⁹⁷. As such, it is important to note that microbial metabolism will operate differently in a host environment. Together, these *in vitro* studies suggest differences in substrate type and complexity alter the ratio of formate to the other major end products and therefore may have impacts *in vivo* on host metabolism. Each of these products may interact with the host through various modalities such as binding receptors on host cells to initiate signal transduction, or transport and metabolism in host tissues. These processes tune the host

metabolic, endocrine and immune system development with each small molecule having distinctive effects.

3.1.1 Acetate

Acetate is one of the most prevalent metabolites observed in the human gut due to its production from dietary fiber by a variety of acetogenic organisms populating the gut lumen. As acetate is a major fermentation product of hexose metabolism by *Bifidobacterium*, acetate content in breastfed infant feces is often high, commensurate with increased bifidobacterial counts^{98,99}. However, previous studies have shown mixed results; preterm infants supplemented with *B. lactis* resulted in significantly higher fecal acetate concentrations relative to the placebo control group¹⁰⁰, while another study found no difference in acetate for preterm infants given a probiotic mixture of *L. acidophilus*, *B. bifidum*, *B. longum* and *B. infantis* and FOS¹⁰¹. However, in full term, exclusively breastfed infants, supplementation of *B. infantis* EVC 001 resulted in significantly higher fecal acetate and *Bifidobacteriaceae* compared to infants not receiving probiotics¹⁴. The discrepancy in fecal acetate between these studies may arise from several differences including species and strain level differences, mixed fed or exclusive breast feeding, and gestational ages (term vs. preterm). Commensurate with the rise in acetate is the decline in fecal pH. The high acid content of infant feces, contributed largely by bifidobacteria¹⁰², reduces pH in the gut lumen resulting in an environment less hospitable to potentially pathogenic microbes^{14,98,100}. This provides an important level of protection against infection during a critical window in which the immune system is still developing. A study using mice monocolonized with various species of *Bifidobacterium* including *B. adolescentis* JCM1275, *B. longum* JCM1217, *B. infantis* JCM1222 and *B. infantis* 157F have shown protection against enteropathogenic *E. coli* (EPEC) mediated by species and strain specific acetate production¹⁶. The authors found that acetate strengthened

colonocyte integrity via increased transepithelial electrical resistance (TER) thereby reducing virulence factor translocation. Notably, a difference in acetate production in the studied species of bifidobacteria was due to *B. adolescentis* JCM 1275T and *B. infantis* JCM 1222 lacking an ABC-type carbohydrate transporter, thereby limiting carbohydrate acquisition and metabolism into acetate. The authors confirmed this by knockout of the carbohydrate transporter in a *B. longum* strain resulting in lower fecal acetate and survivability from challenge with EPEC relative to the wild type strain. To reiterate, protection against pathogenic *E. coli* was only conferred when the strain of bifidobacteria expressed a carbohydrate transporter allowing increased metabolism of fructose enabling release of acetate in sufficient quantity to fortify the intestinal barrier. As such, species and strain dependent metabolic output as a function of carbohydrate transporting capacity would presumably account for variation in both fecal metabolites and related outcomes across studies. Indeed, previous studies have reported differences in growth based on the presence of carbohydrate specific ABC-transporters^{53,89,103}.

Mechanistic studies using animal models and mammalian cell culture have delineated various physiologic impacts of acetate in the gut and other tissues. Acetate functions as a ligand to bind G protein-coupled receptors (GPCRs) on colonocytes and immune cells in the intestine thereby modulating host metabolism and immunity¹⁰⁴⁻¹⁰⁶. Activation of Gpr41 and Gpr43 on gut epithelial cells stimulates secretion of the anorexigenic hormones peptide YY (PYY) and glucagon-like peptide 1 (GLP-1) from gut enteroendocrine cells¹⁰⁷. Mouse models of germ free (GF) and Gpr41 null mice reveal that both a microbiota capable of producing acetate and propionate along with a functional Gpr41 receptor are responsible for increased secretion of PYY with subsequent effects of reducing intestinal transit time and promoting energy harvest of short chain fatty acids (SCFAs) in the gut¹⁰⁸. In human subjects, prebiotic administration stimulates the

gut microbiota to increase GLP-1 that is associated with a favorable postprandial glucose response¹⁰⁹. Moreover, a clinical trial to investigate the effect of a high fiber diet and prebiotics to reduce comorbidities of type 2 diabetes (T2D) found greater abundance of *B. pseudocatenulatum* and increased fecal acetate in the treatment arm alongside improved glucose homeostasis and GLP-1 secretion¹¹⁰. When the authors inoculated mice with *B. pseudocatenulatum* C95 isolated from a participant in the treatment arm and challenged them with a high-fat diet (HFD), they found that the mice given the probiotic were protected against HFD-induced obesity through reduced body weight gain, body fat, insulin resistance and fasting blood glucose compared to mice on the HFD alone.

In addition to interacting with the gut epithelium, acetate in the colon is absorbed via the hepatic portal vein where it comes in contact with the liver before reaching systemic circulation. The absorption of microbial metabolites, including acetate, offers another layer of host-microbe interaction through systemic effects across host tissues. Studies of bifidobacteria supplementation in mouse models have shown increased blood acetate concentrations, presumably due to bifidobacterial acetate production in the colon. Mice enriched with *B. animalis* subsp. *lactis* GCL2505 presented with increased cecal and plasma acetate and were protected against metabolic aberrations in glucose metabolism after a high-fat diet (HFD) challenge¹¹¹. However, this protection was abolished in Gpr43 knockout mice indicating the effect was driven by downstream signaling via Gpr43 activation. The authors observed differences in insulin sensitivity for both adipose and muscle tissue revealing a blunted response in Akt phosphorylation in wild type, but not Gpr43 null mice given *B. lactis* relative to saline treated animals. The effect of insulin binding leads to downstream phosphorylation of Akt and therefore a lower ratio of phosphorylated to non-phosphorylated Akt following an insulin tolerance test, indicating greater insulin sensitivity. In

muscle, the opposite effect was observed; a greater ratio of phosphorylated to non-phosphorylated Akt in mice given *B. lactis*. Overall, the shift away from insulin mediated glucose uptake in adipose and increased insulin sensitivity in muscle is proposed to be responsible for the decreased fat mass and improved glucose control. Similar results have been produced in other mouse studies supplementing *Bifidobacterium* in the face of a HFD challenge lending increased validity and attention to the therapeutic role of bifidobacteria in host metabolism^{110,112}. The basic research describing the relationship between the microbial production of acetate and host physiology is imperative in translating these concepts to infant developmental processes. Understanding the clinical relevance of metabolic outputs from the developing microbiota will help to inform the standard of care and improve gut microbiome targeted therapeutics.

3.1.2 Lactate

Following acetate, lactate is the next major fermentation product of saccharolytic metabolism in *Bifidobacterium* with an approximate mole ratio of 2:3 (lactate to acetate) depending on the carbohydrate source¹¹³. As such, breastfed infants with a greater amount of bifidobacteria in their gut are reported to have increased lactate concentrations relative to formula fed infants^{71,114}. Gpr81 is the only GPCR identified with a specificity for lactate; however, multiple downstream effects have been documented in conjunction with its expression in several tissues types¹¹⁵⁻¹¹⁷. Lactate binding to Gpr81 on adipocytes inhibits lipolysis and therefore plays a role in systemic energy metabolism. However, it is important to consider that blood lactate is heavily regulated and any lactate coming from microbial origin via absorption from the intestine is subject to this regulation¹¹⁸. In the colon, activation of Gpr81 in mice colonized with a lactate producing *Lactobacillus* species promotes stimulation of intestinal stem cell proliferation which is protective

against radiation induced cell death¹¹⁹. This protection may be relevant in preterm infants with developmentally delayed gut tissue.

The biochemical nature of lactate as a central metabolite involved in energy production promotes its use in the host-microbe relationship and across microbes in the gut. The conservation of genes involved with metabolizing lactate gives way to trophic interactions that support growth of lactate utilizing microbes and their metabolites. It is interesting to note that this is counter to the strategy of *Bifidobacterium* in secreting excess organic acids, and has the effect of suppressing sensitive microbes. *In vitro* fermentation studies have identified some of these interactions along with the microbes responsible. One study found that modifying the initial pH with the addition of lactate in fecal inocula dictated the metabolic output of each culture finding that only propionate and butyrate production occurred at pH values of 5.9 and 6.4, with no production at pH 5.2¹²⁰. Moreover, they demonstrated that the microbe largely responsible for butyrate production was *Eubacterium hallii* (*E. hallii*), which was indicated by a 100-fold increase in CFU at in the two higher pH cultures. Further interactions between *E. hallii* and bifidobacteria have been reported using co-culture methods, which found that during growth on HMO, *Bifidobacterium* releases several products utilized by *E. hallii* including acetate, 1,2-PD, as well as lactate¹²¹. Importantly, these two studies show that cross-feeding between these microbes occurs, but only at a pH of 6 or greater. The infant gut pH is variable, but with a high relative abundance of *Bifidobacterium*, the gut luminal pH is in the sub 5.5 range¹⁴. However, intestinal pH appears to be increasing over the past century, possibly as a result of decreased breastfeeding rates and lower *Bifidobacterium* abundance¹²² which may affect cross-feeding dynamics in the gut. Together, the trophic interactions occurring in the gut are dependent on environmental factors that permit microbial

colonization affecting both immediate changes in the host as well as its succession into new steady state ecosystems.

3.1.3 Formate

In mammals, formate feeds into one-carbon metabolism (OCM) through incorporation into tetrahydrofolate (THF) to yield 10-formylTHF (10-fTHF) via 10-formylTHF synthetase (MTHFD1). Cytosolic 10-fTHF proceeds to split into several anabolic pathways which may be shunted toward nucleotide biosynthesis (purines and thymidylate) or fed into the production of SAM via 5-methyl-THF for use in methylation reactions¹²³. Hence, the absorption of microbial derived formate may supply methyl donors to augment OCM and DNA methylation contributing to changes in the host epigenome. Indeed, higher formate concentrations are observed in cord blood relative to maternal plasma that may promote increased OCM activity to provide the necessary precursors during this critical growth window¹²⁴. It is interesting to speculate on the role that *Bifidobacterium* has in generating formate for host metabolism especially during infancy, as this life stage is characterized by high anabolism and thus requires substantial precursors, i.e. nucleotides, for cell replication during this process. Formate is higher in breastfed infant blood compared to FF infants which may be due to a higher level of bifidobacteria¹²⁵. Further evidence to support this comes from mice provided a synbiotic of *B. pseudocatenulatum* and 2'FL, showing that higher proportions of *Bifidobacteriaceae* are associated with elevated formate in both the intestine and serum (**Chapter 2**). However, formate in high concentrations is toxic to mammals^{126,127}, although clearance through folate-dependent detoxification pathways alleviates excess build-up in the presence of acute exposures¹²⁸. As such, contribution from microbial sources are unlikely to accumulate to toxic levels. It is important to state that virtually any substance in excess can lead to homeostatic imbalance and physiological complications. Within this

homeostatic range, formate is integral in human OCM and thus the absorption of microbial produced formate may be able to augment its activity.

3.1.4 1,2-propanediol

Infant associated species of *Bifidobacterium* possessing HMO utilization clusters contain fucose operons that enable its degradation into predominately acetate, lactate, formate and 1,2-PD¹²⁹. Thus, it is not uncommon to observe 1,2-PD in breastfed infant feces at concentrations that rival SCFAs^{71,130}. In addition to fucosylated HMOs, mucus lining the intestine in mammals is another source of fucose. Previous reports show that *B. bifidum* is capable to liberating fucose from mucin to enable cross-feeding by another *B. breve* strain possessing a fucose operon¹³¹. Including bifidobacteria, fucose catabolism occurs in several human gut bacterial species such as *Escherichia coli*¹³², *Roseburia innulinivorans*¹³³, *Bacteroides thetaiotamicron*¹³⁴, *Akkermansia muciniphila*¹³⁵ and others. However, the fucose catabolism pathway in bifidobacteria generates 1,2-PD¹³⁶ without further oxidizing it to propionate, whereas these other species mainly rely on either succinate or propanediol pathways that further metabolizes 1,2-PD into propionate. This conversion may also happen *ex situ* as in the case of excreted 1,2-PD from bifidobacteria taken up by *E. hallii* to produce propionate^{121,137}. Interestingly, syntrophic interaction between the gut symbionts *B. breve* and *Lactobacillus reuteri* revealed a fitness advantage for *L. reuteri* was gained in the presence of *B. breve* produced 1,2-PD¹³¹. In this study germ free mice colonized with *B. breve*, *L. reuteri* or their isogenic mutants demonstrated that a loss of function of either fucose import for metabolism into 1,2-PD by *B. breve* (fucP) or the ability of *L. reuteri* to turnover 1,2-PD (pduCDE operon) abrogated the *in vivo* fitness advantage. These cross-feeding interactions between bacteria are highly relevant to understanding the metabolic output of microbial consortia in the infant gut and how we may modify this metabolic network to favor production of specific small molecules.

The biochemical role of 1,2-PD in mammals may be linked to ketone metabolism as evidenced in humans with diabetic ketoacidosis¹³⁸. In this small study (n = 7) intravenous infusion of 2-[¹⁴C]acetone was administered while blood was monitored for changes in the incorporation of ¹⁴C into acetone, acetol, 1,2-PD and glucose. The authors observed a direct relationship between acetone and 1,2-PD ($r = 0.97$) and incorporation of the radiolabel into 1,2-PD. Moreover, incorporation of the ¹⁴C label from acetone into blood glucose and liver glycogen results in labeling of carbons 1, 2, 5 and 6 which had been previously observed with administration of 2-[¹⁴C]pyruvate^{139,140}. This indicates acetone is metabolized into 1,2-PD as an intermediate and subsequently into pyruvate before conversion to glucose. Animal experiments using rats have corroborated these findings; isolated hepatocytes incubated with 5 mM of 1,2-PD from rats maintained on 1% acetone v/v in their drinking water had an increased rate of glucose synthesis relative to hepatocytes with no substrate added¹⁴¹. In addition to the liver, gluconeogenesis occurs in the intestine where microbial metabolites such as propionate and succinate can be used as substrates for *in situ* glucose production^{142,143}. In light of this, it is possible that 1,2-PD could be fed into intestinal glucose production directly, or indirectly by microbial transformation into propionate. Together, these studies show 1,2-PD is glucogenic and thus could contribute to energy harvest via absorption of microbe derived 1,2-PD. The degree to which 1,2-PD supplements total caloric intake is yet undetermined but will depend on the amount of fucose or rhamnose in the diet and bacteria capable of converting it into 1,2-PD, such as bifidobacteria. High fecal 1,2-PD, as observed in infancy, may provide a nontrivial contribution to overall energy metabolism as an added benefit of this symbiosis.

3.1.5 Folate

Vitamin synthesis has long been recognized as a function of the human gut microbiota. Species of *Bifidobacterium*, notably *B. pseudocatenulatum* and *B. adolescentis*, have been shown to synthesize and release folate *in vitro*¹⁴⁴ and *in vivo*¹⁴⁵. An experiment using rats fed human milk solids led to an increase in cecal bifidobacteria that was associated with higher cecal and plasma folate¹⁴⁶. Germ free mice monocolonized with either *B. longum* BB536 or *B. animalis* subsp. *lactis* resulted in higher fecal folate concentrations relative to mice not receiving the inoculum¹⁴⁷. In humans, one study observed increased levels of *B. longum* and enriched synthesis of tetrahydrofolate in breastfed infant microbiomes compared to formula fed infants¹⁴⁸. Moreover, the microbiome in breastfed babies have increased capacity to synthesize folate *de novo* compared with formula-fed infant and adult microbiomes whose functions are geared toward metabolism of dietary folates¹⁴⁹. This may reflect dietary differences in folate fortified formula and foods compared with sources coming from human milk. The added benefit of providing endogenous folate reduces the need to obtain dietary folate sources especially in areas where the food supply is not fortified with folate.

3.1.6 Indole-3-lactate

Several indole derivatives are produced via microbial metabolism of tryptophan (Trp) with many human gut microbes capable of producing these metabolites¹⁵⁰. Thus, bifidobacteria are able to convert Trp to indole-3-lactate (ILA)¹⁵¹. As such, infants with higher relative abundance of *Bifidobacterium* have increased fecal concentrations of ILA¹⁵² (**Chapter 3**). The importance of ILA is linked to its anti-inflammatory property which is mediated by the activation of the aryl hydrocarbon receptor (AhR) in intestinal epithelial cells (IECs) and innate lymphoid cells (ILCs)¹⁵³. The signaling cascade resulting from activation of AhR by agonists such as ILA promotes survival of ILCs which negatively regulate the pro-inflammatory Th17 cell response¹⁵⁴.

In IECs, pretreatment with ILA activates the AhR, affecting a signal transduction cascade to inhibit transcription of pro-inflammatory cytokines following incubation with an inflammatory stimulus^{152,155}. Importantly, studies in mice have demonstrated that altering the microbiota composition can tune the inflammatory response mediated through AhR activation. In mice genetically susceptible to DSS-induced colitis, microbiota engraftment with Lactobacilli producing AhR-activating indole compounds or administration of an AhR agonist induce the expression of IL-22 and protect from DSS challenge¹⁵⁶. Mice colonized with a mutant *L. reuteri* strain incapable of producing the AhR agonist found that IL-22 was significantly reduced compared to mice colonized with the wild type *L. reuteri*, abrogating protection from a pathogen challenge¹⁵⁷. Additionally, AhR deletion in IECs renders mice more susceptible to the pathogen *Citrobacter rodentium* by impairing intestinal barrier function via reduction in IL-22 and reduced goblet cell differentiation¹⁵⁸. The action of IL-22 in promoting IEC renewal and therefore barrier function is relevant in the context of preterm infants whose underdeveloped gut tissues are more susceptible to necrotizing enterocolitis (NEC)¹⁵⁹. Interestingly, one of the hallmarks of NEC is the increased expression of Toll-like receptor 4 (TLR-4), whose aberrant activation in the preterm gut results in increased enterocyte apoptosis and reduced barrier function¹⁶⁰. Additionally, expression of the neutrophil chemoattractant IL-8 is reduced in IECs via activation of AhR. Notably, this reduction in neutrophil recruitment in the gut measured by fecal calprotectin is reduced in *Bifidobacterium* supplemented preterm infants with increased fecal ILA (**Chapter 3**). Therefore, it is conceivable that part of the immune stimulating properties of *Bifidobacterium* could be associated with ILA induced AhR signaling in the host. In summary, the provision of *Bifidobacterium* to produce the AhR ligand ILA may be a rational means to provide

immunomodulatory function in the gut by fortifying the intestinal barrier and augmenting mucosal immunity.

4. Dissertation focus

There is a copious amount of information on the symbiotic role of *Bifidobacterium* in the human gut with many of the salient articles highlighted in the preceding discussion. This dissertation furthers our understanding of the host-*Bifidobacterium* co-metabolic relationship through studying colonization in a mouse model (Chapter 2), and in preterm infants (Chapter 3). Chapter 2 details the HMO driven persistence of *B. pseudocatenulatum* in the murine gut, and evaluates the metabolic output locally and in peripheral tissues. In Chapter 3, a cohort of human preterm infants supplemented with *B. infantis* provides clinical data on metabolic and immunologic effects in response to probiotic treatment. By highlighting the strengths of each approach, a more comprehensive picture of the dynamic and complex interactions between *Bifidobacterium* and humans is illustrated. There is a great impetus to develop new strategies to improve the standard of care for future generations and reduce the incidence of non-transmissible diseases related to development of the gut microbiota.

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6. Figures

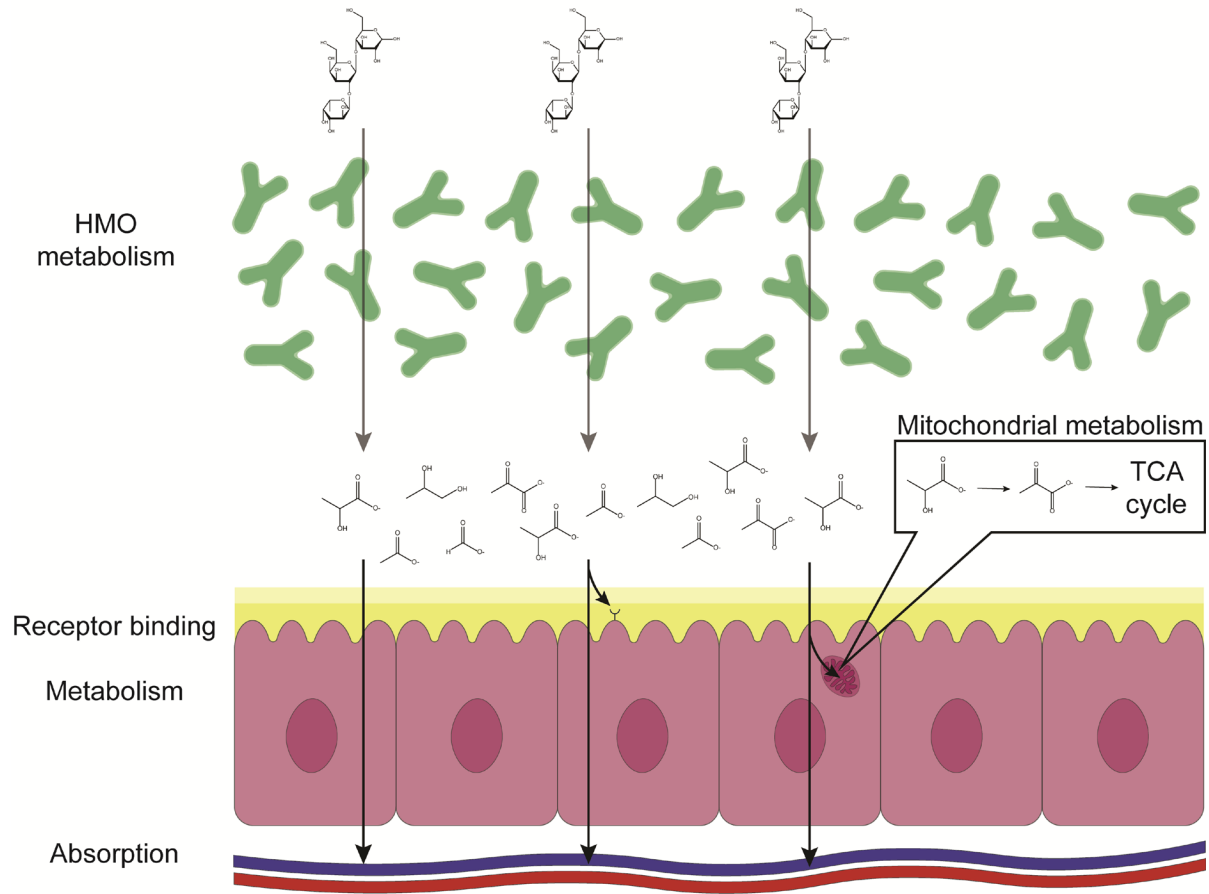


Figure 1. Fermentation of 2'-FL by *Bifidobacterium* in the gut lumen. Metabolism of 2'-FL results in secretion of organic acids and other products that may interact with the host by (1) receptor binding on the apical colonocyte surface (2) mitochondrial metabolism or (3) absorption into host circulation.

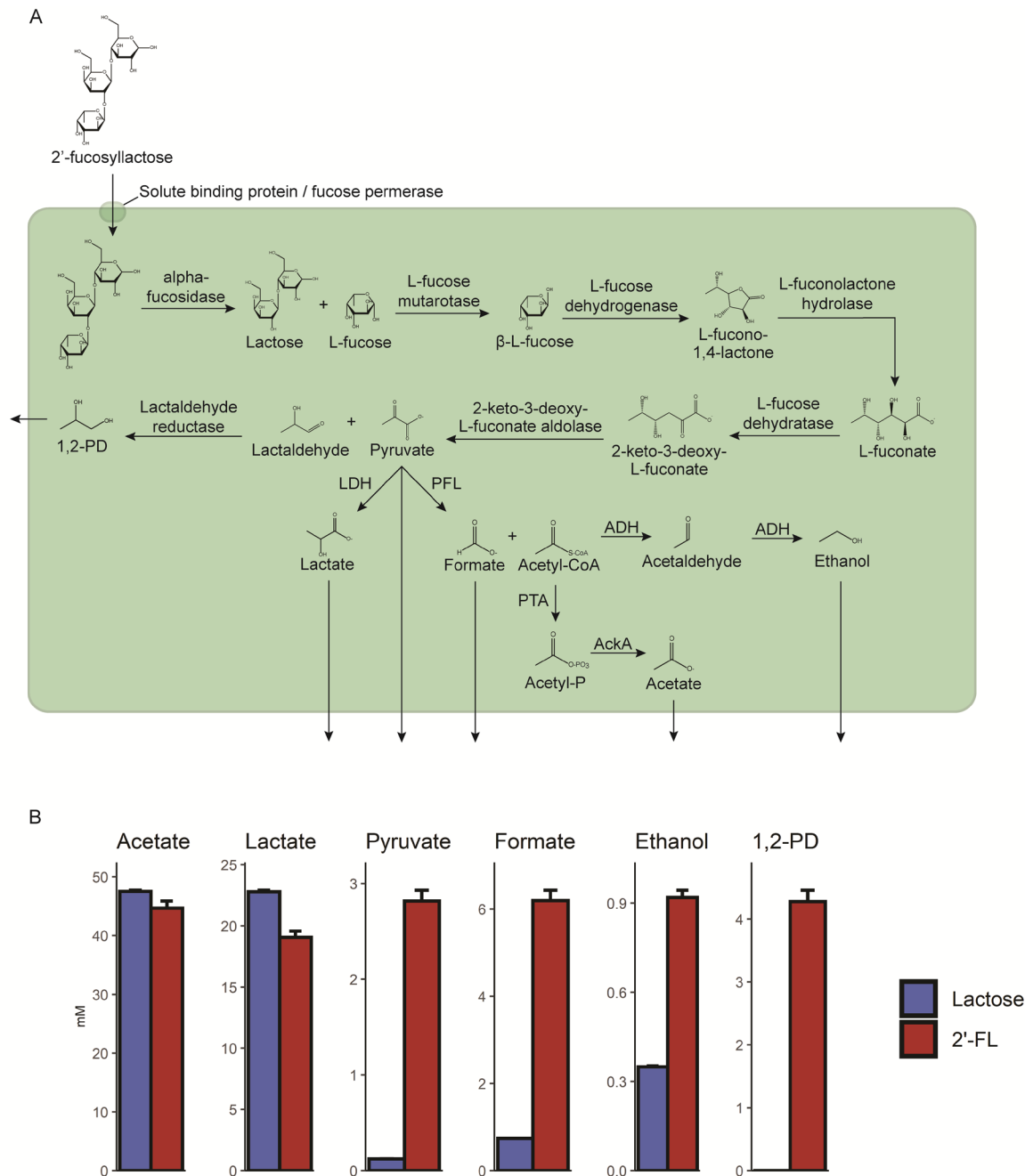


Figure 2. Fucose metabolism in *Bifidobacterium*. Pathway depicting putative fucose intermediates and end products (A) and the difference in major secreted end products during fermentation of either lactose or 2'-fucosyllactose (1% w/v) by *B. pseudocatenulatum* MP80 (B) LDH, lactate dehydrogenase; PFL, pyruvate formate lyase; ADH, alcohol dehydrogenase; PTA, phosphotransacetylase; AckA, acetate kinase.

Chapter 2: Persistence of *Bifidobacterium pseudocatenulatum* modulates local and systemic microbial metabolites upon synbiotic treatment in conventionally colonized mice

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1. Introduction

Early, dominant, infant borne *Bifidobacterium* colonization in breastfed infants is favored by the consumption of human milk oligosaccharides (HMOs) due to their prebiotic nature¹. These structurally-complex oligosaccharides comprised of a range of monomers and linkages^{2,3} establish a nutrient niche in the gut that selectively enrich several *Bifidobacterium* species^{4,5}. In infant cohort studies, associations between human milk oligosaccharide (HMO) degradation, enrichment of select *Bifidobacterium* species, higher fecal acetate and lactate, and advantageous health outcomes have been observed⁶⁻¹¹. As such, robust colonization of *Bifidobacterium* during infancy has been linked with improved markers for T1D¹², reduced likelihood of obesity^{13,14}, robust vaccine responses¹⁵ and lower antimicrobial resistance gene carriage^{8,16,17}.

Given the associations of *Bifidobacterium* and health outcomes in infants, there is an increased interest to promote *Bifidobacterium* populations in later human life stages and model colonization of this organism to scrutinize mechanisms of action. Probiotic supplementation is a commonly used strategy to manipulate the gut microbiota, however, efficacy is influenced by inter-individual variation of host related factors including genetics, diet, and microbiome composition¹⁸⁻²². As such, deriving health benefits from the probiotic may be case specific in which only certain diet and/or microbiome configurations promote metabolic or other microbial activities responsible for the benefit²³. While probiotic bacteria are often capable of surviving passage through the gastrointestinal tract, most probiotics do not colonize, and little is known about their interaction with indigenous microbiota and gut accessible nutrient resources. Furthermore, to produce detectable metabolic changes in the gut requires the accumulation of biomass which is constrained by the competition with indigenous microbes. However, synbiotics, comprised of both probiotic and prebiotic components may act synergistically to enhance colonization and functionality in the

gut²⁴. With this targeted enrichment strategy, there is a greater likelihood of colonization through which health outcomes can be achieved.

A prior synbiotic rodent model pairing a fermented milk product and five food-borne bacterial strains found that the supplemented bacteria were quantifiable in the feces during the feeding periods with only a subset of rats continuing to shed one of the five strains 2 days post-supplementation²⁵. Researchers concluded that a subset of rats were permissive to probiotic persistence while others were resistant. Alternatively, by exploiting the established, evolutionary-selected, complementary milk glycan-bacterial synbiotic pairing, we established a persistent population of *Bifidobacterium pseudocatenulatum* MP80 (*B. p.* MP80), a breastfed infant bacterial isolate, with continuous supplementation of the HMO 2'-fucosyllactose (2'-FL)²⁶. When mice were subjected to a chemically induced colitis model, synbiotic treatment improved health outcomes and reduced inflammation suggesting a synergistic protective effect. Given the evidence of *Bifidobacterium* engraftment among probiotic supplemented breastfed infants^{27,28}, this mouse persistence model was designed to recapitulate the critical role HMOs play in the colonization of HMO-consuming *Bifidobacterium*.

Here, we sought to investigate how provision of 2'-FL may augment the colonization and metabolic output of *B.p.* MP80 in the murine gut. We approached this question by evaluating the gut microbiota and metabolic profiles of mice provided synbiotic treatment containing 2'-FL and *B. p.* MP80 compared with supplementation of either 2'-FL or *B. p.* MP80 alone. This allowed us to gauge the effect of 2'-FL on sustaining *Bifidobacterium* populations in the gut and the corresponding metabolite profiles. Determining how the indigenous microbiota can be modulated by probiotic or synbiotic colonization and the resultant metabolic outputs are critical to understanding how synbiotics may facilitate health outcomes.

2. Results

To measure changes in metabolism upon supplementation of the bifidobacterial strain, *B.p.* MP80, a total of 39 mice were divided into four groups and provided either the *B.p.* MP80 probiotic (n=9; PRO), 2'-FL as a prebiotic (n=9; PRE), both *B.p.* MP80 and 2'-FL (n=12, SYN), or a saline control (n=9; CON). The probiotic was provided as an oral gavage each day for four days for the PRO and SYN groups and the prebiotic was provided in their drinking water as a 10% solution of 2'-FL for the PRE and SYN groups every day throughout the experiment (**Figure 3**). A total of three experimental trials were conducted for this study with the following sample sizes; trial 1 SYN and CON groups n = 3; trial 2, PRO, PRE, SYN and CON n =3; and trial 3, CON (n = 3) and PRO, PRE and SYN (n = 6).

Persistence of B.p. MP80 is achievable when supplemented as a synbiotic but not as a probiotic.

To assess *B.p.* MP80 persistence in mice, the fecal microbial community structure was evaluated using 16S rRNA amplicon sequencing. Community structure in the PRO treated mice corresponded to small, non-significant shifts during the experiment (**Figure 4A**, PERMANOVA, $p < 0.181$) that did not persist. However, mice in the SYN group had microbial community shifts across each sample day from baseline to the final time point (**Figure 4B**, PERMANOVA, $p < 0.001$). Post hoc testing revealed statistical significance between baseline and the subsequent time points ($p < 0.005$). Despite a large variance, persistence of *Bifidobacteriaceae* was observed in the SYN group with significantly higher proportions of *Bifidobacteriaceae* relative to the PRO group at completion of the experiment (**Figure 4C**, Wilcoxon rank-sum test, $p < 0.01$). Pairwise comparison of the weighted UniFrac distance revealed that the microbial community structure was statistically different on the final day between each group except for the CON and PRO groups (**Table 1**, PERMANOVA, $p < 0.05$), suggesting that provision of *B.p.* MP80 alone failed to impact

the membership of the microbial community. Notably, β -diversity at baseline differed significantly between the PRE, CON and SYN treatment groups (**Table 1**, PERMANOVA, $p < 0.001$) and the three experimental trials (**Supplementary Table 1**, PERMANOVA, $p < 0.001$).

Gut microbial communities shift dynamically in response to synbiotic treatment

Following oral gavage on day 4, we observed a high coefficient of variation in proportions of *Bifidobacteriaceae* in the synbiotic treatment arm (**Figure 2c**). We therefore decided to evaluate the microbial diversity within these mice by parsing the group into high and low bifidobacterial persistence based on the median relative abundance of *Bifidobacteriaceae* (50.5%). Microbiota composition was not significantly different at baseline as assessed through α -diversity (t-test, $p = 0.221$) and β -diversity (weighted UniFrac, $p = 0.256$), suggesting similar initial microbial distribution within and between synbiotic treated mice (**Supplementary figure 1**). Using a linear mixed effects model with sandwich variance to account for baseline Shannon index, and day of gavage, we examined α -diversity over the course of 2'-FL supplementation. Interestingly, mice with high bifidobacterial persistence had significantly reduced α -diversity compared to mice with low bifidobacterial persistence (**Table 2**). To capture the stability of the gut microbial communities in mice with high versus low persistence we used linear regression with sandwich variance to estimate changes in Morisita-Horn distance from baseline to the day following oral gavage. Mice with high *Bifidobacteriaceae* persistence had a significantly decreased Morisita-Horn distance compared to mice with low persistence (t-test, $p = 0.049$) indicating a reduced community stability over the course of *B.p.* MP80 gavage (**Figure 5A**). At the final time point, one week following oral gavage, β -diversity was significantly different in mice with high compared to low persistence of *Bifidobacteriaceae* (**Figure 5B**, PERMANOVA, $p = 0.002$). Furthermore, differential abundance testing indicated a significantly higher log ratio of *Bifidobacteriaceae* to

Lachnospiraceae and *Ruminococcaceae* in mice classified with high bifidobacterial persistence compared to low persistence (**Figure 5C**, t-test, $p = 0.001$). The overall median proportions of *Lachnospiraceae* in the high and low persistence groups were 0.1% and 12.4% respectively while *Ruminococcaceae* was less than 0.05% and 4.1% respectively (**Supplemental figure 2**).

Metabolic changes in the gut occur with pre- and syn- but not pro-biotic treatment

The metabolic output of the gut microbiota inherently depends on the composition of microbes, substrate availability, and their related metabolic activities. Using proton NMR spectroscopy we interrogated the metabolome of the gut lumen by sampling colon content at the final time point. Non-metric multidimensional scaling (NMDS) revealed considerable overlap of PRO compared with CON group mice suggesting the probiotic alone does not have an effect on microbial metabolites within the gut lumen one week after supplementation (**Figure 6A**). Prebiotic treated mouse metabolite profiles were distinct from others due to changes driven primarily by monomeric fucose, propionate and succinate. Synbiotic treatment resulted in high dispersion in which five mice had distinct gut metabolite profiles that correlated with high lactate, pyruvate, formate and 1,2-propanediol (1,2-PD). Notably, these mice corresponded to those with high persistence ($> 50.5\%$ *Bifidobacteriaceae*), indicating a relationship between these metabolites and the degree of colonization by bifidobacteria. Using the cutoff of 50.5% *Bifidobacteriaceae*, we compared several discriminating metabolites indicated by NMDS. Lactate, formate and 1,2-PD were all significantly higher in mice with high persistence, whereas acetate, propionate and butyrate were significantly greater in mice with low persistence of *Bifidobacteriaceae* (**Figure 6B**, Wilcoxon rank-sum test, $p < 0.05$). Next, we summed the seven highest concentrated fecal organic acids and divided by their total to visualize the organic acid composition in the gut and provide an overview of the relative metabolic makeup by treatment (**Figure 6C**). Acetate was the dominate metabolite

comprising over 60 percent of the total organic acid content for all except the SYN group, which was approximately 40 percent of the total. Both propionate and succinate were higher in the PRE group relative to the other groups. The CON and PRO treated mice had similar organic acid profiles to each other which featured higher butyrate concentrations compared to PRE and SYN treated mice.

Metabolic changes in the gut are associated with modulation of the gut microbiota

To evaluate associations between gut luminal metabolites and corresponding microbiota composition we used median metabolite concentrations as a cutoff to assess differences in overall microbial community structure and differential abundance of selected bacterial families at the final time point. Mice with higher butyrate concentrations had a significantly higher log ratio of *Lachnospiraceae* and *Ruminococcaceae* relative to *Bifidobacteriaceae* (**Figure 7A**, Wilcoxon rank-sum test, $p = 0.003$). In mice provided 2'-FL (PRE and SYN), colonic propionate at the median cutoff was discriminatory for microbial community structure (**Figure 7B**, PERMANOVA, $p = 0.004$). Additionally, a significant log ratio increase of *Bacteroidaceae* to *Bifidobacteriaceae* was observed in the high propionate group (**Figure 7C**, Wilcoxon rank-sum test, $p = 0.015$). For mice receiving 2'-FL (PRE and SYN), the metabolite 1,2-PD produced during microbial fermentation of fucose was associated with differences in microbial communities (**Figure 7D**, PERMANOVA, $p = 0.028$), and a significant increase in the ratio of *Bifidobacteriaceae* to *Lachnospiraceae* and *Ruminococcaceae* (**Figure 7E**, t-test, $p = 0.01$). In addition to differential abundance testing, groups were compared across treatment arms for the purpose of assessing changes in the microbiota associated with probiotic, prebiotic or synbiotic administration. Proportions of *Lachnospiraceae* and *Ruminococcaceae* were observed to be significantly higher in CON and PRO groups at the final day of sample collection (**Figure 8A, B**, Dunn's test, $p <$

0.05). The proportion of *Bacteroidaceae* in PRE treated mice was significantly enriched at the final day of sample collection relative to the CON group with concomitantly higher concentrations of free fucose (**Figure 8C, D**, Dunn's test, $p < 0.01$). Furthermore, a high correlation between *Bacteroidaceae* and propionate was detected in mice provided 2'-FL (PRE and SYN groups) (Figure 6e, Pearson's r , $r^2 = 0.741$, $p = 0.00019$), strengthening the link between the co-occurrence of *Bacteroidaceae* with high propionate levels (**Figure 7C**).

Synbiotic treatment affects systemic metabolism

Serum obtained on the final day was analyzed for metabolomics to discern changes in systemic metabolites related to treatment. Ordination by NMDS showed similar results as observed with colon content metabolites; overlap of CON and PRO groups followed by some separation with PRE treatment and diffuse SYN group dispersion (**Figure 9A**). Notably, the five mice exhibiting the greatest separation away from the control groups were identified as those with high persistence ($> 50.5\%$ *Bifidobacteriaceae*) and distinct gut metabolite profiles (**Figure 6A**). Metabolic features related to separation in serum were predominately fucose, formate and 1,2-PD. The higher concentrations of the fucose metabolite 1,2-PD found in the colon contents of mice with high bifidobacterial persistence were also significantly higher in the serum of these same animals (**Figure 9B**, t-test, $p < 0.05$) suggesting increased absorption across the gut epithelium. Lastly, we examined the liver and brain to determine if the perfusion of blood with enriched microbial metabolites equilibrated with these organs. Notably, only synbiotic treated mice showed detectable concentrations of 1,2-PD in liver and brain samples with none detected in the control animals (**Figure 9C**). Together, only persistent colonization corresponded to an increase in the microbial fermentation products 1,2-PD observed in circulation. The presence of 1,2-PD in liver and brain further indicates a systemic distribution of metabolites that occurs during synbiotic treatment.

3. Discussion

Previous work established that the HMO 2'-FL is sufficient to facilitate persistence of *B.p.* MP80 in a competitive environment²⁶. Concordant with that study, we observed synbiotic treatment, rather than the probiotic alone, resulted in persistent colonization of *Bifidobacterium*. However, in this study amongst the synbiotic treated mice, *Bifidobacterium* populations were more heterogeneous than prior mouse trials, resulting in mice having either a high or low bifidobacteria count. The inability of a novel intestinal microbe to compete with the indigenous microbial community is well established^{19,29-31}, with studies indicating the degree of individual permissiveness to an invading microbe is contingent on the baseline microbial composition^{18,19,29}. However, in this study no baseline microbial community distinction (α -diversity or β -diversity) was found between groups with high and low persistence of *Bifidobacteriaceae*, although absolute bacterial values and functional capacity via metagenomics remains to be investigated. Stratification into high and low proportions of bifidobacteria were distinct throughout the experiment by α -diversity, exemplifying the diversity-invasion effect where a survival of an invader, *B.p.* MP80, is inversely correlated to species richness and evenness^{30,31}. At the final time point, the microbial community structure (β -diversity) was distinct within the SYN group, resulting from the division of high and low *Bifidobacteriaceae* persistence.

In infants, the functional capacity to catabolize HMOs is associated with high levels of *Bifidobacterium* and their metabolites^{4,17,27,32,33}. In our mouse model, a persistent, predominant *B.p.* MP80 population generated discrete metabolic profiles defined by elevated lactate, formate and 1,2-PD in the colon. Additionally, this metabolic capacity aligns with prior *in vitro* analysis of *B.p.* MP80 2'-FL catabolism, leading us to conclude that the degree of colonization *in vivo* is commensurate with the enrichment of 2'-FL metabolites produced by *Bifidobacterium* including lactate and 1,2-PD. These products are found in considerably lower quantities in the adult gut due

to either a reduced capacity to produce these metabolites and/or their utilization by other microbial inhabitants³⁴. As such, a lower diversity ecosystem dominated with bifidobacteria as observed in the mice with high persistence of bifidobacteria has greater potential to accumulate these products.

Diet-driven microbial metabolic effects have been widely studied in humans and animal models due to associated health benefits³⁵⁻³⁷. Here, we identified gut microbiota compositions are distinguished by their metabolic output (butyrate, propionate, and 1,2-PD). Butyrate-producing bacteria create a functional cohort where the two most abundant groups include *Eubacterium rectale/Roseburia spp.* (*Lachnospiraceae*) and *Faecalibacterium prausnitzii* (*Ruminococcaceae*)³⁸. As butyrate production is due to the breakdown of complex polysaccharides that reach the colon, it is appropriate that we found butyrate concentrations associated with enrichment of *Lachnospiraceae* and *Ruminococcaceae* families in mice where chow was the predominant fiber source. However, in mice with 2'-FL supplementation, butyrate concentrations were proportionally lower, coinciding with a previous report in which 2'-FL supplementation in mice was associated with reduced butyrate³⁹. Following baseline, we observed provision of 2'-FL in the PRE and SYN groups corresponded with enrichment of *Bacteroidaceae* in the murine gut at each sampling period. *Bacteroides* species typically possess several polysaccharide utilization loci in their genomes that enable cleavage of a variety of glycosidic linkages including HMOs⁴⁰. Metabolically, *Bacteroides* is a primary producer of propionate in the gut microbiome via the succinate pathway^{41,42}. Therefore, in cases where 2'-FL provision resulted in high propionate, indigenous *Bacteroidaceae* likely outcompeted the autochthonous microbial community in prebiotic fed mice and *B.p.* MP80 in those receiving the synbiotic. This model draws parallels with previous reports that describe the high prevalence of *Bacteroides* in the gut microbiota of breastfed infants⁴³ which likely arises from the opportunistic utilization of HMOs. Competition between

Bacteroides and *Bifidobacterium* for the HMO nutrient niche⁴⁴ is recapitulated in this model, as evidenced by the greater enrichment of *Bacteroidaceae* in the mice receiving only 2'-FL (PRE) relative to those provided the synbiotic which had higher proportions of *Bifidobacteriaceae*. Additionally, mice with high concentrations of 1,2-PD in the gut were enriched in *Bifidobacteriaceae*. The propanediol pathway, common to *Bifidobacterium*, characteristically produces 1,2-PD from the metabolism fucose which is found to be elevated in infants enriched with *Bifidobacteriaceae*^{45,46}. In our model, high concentrations of 1,2-PD were observed only in mice with high *Bifidobacteriaceae* suggesting a related source of this metabolite.

Some microbial metabolites produced in the intestine can be absorbed across the gut epithelium into systemic circulation. We found that each treatment group exhibited a similar pattern of serum and colon content metabolite profiles. Notably, significantly higher concentrations of 1,2-PD in serum was observed in mice with high persistence of *Bifidobacteriaceae*, suggesting that in this model, probiotic persistence (and hence synbiotic supplementation) is necessary to produce metabolites at a sufficient concentration to become systemically enriched. Evidence of this relationship has been shown in human adults during which the provision of *Bifidobacterium lactis* (*B. lactis*) and fructooligosaccharides had a more pronounced effect on serum metabolites compared to *B. lactis* alone⁴⁷. Moreover, the absorption of microbial metabolites into circulation have the potential to interact with peripheral tissues and influence their metabolism. We collected liver and brain tissue from three mice in the CON and SYN group (which had high persistence of *Bifidobacteriaceae*) from our first experimental trial to compare changes to metabolite concentrations in these tissues. Metabolic profiles in these tissues were similar across groups with the exception of 1,2-PD which was only detected in the liver and brain of SYN treated mice. Based on these data, we conclude that the high concentration of 1,2-

PD in the gut generated by microbial fermentation of fucose is absorbed via portal circulation passing through the liver and subsequently reaching other peripheral tissues such as the brain. Prior work using ^{13}C -labeled 2'-FL orally administered to mice showed that ^{13}C enrichment occurred in tissues including liver and brain⁴⁸. Moreover, in their study, 2'-FL administered to germ-free mice failed to retain the ^{13}C label in their tissues indicating that the gut microbiota is fundamental to enrichment of 2'-FL. Additionally, intravenous administration resulted in ^{13}C being excreted in urine further implicating that microbial metabolism is a precursor to tissue incorporation. Our work provides evidence that fermentation of 2'-FL by gut microbes produce metabolites that enter circulation. It is likely that these metabolites play an important role in metabolism in peripheral tissues, although this has not been shown to date. This is potentially important considering the assembly of the gut microbiota in early life could tune host metabolic processes that impact cognitive and metabolic development. Understanding the contribution of microbial metabolites at this stage of life will be instrumental in the development and use of biologics to confer well-being throughout the lifespan.

In summary, we found that introducing *B.p.* MP80 into the colonized murine gut environment requires concomitant provision of a nutrient niche (2'-FL) to modulate metabolism at the local and systemic level. Without this advantage, colonization resistance cannot be overcome as the community structure reconfigures to the pre-treatment condition following probiotic inoculation. Additionally, this reinforces the finding that HMOs act as a privileged nutrient resource for a competitive population of *Bifidobacterium*, although the permissiveness to colonization does have variability. Moreover, enrichment of microbial metabolites are dependent on persistent, rather than transient, colonization of *B.p.* MP80, and are reflected by bifidobacterial products of 2'-FL catabolism throughout the host organism.

Our study does have limitations; although mice were ordered through the same facility, the baseline microbial community structure was distinct between trials. This limitation directly affected differential abundance testing where ASVs were grouped by bacterial family for data processing. While this reduced analysis granularity, the fact that differential abundance at the bacterial family level was statistically significant demonstrates how strongly associated diet and microbial communities were within this trial. Additionally, the number of mice in each group were not evenly distributed amongst experimental trials that may contribute to microbial community differences between treatments at baseline. The established microbial community of the mouse gut does not perfectly exemplify the human gut microbiota nor identically capture the competition for resources and available physical niches. This model assessed how infant-borne *Bifidobacterium* strains are competitive, persistent and metabolically active when a privileged nutrient source is provided, even in a non-indigenous environment. Developing an ecological framework through the use of synbiotics or their components is crucial for discovering the underlying mechanisms of synbiotic-associated health outcomes. Overall, such findings will aid in pinpointing synbiotic pairings that possess a higher likelihood of conferring health benefits to the host.

4. Methods

Mouse study design

Animals were maintained in accordance with IACUC Protocol 21900 approved by the Institutional Animal Care and Use Committee of University of California, Davis. Male C57BL/6J mice (5-6 weeks old, Jackson Labs) were group housed (3 per cage) and maintained at 22 °C with a 12 hour light-dark cycle. Before commencing experiments, mice were acclimated for a minimum of one week at the facility. Food (5058 Irradiated Pico Mouse Lab Diet) and water were provided *ad libitum*. 2'-FL was provided in the drinking water as a 10% (w/v) solution. Under anaerobic

conditions at 37 °C, *B.p.* MP80 was grown in de Man, Rogosa, and Sharpe media (BD Difco Microbiology, Houston, TX) supplemented with 0.05% w/v L-cysteine (Sigma-Aldrich, St. Louis, MO). *B.p.* MP80 (10^9 cfu/ml in PBS) or phosphate buffered saline was administered via oral gavage (100 μ l) for three days. Within 1 hour of the light cycle's start, fecal samples were collected from individual mice. For validation, three experimental trials were conducted at separate time points. While experimental protocols across trials were preserved, the number of sampling days and final time point did vary between cohorts. For experiments 1 and 2, samples were collected at baseline and days 2, 4, 6 and a final time point at either day 9 or 10, respectively. For experiment 3, samples were collected at baseline and days 4 and 10. Mice were euthanized via CO₂ asphyxiation.

Fecal extraction and microbial DNA sequencing

DNA was extracted from stool samples (30-100 mg) using the Quick-DNA Fecal/Soil Microbe Miniprep Kit, Catalog No. D6010 (ZYMO, Irvine, CA, USA). Following the manufacturer's instructions, the extraction protocol included a bead-beating step using a FastPrep-24 Instrument (MP Biomedicals, Santa Ana, CA, USA) for a total of 2 min at 25 °C at a speed of 6.5 m/s. The V4 region of the 16S rRNA gene was amplified in triplicate with barcoded PCR primers F515 (5'-CACGGTCGKCGGCCATT-3') and R806 (5'-GGACTACHVGGGTWTCTAAT-3')⁴⁹ modified to contain an adapter region for sequencing on the Illumina MiSeq platform. Amplicons were verified by gel electrophoresis, combined, purified, and sent to the UC Davis Genome Center for library preparation and high throughput 250-bp paired-end sequencing. Raw sequencing data was demultiplexed and quality filtered before import into QIIME2-2019.7⁵⁰. Samples with poor quality data were excluded from analysis. After trimming, reads were processed with DADA2⁵¹. Filtered sequences were aligned and taxonomy was assigned using the 99% SILVA naïve Bayesian

classifier⁵². Samples were rarified to 3000 sequences. The NCBI BioProject ID for raw 16s sequencing data is PRJNA725904.

Statistics (Microbial ecosystem)

Microbial community statistical analysis was performed in R (version 4.0.2)⁵³. For each fecal sample, α -diversity was measured with Shannon Index values (vegan::diversity). A linear regression was used to test α -diversity differences between high and low bifidobacteria groups amongst *B.p.* MP80 + 2'-FL treated mice. Included in the LME analysis (lme4::lmer)⁵⁴ were robust sandwich variance estimates (clubSandwich::vcovCR)⁵⁵ and a degrees of freedom Satterthwaite correction (clubSandwich::coef_test). The GLM analysis (lme4::glm) for Morisita-Horn stability (vegan::vegdist, method = "horn") also included robust sandwich variance estimates and a degrees of freedom Satterthwaite correction. β -diversity was measured by UniFrac distances (GUniFrac) and visualized using non-metric multidimensional scaling (NMDS) (vegan::metaMDS, k=2) with ellipses (vegan::veganCovEllipse)⁵⁶. β -diversity statistical analysis consisted of checking dispersion (vegan::betadisper), a permutational multivariate ANOVA (vegan::adonis2, 999 permutations), and post hoc testing (RVAideMemoire::pairwise.perm.manova, nperm = "500")⁵⁷. The strata argument was used to constrain by mouse subject when longitudinal data was examined. Songbird was employed for differential abundance testing which ranks the log-fold changes between selected features⁵⁸. Due to a lack of ASV overlap between cohorts, ASVs were aggregated by bacterial family for Songbird analysis. Songbird analysis to differentiate between high and low bifidobacteria categories included samples from SYN treated mice at the final time point and accounted for experimental trial differences. Songbird formulas tested the association between final time point microbial communities and metabolites (butyrate, propionate, and 1,2-PD) while accounting for experimental trials. For statistical testing, the metabolite median was used as a cut

off for high or low metabolite production. Based on being the highest or lowest ranked features, bacterial families *Bifidobacteriaceae*, *Bacteroidaceae*, *Lachnospiraceae* and *Ruminococcaceae* were chosen as the numerator or denominator for respective analyses. Differential abundance associations with propionate resulted in a lower number of log ratios because two samples failed to have any *Bacteroidaceae* reads, resulting in no log ratio for two fecal samples. Differential abundance log ratios were assessed for normality using a Shapiro-Wilk test which determined whether a Student's t-Test or Wilcoxon Rank Sum test was employed. Corrections were applied for multiple comparisons by Benjamini-Hochberg.

Metabolomics sample preparation

Colon contents were weighed and combined with 500 μ L aliquots of ice-cold PBS. Samples were then vortexed for 2 minutes, incubated on ice for 5 minutes and vortexed for 2 additional minutes before centrifugation (6000 x RCF, 15 minutes, 4° C). Supernatant was transferred to a new tube and the pellets were dried in a miVac sample concentrator to determine dry weight. After an additional centrifugation step (14k RCF, 10 minutes, 4° C), the supernatant was transferred to 3 kDa filters and centrifuged again (14k RCF, 60 minutes, 4° C). 207 μ L of filtrate was transferred to a new tube, and combined with 23 μ L of internal standard consisting of 4.8 mM DSS-d₆ containing 0.2% NaN₃ (to inhibit bacterial growth) in 99.8% D₂O to serve as a field frequency lock. pH was adjusted to 6.7-6.9 with NaOH or HCl prior to transfer to 3mm NMR tubes. Sera were thawed on ice and transferred to 3 kDa filters. After 60 minutes of centrifugation (4° C, 14000 x RCF), 207 μ L of filtrate was combined with 23 μ L of 4.8 mM DSS-d₆. pH was adjusted to 6.7-6.9 with NaOH or HCl. Thawed liver or brain samples were weighed and combined with either 900 μ L (liver) or 550 μ L (brain) of ice-cold PBS in an MB Bio Lysing matrix D bead beating tube (MP Biomedicals, USA), and homogenized using a FastPrep-24 bead beater (MP Biomedicals,

USA) for 60 s at 6 m/s and repeated for a total of two minutes. Tissue homogenates were centrifuged for 10 seconds and incubated on ice for 1 min followed by centrifugation for 15 minutes (14k RCF, 4° C). Supernatants were transferred to a new tube and spun down for 10 min at 14k RCF and 4° C, and subsequently filtered through a 3 kDa molecular weight filter using centrifugation for 45 minutes at 14k RCF and 4° C. 207 µL of filtrate was combined with 23 µL of 4.8 mM DSS-d₆, and pH was adjusted to between 6.7 and 6.9.

Acquisition parameters for ¹H-NMR

¹H NMR spectra were acquired at 298K using the NOESY ¹H pre-saturation experiment ('noesypr1d') on a Bruker Avance 600 MHz NMR spectrometer (Bruker BioSpin, Germany). Data acquisition was achieved with the following parameters; 8 dummy scans and 32 transients over a spectral width of 12 ppm and a total acquisition time of 2.5 s. Water saturation was applied during relaxation delay (2.5 s) and mixing time (100 ms). The resulting spectra were Fourier transformed with zero filling to 128k data points and the Free Induction Decays (FIDs) were transformed with an exponential apodization function corresponding to a line broadening of 0.5 Hz. Chenomx NMR Suite v8.4 (Chenomx Inc, Edmonton, Alberta, Canada) was used to manually phase and correct baseline spectra. Each metabolite was assigned manually and quantified using Chenomx Profiler.

Statistics (Metabolites)

All metabolite statistical analyses and graphics were generated using R (v4.0.2). Non-metric multidimensional scaling (NMDS) plots were generated using `vegan::metaMDS`, `k = 2`, `distance = "euclidian"`. Normality was assessed using the Shapiro-Wilk test in addition to observing deviations in the residuals of Quantile-Quantile plots. Group comparisons were evaluated using Student's t-test. Corrections were applied for multiple comparisons by either false discovery rate

correction or Benjamini-Hochberg when appropriate. Statistical significance was considered as $\alpha < 0.05$ and statistical trends for $\alpha < 0.1$.

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6. Tables

Table 1. Pairwise comparisons of weighted UniFrac measures between treatment groups at the baseline and final time points.

	<i>Baseline</i>		
	SYN	PRO	PRE
CON	0.567	0.311	0.040
PRE	0.024	0.40	
PRO	0.311		
	<i>Final</i>		
	SYN	PRO	PRE
CON	0.024	0.076	0.006
PRE	0.008	0.006	
PRO	0.009		

Comparisons were evaluated using PERMANOVA and FDR adjustment.

Table 2. Linear regression model of Shannon Index (α -diversity) values for SYN treated mice.

	Beta Coefficients	t-statistic	Lower 95% CI	Upper 95% CI	<i>p</i> -value*
High Bif. Persistence	-0.578	-2.74	-1.07	-0.09	0.0271
Baseline Shannon Index	1.393	3.06	0.27	2.52	0.0232
Day of Gavage	0.651	4.33	0.32	0.98	<0.001

*Treatment or interaction between treatments is considered significant if $p < 0.05$ and are shown in bold.

7. Figures

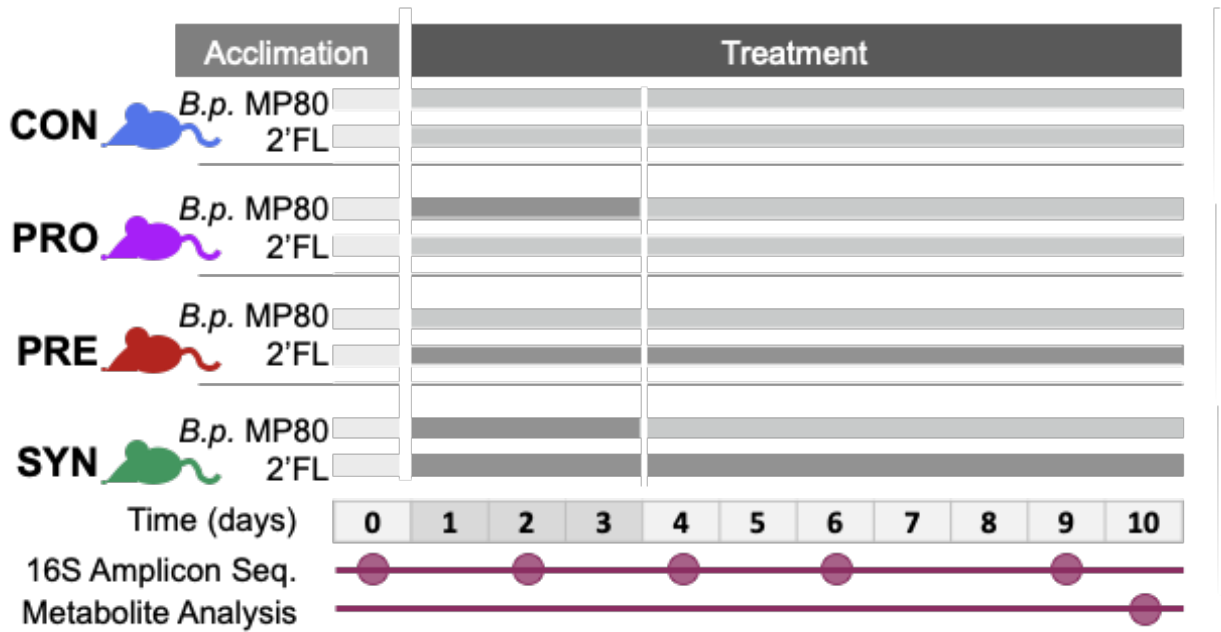


Figure 3. Mouse trial experimental timeline. Treatment groups consisted of: Control (CON) mice; Probiotic (PRO) mice, provided *B.p.* MP80 for three days by oral gavage; Prebiotic (PRE) mice, supplemented with 2'-FL for the duration of the experiment; and Synbiotic (SYN) mice, provided *B.p.* MP80 for three days and supplemented with 2'-FL for the duration of the experiment.

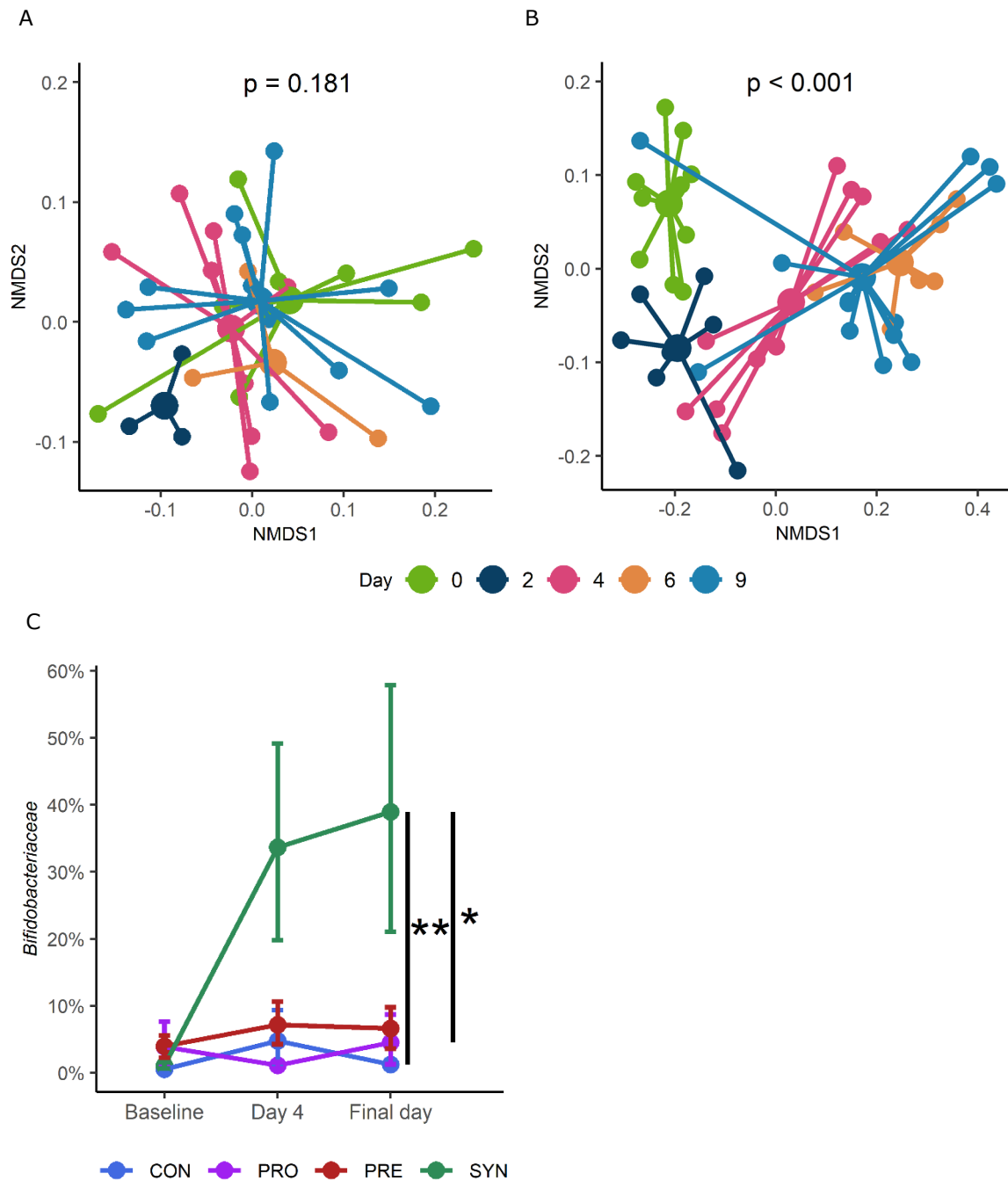


Figure 4. Microbial community structure changes during synbiotic treatment. (A) NMDS plot of β -diversity index weighted UniFrac for probiotic treated mice throughout the experiment (days 0 to final time point) colored by day ($n = 9$); (B) weighted UniFrac for synbiotic treated mice throughout the experiment ($n = 12$); and (C) relative abundance of Bifidobacteriaceae in synbiotic ($n = 12$) and probiotic ($n = 9$) groups from baseline, after oral gavage (day 4) and one week following (final day). Final day, while grouped as day 9 for A, consists of day 9 or 10 depending on the experimental trial. Error bars for relative abundance data is represented as mean and standard error from bootstrapped confidence intervals with 1000 iterations. ** $p < 0.01$, * $p < 0.05$.

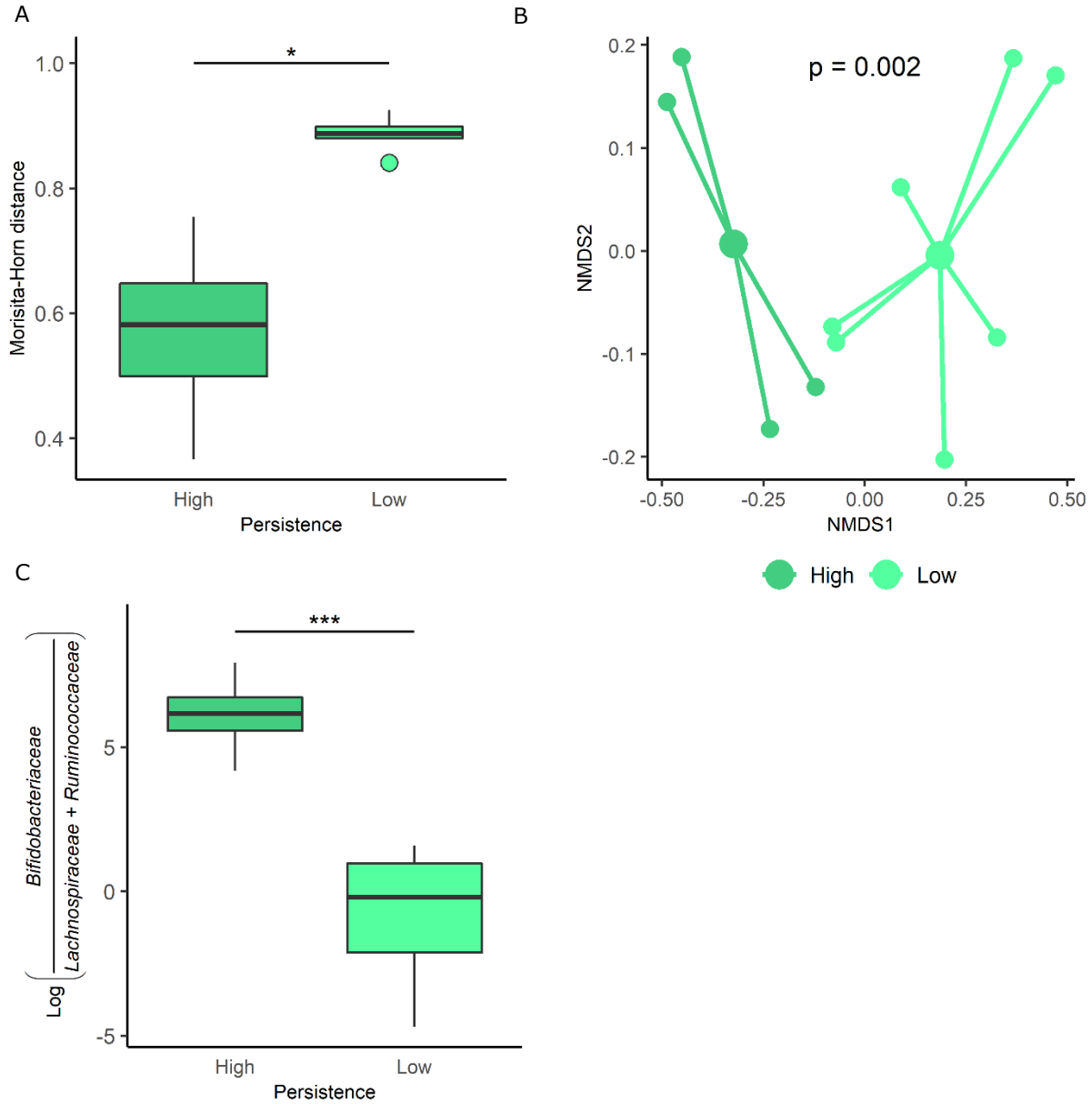


Figure 5. Microbial community differences between high and low bifidobacteria categorizations within the synbiotic treated mice. (A) Morisita-Horn distances for synbiotic treated mice grouped as high ($n = 4$) and low ($n = 7$) bifidobacteria; (B) NMDS plot of β -diversity index weighted UniFrac for high and low bifidobacteria groups at the final time point, colored by high and low; and (C) log ratio of *Bifidobacteriaceae* relative to *Lachnospiraceae* and *Ruminococcaceae* on the final day of the experiment. Boxplots represent medians and interquartile range (IQR) with whisker end points equal to the maximum and minimum values below or above the median at 1.5 times the IQR. *** $p < 0.001$, * $p < 0.05$.

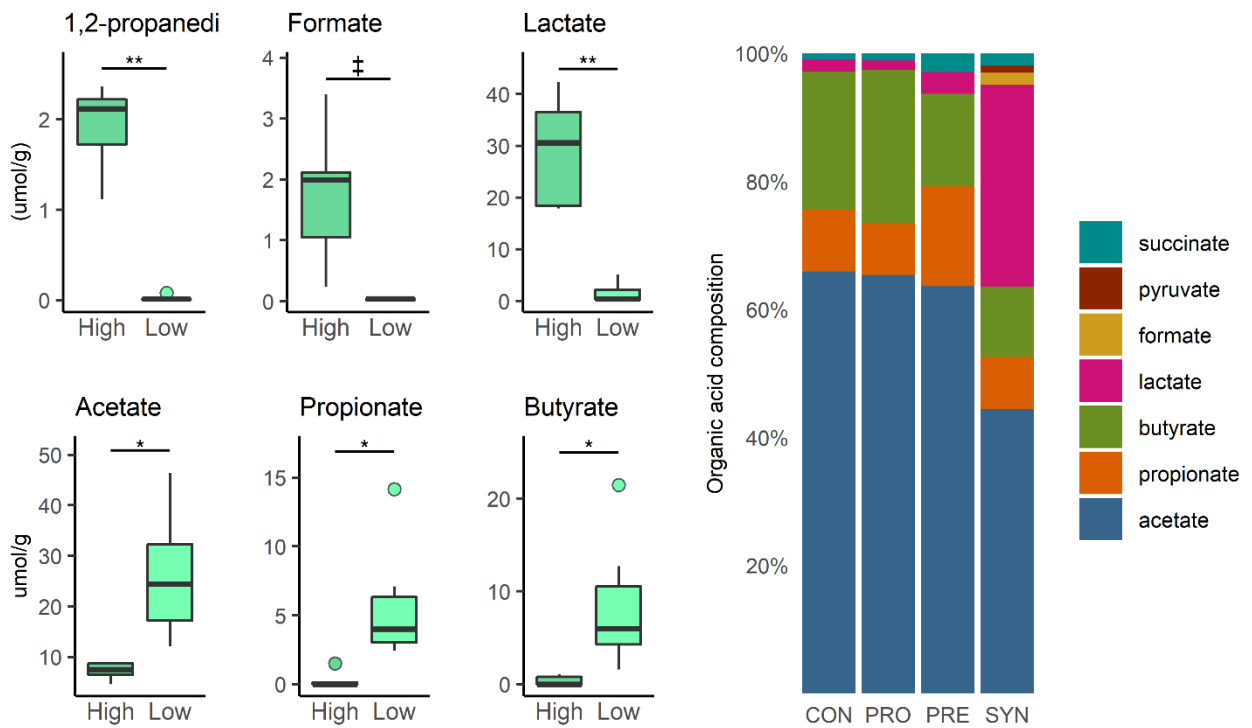
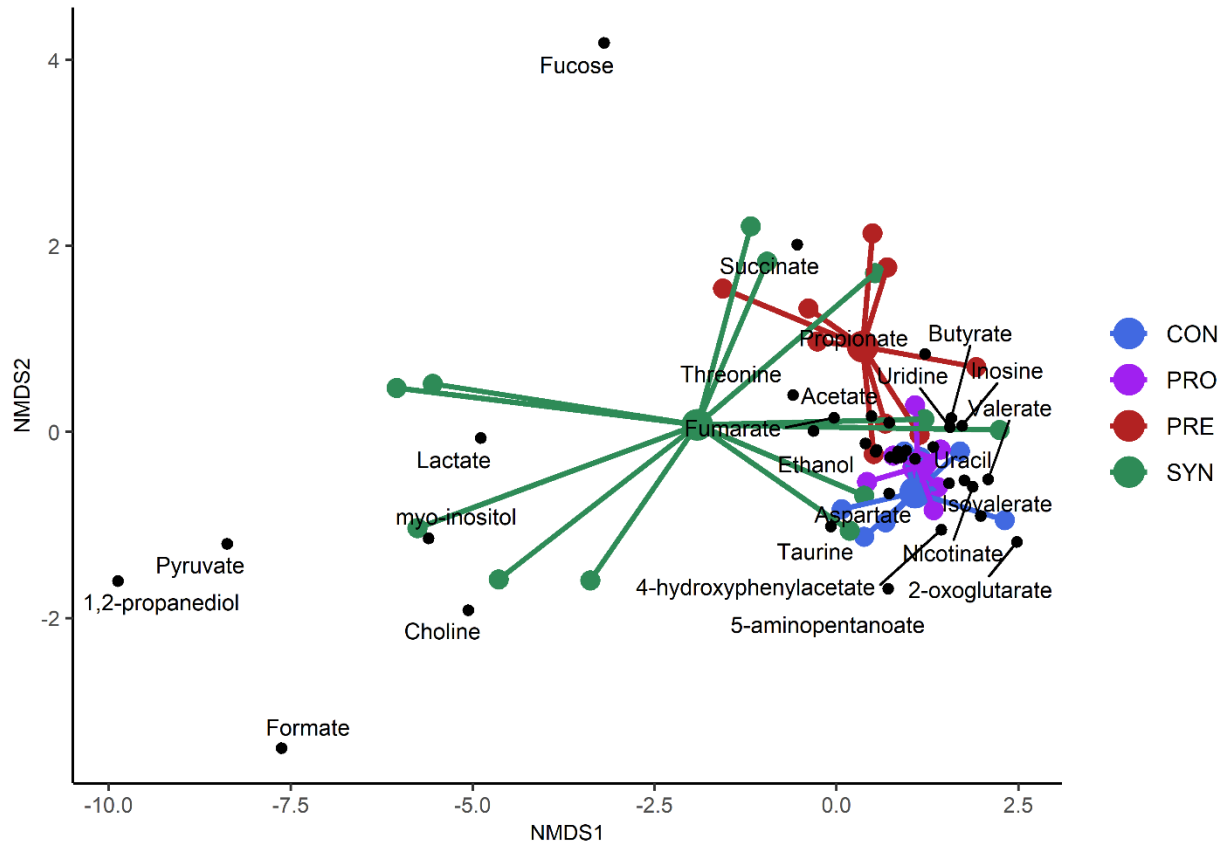


Figure 6. Metabolic profiling of colon contents reveal distinct compositions in synbiotic treated mice with high persistence of bifidobacteria. (A) NMDS plot of mouse colon content metabolome at the final experimental time point; (B) colon content metabolites differ across high and low persistence of *Bifidobacteriaceae* by high ($n = 4$) and low ($n = 7$); and (C) organic acid composition across treatment groups. Boxplots represent medians and interquartile range (IQR) with whisker end points equal to the maximum and minimum values below or above the median at 1.5 times the IQR. Organic acid composition was derived by the sum of each acid divided by the total. ** $p < 0.01$, * $p < 0.05$, ‡ $p < 0.1$.

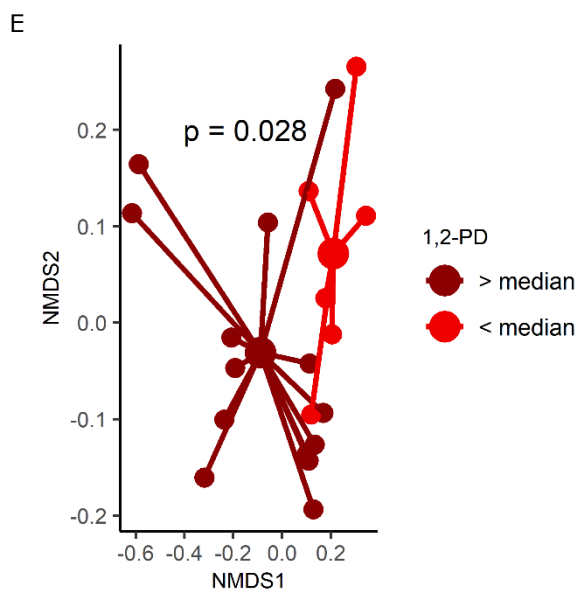
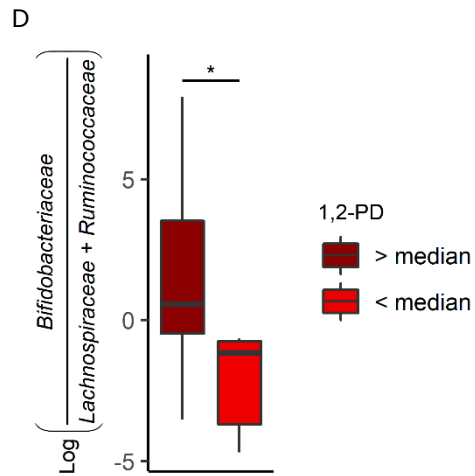
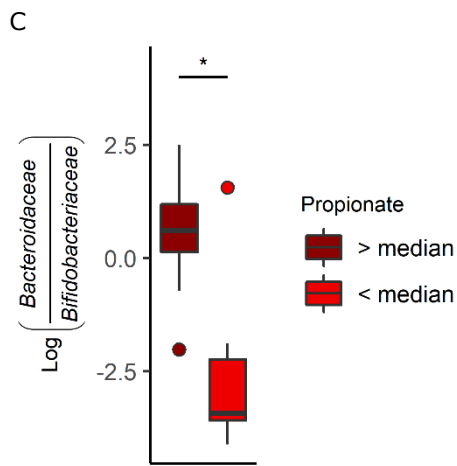
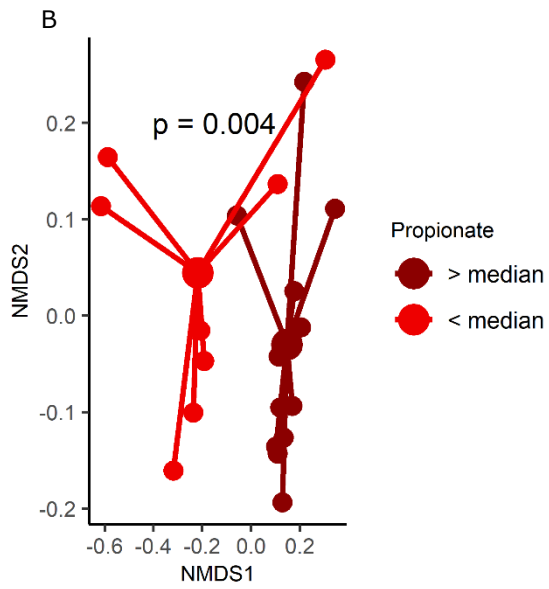
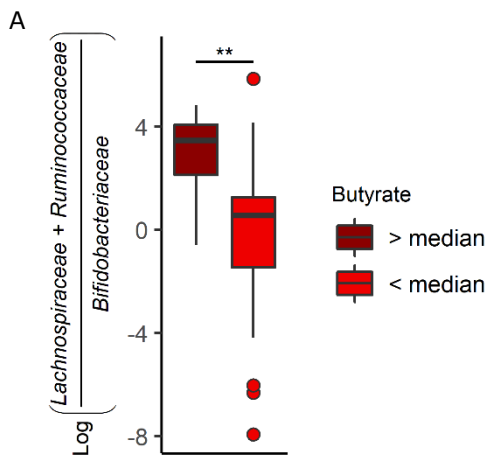
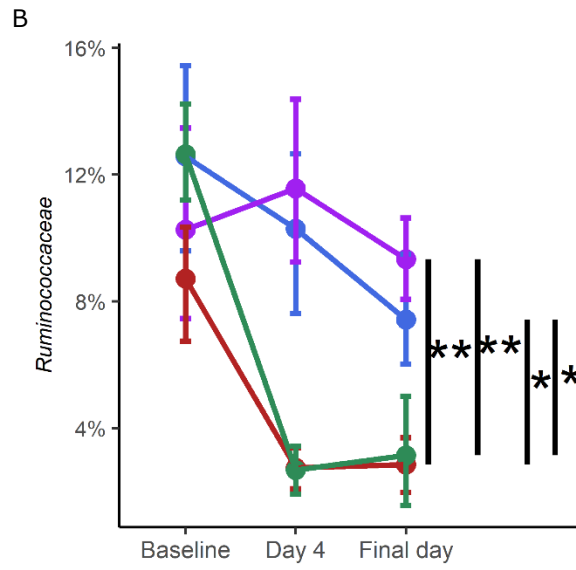
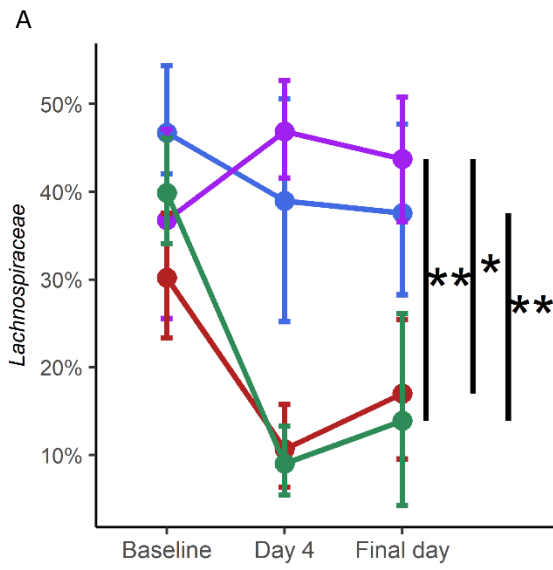


Figure 7. Microbial community differences are associated with distinct metabolite profiles at the final time point. (A) Log ratio of *Lachnospiraceae* and *Ruminococcaceae* relative to *Bifidobacteriaceae* for all treatments of mice, grouped as either above ($n = 19$) or below ($n = 19$) the median butyrate concentration; (B) log ratio of *Bacteroidaceae* relative to *Bifidobacteriaceae* for all 2-FL treated mice, grouped as either above ($n = 12$) or below ($n = 8$) the median propionate concentration; (C) NMDS plot of β -diversity index weighted UniFrac for above and below the median propionate concentration; (D) log ratio of *Bifidobacteriaceae* relative to *Lachnospiraceae* and *Ruminococcaceae* for all 2-FL treated mice, grouped as either above ($n = 14$) or below ($n = 6$) the median 1,2-propanediol (1,2-PD) concentration; and (E) NMDS plot of β -diversity index weighted UniFrac for above and below the median 1,2-PD concentration. Boxplots represent medians and interquartile range (IQR) with whisker end points equal to the maximum and minimum values below or above the median at 1.5 times the IQR. ** $p < 0.01$, * $p < 0.05$.



● CON ● PRO ● PRE ● SYN

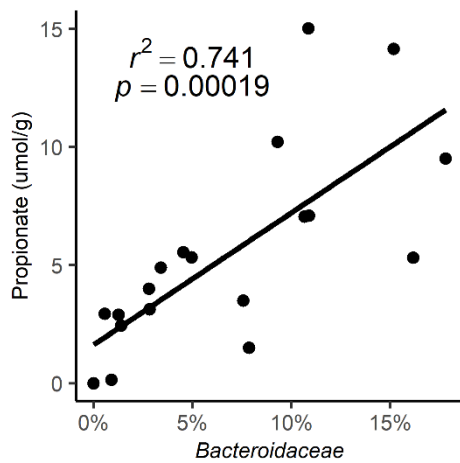
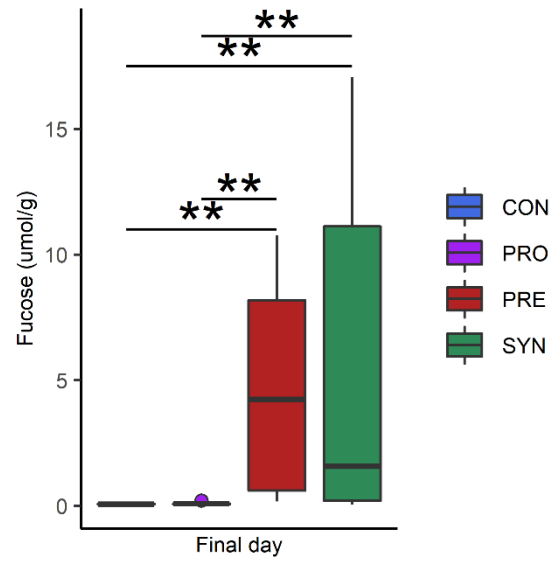
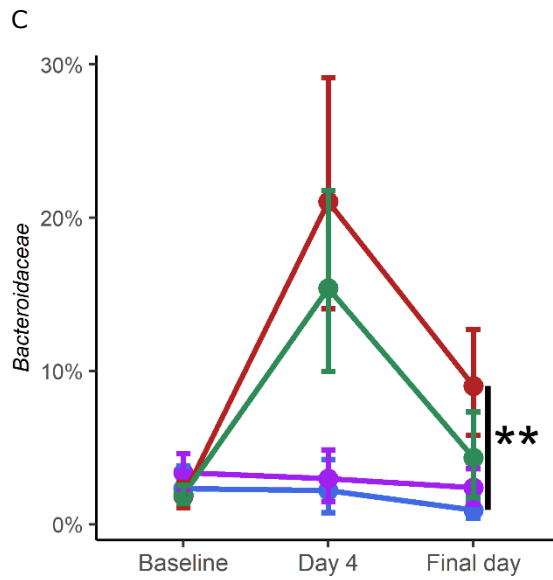


Figure 8. Bacterial relative abundances differ by treatment group. Relative abundance of (A) *Lachnospiraceae*, (B) *Ruminococcaceae*, and (C) *Bacteroidaceae* in control ($n = 9$), probiotic ($n = 9$), prebiotic ($n = 9$), and synbiotic ($n = 12$) groups from baseline, after oral gavage (day 4) and one week following (final day); (D) free fucose ($\mu\text{mol/g}$) at the final day for all treatments and (E) Pearson correlation between *Bacteroidaceae* and propionate in the PRE and SYN mice on final day. Boxplots represent medians and interquartile range (IQR) with whisker end points equal to the maximum and minimum values below or above the median at 1.5 times the IQR. Error bars for relative abundance data is represented as mean and standard error from bootstrapped confidence intervals with 1000 iterations. ** $p < 0.01$, * $p < 0.05$.

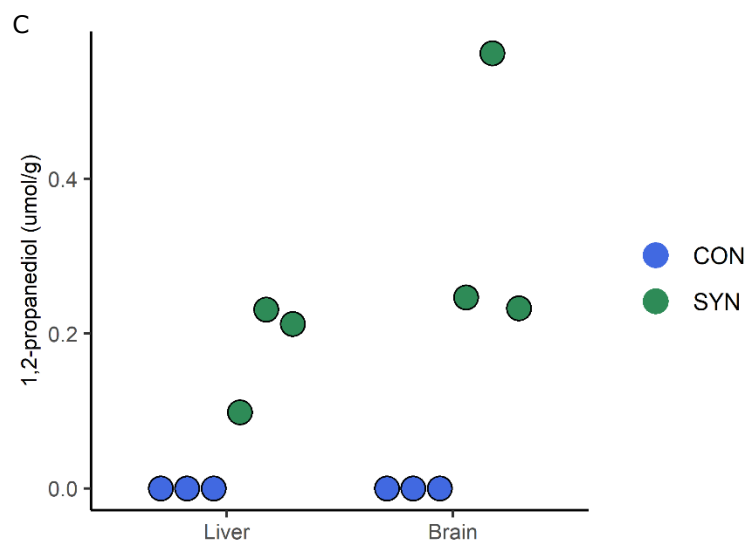
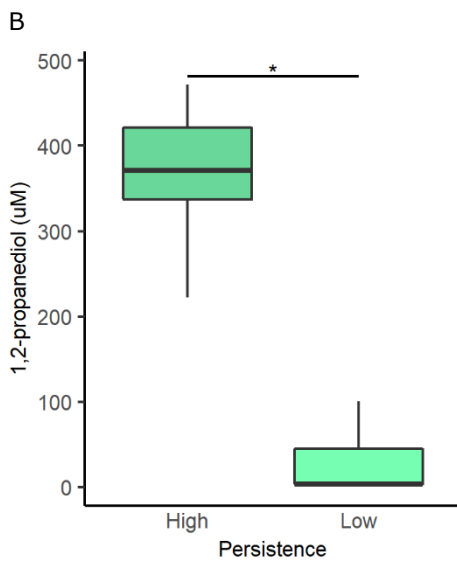
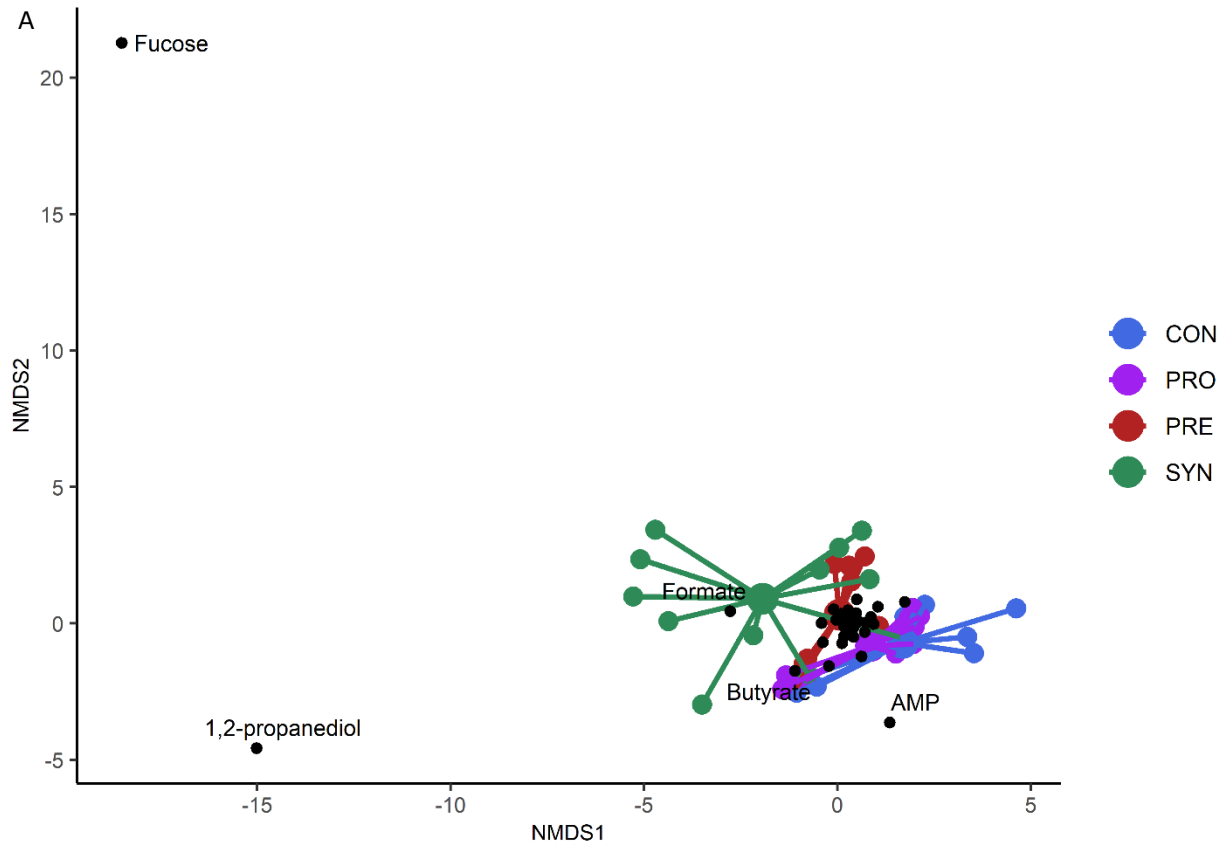


Figure 9. Metabolite profiling reveals enrichment of 1,2-PD in serum liver and brain in synbiotic treated mice. (A) NMDS plot of mouse serum metabolome at the final experimental time point; (B) serum 1,2-propanediol ($\mu\text{mol/g}$) by high and low bifidobacteria categorization of synbiotic treated mice; and (C) liver and brain 1,2-propanediol for control ($n = 3$) and synbiotic ($n = 3$) treated mice from experimental trial 1. Boxplots represent medians and interquartile range (IQR) with whisker end points equal to the maximum and minimum values below or above the median at 1.5 times the IQR. * $p < 0.05$.

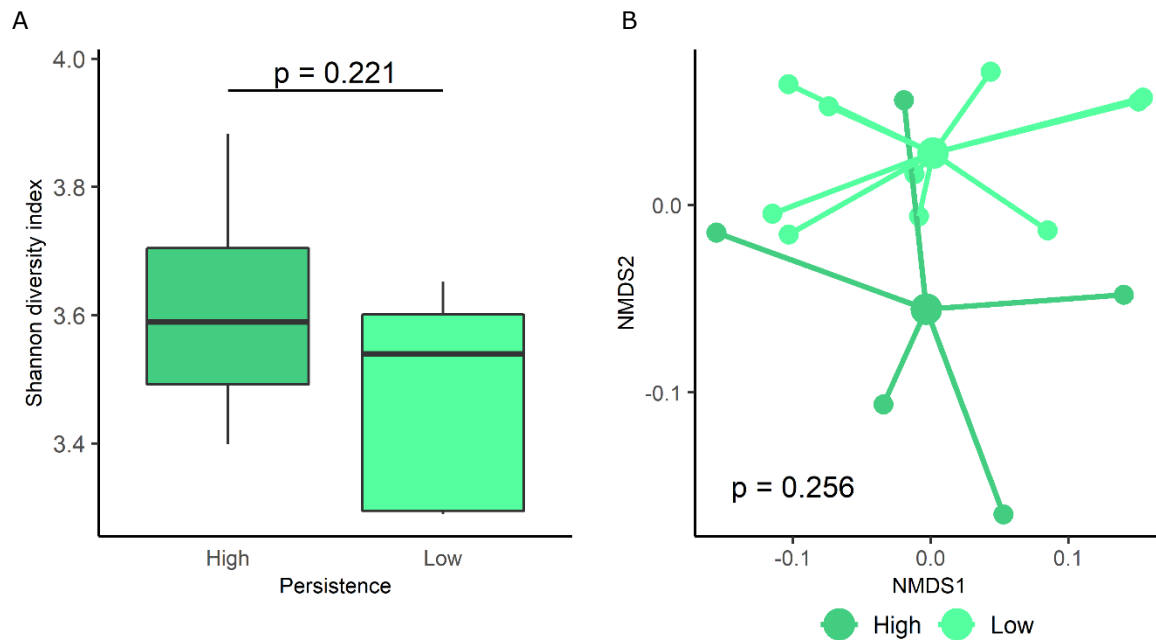
8. Supplementary Information

Supplementary Table 1. Pairwise comparisons* of weighted UniFrac measures between experimental trials at baseline.

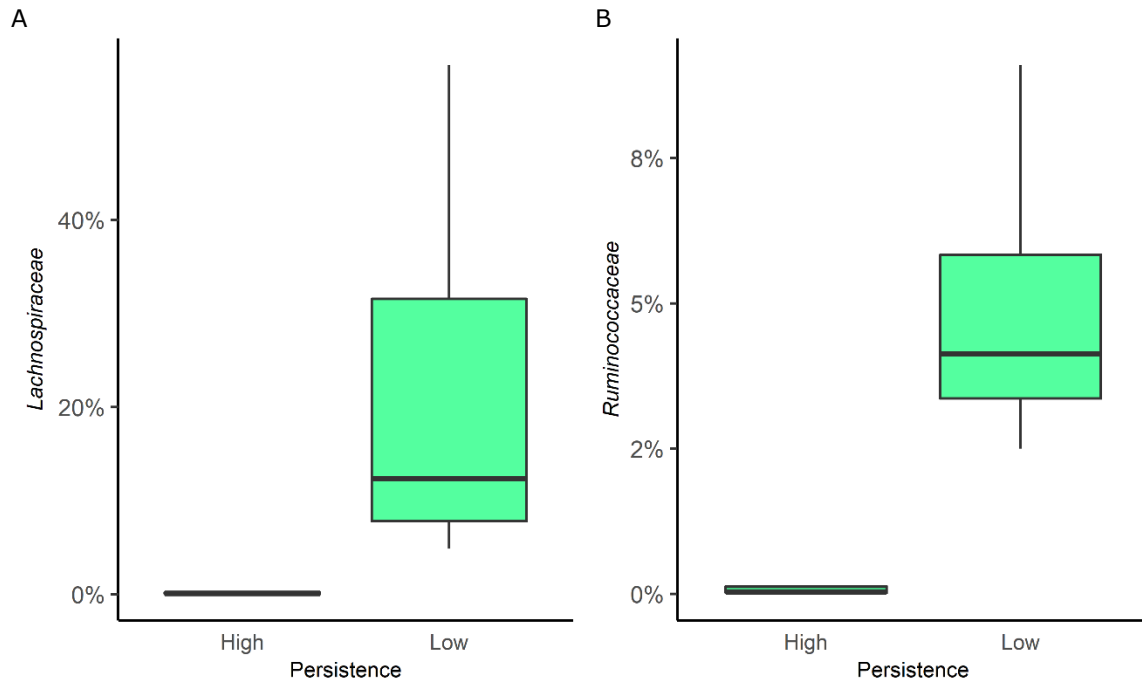
	First Exp. Trial	Second Exp. Trial
Second Experimental Trial	0.021	
Third Experimental Trial	0.021	0.148

*Comparisons were evaluated using PERMANOVA and FDR adjustment.

P-values with statistical significance are denoted in bold.



Supplementary Figure 1. Baseline differences not significant between high and low bifidobacteria categorizations of synbiotic treated mice. (A) Shannon α -diversity index values at baseline for synbiotic treated mice grouped as high ($n = 4$) and low ($n = 7$) bifidobacteria; and (B) NMDS plot of β -diversity index weighted UniFrac for high and low bifidobacteria groups at baseline. Boxplots represent medians and interquartile range (IQR) with whisker end points equal to the maximum and minimum values below or above the median at 1.5 times the IQR.



Supplementary Figure 2. Relative abundance of (A) Lachnospiraceae and (B) Ruminococcaceae at the final time point for synbiotic treated mice. High persistence ($n = 4$) low persistence ($n = 7$). Boxplots represent medians and interquartile range (IQR) with whisker end points equal to the maximum and minimum values below or above the median at 1.5 times the IQR.

Chapter 3: Preterm infant fecal microbiota and metabolite profiles are modulated in a probiotic specific manner^a

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^aA version of this work is being submitted to *The Journal of Pediatrics*.

1. Summary

Probiotic supplementation in preterm infants has been shown to effectively reduce clinical complications related to aberrant gut microbial community development. Characterizing the ecologic, molecular interactions by which probiotic organisms reduce enteric disease burden will aid development of probiotic therapies. In this single-center observational cohort study of 45 low birth weight preterm infants, we assessed the effects of *Bifidobacterium longum* subsp. *infantis* or *Lactobacillus reuteri* supplementation on the infant gut microbiota by 16S rRNA gene marker sequencing and fecal metabolites by ¹H NMR spectroscopy. Human milk oligosaccharides (HMOs) were quantified by NMR in each milk and fecal sample during sample collection. Comparatively, each probiotic treatment was associated with increased proportions of the respective taxon. Fecal HMOs were significantly higher in *L. reuteri* fed babies despite similar HMO content in the milk consumed. Fecal metabolites associated with bifidobacteria fermentation products were significantly increased in *B. infantis* supplemented infants. Intestinal inflammation was decreased in *B. infantis* relative to *L. reuteri* treatment and was associated with the microbial metabolite indole-3-lactate. This study demonstrates supplementing an HMO-catabolizing *Bifidobacterium* probiotic corresponds to increased microbial metabolism and reduced inflammation relative to a non-catabolizing *Lactobacillus* probiotic in the human milk fed preterm infant gut. In this context, *Bifidobacterium* may provide greater benefit via activation of the microbiota-metabolite-immune axis.

2. Introduction

Early microbial colonization of the intestine is fundamental for neonatal development, as it has the capacity to influence the metabolic and immune function of the host. However, factors including antibiotic exposure^{1,2} and delivery by caesarian section³ perturb the gut microbiota in preterm

infants. While the increased frequency of antibiotic use in newborns and caesarian delivery are aimed at reducing mortality, delayed maturation of the microbiota creates collateral complications associated with increased risk of asthma⁴, diabetes⁵, and obesity⁶ at later life stages. Moreover, development of necrotizing enterocolitis (NEC), a devastating enteric disease primarily afflicting preterm infants, is linked to disruption in the assembly of the gut microbial community⁷. Probiotic administration in preterm infants has been shown to be an effective therapy for reducing risk of NEC by rectifying gut dysbiosis⁸⁻¹⁰.

Several common probiotics from the genera *Bifidobacterium* and *Lactobacillus* have been widely studied¹¹. Selection of probiotic species and strain is an important consideration as the beneficial effects depend on the probiotic's ability to compete with other microbial community members and secrete bioactive compounds in quantities to elicit a host response. Human milk oligosaccharides (HMOs) represent a major source of fermentable substrate in early life, exerting a prebiotic effect on microbes able to catabolize these complex sugars. Gene clusters involved in HMO utilization are observed in the genome of some *Bifidobacterium* species¹², supporting their colonization in breastfed infants¹³. Members of the *Lactobacillus* genus have a minimal capacity for HMO utilization¹⁴, limiting their ability to use this privileged resource and limiting colonization potential during provision of breastmilk.

We sought to compare the impact of two probiotics, *Bifidobacterium longum* subsp. *infantis* EVC001 (*B. infantis*) and *Lactobacillus reuteri* DSM 17938 (*L. reuteri*) in an observational prospective cohort study. The outcomes measured were the efficacy of colonization fecal metabolites and intestinal inflammation in preterm infants. To our knowledge this is the first study to explore the differences these organisms exert on preterm infant intestinal microbiota, microbial metabolism and inflammation in a single center study.

3. Results

Infant characteristics

Demographic data from the cohort are summarized in **Table 3**. Overall, birthweight, gestational age, infant sex, and percentage of infants delivered by cesarean section were not significantly different between the cohorts. Additionally, the number of days infants received antibiotics and infant morbidity and mortality were similar between groups. Infants in the *B. infantis* probiotic treatment group had significantly higher one-minute Apgar scores ($p < 0.05$); however, at five minutes, the Apgar scores of infants in both groups were similar. The *L. reuteri* probiotic was started on admission whereas the *B. infantis* probiotic was started when 3 mL feeding volumes were reached resulting in an earlier Day-of-life (DOL) for the first probiotic dose in infants receiving *L. reuteri* compared to infants receiving *B. infantis* (median DOL one and three respectively, $p < 0.05$).

Feeding mode and Milk HMO profiles are similar across probiotic groups

For the duration of the study, all infants consumed either mother's own milk (MOM) or pasteurized donor human milk (PDHM) (See Methods for details on feeding protocol). Human milk oligosaccharides were assessed in samples of milk consumed by each infant at 30 and 32 weeks CGA. Comparison of the milk received by both groups showed no significant difference in the HMO composition between groups (**Figure 10**). In each group, infants were categorized as receiving MOM, PDHM, or a combination (**Table 3**). The proportion of infants receiving predominantly or exclusively MOM's milk was similar for the two cohorts (82% and 85% for the *L. reuteri* and *B. infantis* groups respectively).

Probiotics influence the preterm infant gut microbiota

Taxonomic composition of the fecal microbiota for each infant is displayed in **Figure 11A, B**. Supplementation with *B. infantis* significantly increased the proportion of *Bifidobacteriaceae* compared to infants provided *L. reuteri* at 30 and 32 weeks CGA (**Figure 11C**, Wilcoxon rank-sum test, $p < 0.01$). Conversely, supplementing with *L. reuteri* resulted in a significantly higher percentage of *Lactobacillaceae* relative to infants provided *B. infantis* at both 30 and 32 weeks CGA (**Figure 11D**, Wilcoxon rank-sum test, $p < 0.01$). Large proportions of *Enterobacteriaceae* were present in many preterm infant gut microbiota at each gestational age. Supplementation of *B. infantis* revealed a trend toward lower *Enterobacteriaceae* abundance compared to infants supplemented with *L. reuteri* at 30 weeks CGA; however, no significant difference was observed at 32 weeks CGA (**Figure S3**). To determine whether probiotic supplementation impacted fecal microbial β -diversity, treatment groups were compared using permutational multivariate ANOVA (PERMANOVA). The fecal microbiota of infants provided the *B. infantis* and *L. reuteri* probiotics showed distinct compositions by 30 weeks CGA; however, due to the small sample size did not reach statistical significance (**Figure 11E**, Adonis, $p = 0.37$). At 32 weeks CGA microbial communities separated by probiotic treatment and were found to be statistically different (**Figure 11F**, Adonis, $p = 0.04$).

Probiotic supplementation affects fecal HMO and metabolite profiles

HMOs passing through the intestinal tract and present in the feces have not been digested by intestinal microbes. Univariate analysis of individual fecal HMOs showed that infants provided *L. reuteri* had higher 3-FL, LNFP II, LNFP III, 3'-SL and 6'-SL ($p < 0.01$) and a trend for higher 2'-FL, LNFP I, LDFT and LNT ($p < 0.1$) compared to infants provided *B. infantis* at 30 weeks CGA (**Figure 12A**, Wilcoxon rank-sum test). At 32 weeks CGA, with the exception of LNnT, all fecal HMOs were significantly elevated in the *L. reuteri* group compared to the *B. infantis* group ($p <$

0.01) suggesting increased utilization of HMOs by *B. infantis* (**Figure 12B**, Wilcoxon rank-sum test). Analysis of HMO fermentation products revealed only a trend for increased 1,2-propanediol in the *B. infantis* group ($p < 0.1$) at 30 weeks CGA (**Figure 12C**, Wilcoxon rank-sum test), and significantly higher 1,2-propanediol, formate, acetate, pyruvate and indole-3-lactate (ILA) in the *B. infantis* group at 32 weeks CGA (Acetate; $p < 0.05$, others; $p < 0.01$) (**Figure 12D**, Wilcoxon rank-sum test). Fecal short chain fatty acids (SCFAs), propionate and butyrate, trended higher ($p = 0.1$) in the group receiving *L. reuteri* at 30 weeks CGA compared to the group receiving *B. infantis* (**Figure S4A, C**, Wilcoxon rank-sum test); however, by 32 weeks, similar concentrations of propionate and butyrate were observed (**Figure S4B, D**). Total organic acids in feces were also compared to examine the fermentative activity in each probiotic group along with five infants who did not receive a probiotic. The *B. infantis* group had significantly higher total fecal acids compared to infants that received *L. reuteri* and those who received no probiotic (**Figure S5**, $p < 0.05$, Kruskal-Wallis H test).

Fucosylated HMOs (FHMO) contain one or more fucose residues. A very low abundance of HMOs with an $\alpha 1,2$ fucosyl linkage is characteristic of mothers who are homozygous for one of the common mutations in the FUT2 gene, and these individuals are commonly referred to as non-secretors. Excluding infants of non-secretor mothers, correlations were observed between total milk HMO and fecal metabolites, with trends for acetate and ILA in infants provided *B. infantis* at 32 weeks CGA (**Figure 13A**, Spearman's rho, $p < 0.1$). Plasma remaining for routine clinical laboratory analyses provided an opportunity to assess the relationship of microbial metabolites in the gut and circulation. Plasma acetate and 1,2-propanediol correlated with their respective fecal metabolites for infants in the *B. infantis* group at 32 weeks CGA indicating absorption of these compounds across the gut epithelium (**Figure 13B**, Spearman's rho). With the exception of a trend

for 1,2-propanediol and total HMO at 30 weeks CGA, no correlations were found to be significant in infants receiving *L. reuteri* (**Figure S6**, Spearman's rho) suggesting the associations between fecal acetate or ILA and milk HMOs are stronger in infants receiving *B. infantis*.

Bifidobacterium treatment is associated with reduced intestinal inflammation

Microbial metabolites including SCFAs and indole derivatives have demonstrated potent anti-inflammatory activity^{15,16}. Given that infants supplemented with *B. infantis* had significantly higher levels of fecal ILA ($p < 0.01$) (**Figure 14A**, Wilcoxon rank-sum test), we decided to investigate the effect of probiotic treatment on gut inflammation by examining fecal calprotectin in a subset of infants between 30 and 32 weeks CGA. Infants supplemented with *B. infantis* had significantly lower fecal calprotectin ($p < 0.01$) compared to infants provided *L. reuteri* (**Figure 14B**, Wilcoxon rank-sum test). After correcting for gestational age at birth we observed a modest negative correlation for ILA and calprotectin (**Figure 14C**, partial spearman's correlation, $\rho = -0.385, p = 0.02$).

4. Discussion

Probiotic supplementation of preterm infants is becoming a standard of care in neonatal intensive care units; however, there is still debate about what probiotic organism(s) should be provided to the premature neonate. This study shows that providing premature infants with a *B. infantis* probiotic combined with either MOM or donor milk is associated with greater concentrations of HMO fermentation products and lower levels of intestinal inflammation compared to infants provided an *L. reuteri* probiotic. Manipulation of the gut microbiota to increase the presence of HMO catabolizing microbes may exert an immunomodulatory role in the infant via a microbiota-metabolite-immune axis.

Supplementation of *B. infantis* and *L. reuteri* each increased the relative abundance of *Bifidobacteriaceae* and *Lactobacillaceae* in the respective probiotic groups. However, the median relative abundance of *Bifidobacteriaceae* in the *B. infantis* supplemented group was higher than *Lactobacillaceae* in the *L. reuteri* supplemented group at each time point (38.2% vs. 3.4% at 30 weeks CGA and 17.5% vs 7.1% at 32 weeks CGA). This may reflect the adaptation of *B. infantis* in the infant gut through utilizing HMOs to confer a growth advantage¹⁷. In contrast, *L. reuteri* strains are unable to consume many HMOs^{14,17,18}. Previous cell culture growth assays of *L. reuteri* evaluated for growth on five separate HMOs resulted in only minimal growth on LNnT¹⁴. Interestingly, we found all fecal HMOs to be lower in *B. infantis* treated infants at each time point with the exception of LNnT, which may indicate catabolism of this HMO by *L. reuteri*. However, in the absence of a privileged nutrient source, increased competition between *L. reuteri* and other members of the developing microbial community limit its growth potential as shown by the relatively small proportions of *Lactobacillaceae* in this cohort. A recent observational study where preterm infants were supplemented with Infloran, a probiotic containing *Lactobacillus acidophilus* and *Bifidobacterium bifidum*, showed a much greater average proportion of the HMO-consuming *Bifidobacterium* compared with *Lactobacillus*¹⁹.

The majority of infants in this study were receiving human milk supporting the observed bifidogenic effect. Pairing strains of *Bifidobacterium* that harbor HMO gene clusters with human milk may diminish colonization by pathobionts such as members of the *Enterobacteriaceae* family, which contain taxa associated with gut dysbiosis and NEC^{20,21}. *Enterobacteriaceae* are often observed at high proportions in preterm infants exposed to antibiotics early in life^{1,3,22}, and their dominance in this population is further strengthened by the fact that several members in this family carry antibiotic resistance genes^{23,24}. Importantly, in term breastfed infants, higher

proportions of stool bifidobacteria were observed to be associated with a reduction in antimicrobial resistance gene carriage²⁵. This finding supports that antimicrobial resistant microbes could be reduced in the preterm gut through supplementation of bifidobacteria and human milk. Indeed, antibiotic treatment associated increases in *Enterobacteriaceae* were ameliorated in infants administered a multistrain probiotic supplement containing *Bifidobacterium breve*, *Propionibacterium freundenreichii* subsp. *shermanii* and *Lactobacillus rhamnosus*, only when at least partial breastfeeding occurred, which may be in part mediated by the prebiotic effect of HMOs²⁶. In this study, the proportion of *Enterobacteriaceae* trended lower in infants supplemented with *B. infantis* compared to *L. reuteri* at 30 weeks CGA; however, no difference was seen at 32 weeks CGA.

Human milk oligosaccharides provide a fermentable carbon source to microorganisms for generating organic acid metabolites that reduce fecal pH. The more acidic gut environment is less hospitable to pathobionts as described by the inverse relationship between fecal pH and *Proteobacteria* in human infants²⁷, and inhibition of enterohemorrhagic *Escherichia coli* by acetate producing bifidobacteria in mice²⁸. We found that supplementation of *B. infantis* to premature infants along with either MOM or donor milk was associated with higher levels of several HMO fermentation products including formate, acetate, pyruvate and 1,2-propanediol (a metabolite specific to fucose catabolism) and lower levels of fecal HMOs (Figure S3). Although each of these metabolites are not exclusively produced by bifidobacteria, they are known to be secreted by *Bifidobacterium* spp. during metabolism of HMOs²⁹. The total organic acid content in feces of infants supplemented with *B. infantis* was significantly higher than those that had received the *Lactobacillus* probiotic. Interestingly, fecal acetate and 1,2-propanediol in infants given *B. infantis* were correlated with their concentrations in plasma suggesting absorption of these

microbially produced molecules into circulation. As such, the relationship between increased proportions of *Bifidobacteriaceae* and concentrations of metabolites in the gut and circulation support the inference that supplementation of *B. infantis* can have a systemic impact on host metabolism.

Standard of care at the UC Davis NICU dictates that infants born with birth weight > 1500 grams and/or gestational age greater than 32 weeks receive no probiotic supplementation. In this study, 5 infants were thus ineligible for probiotic supplementation providing an opportunity to compare intestinal metabolism at 32 weeks CGA. Total fecal organic acids in non-supplemented infants were significantly lower compared to infants that had been supplemented with *B. infantis*. The low acid content in infants not receiving probiotics likely reflects a nascent microbiome structure represented by few microbial taxa as a result of sampling during the first week of life³⁰. Indeed, many studies suggest that gestational age is a primary determinate of microbiome assembly³¹⁻³³. However, probiotic supplementation has the potential to augment the durability of the microbiota by conferring colonization resistance mediated through increased organic acid production²⁷.

Human milk has been associated with reducing the burden of infectious disease in preterm infants³⁴, with additional studies showing protection from NEC and death^{35,36}. The mechanisms involved in providing this protection is multifaceted due to the myriad of bioactive components in the human milk matrix. However, the well-documented relationship between HMOs and the developing microbiota has implications regarding the impact of human milk on microbial colonization and subsequent clinical outcomes related to gut dysbiosis. For example, a study of preterm infants provided Infloran resulted in a significant reduction in the incidence of NEC, but only in those that had consumed breast milk; no difference in NEC was observed in infants exclusively formula fed despite receiving probiotic treatment³⁷. Similarly, in a large retrospective

cohort study of preterm infants, significant reduction in the incidence of NEC and mortality was associated with probiotic treatment⁹. Interestingly, in their logistic regression model, the adjusted covariates, which included gestational age, Score for Neonatal Acute Physiology, outborn status and human milk intake, any human milk intake had the highest Wald Chi-square statistic, highlighting its importance as a mediator in their modeled risk of NEC development. That study also found that a multi-strain cocktail was more protective against NEC and mortality compared to a single probiotic strain. Notably, the multistrain probiotic included four *Bifidobacterium* (including the HMO-catabolizing *B. infantis*) and one *Lactobacillus* strain, whereas the single strain probiotic contained *L. reuteri*. As such, it can't be determined if the reduction in NEC is afforded by multiple stains or inclusion of bifidobacteria in the probiotic supplement. In the present study the cases of NEC or death were too few to draw any conclusions regarding risk reduction relative to probiotic species. Additional research is needed to assess the protections afforded by select probiotic species and strains.

Metabolites produced by gut microbes in the gut lumen are able to interact with epithelial and immune cells by acting as ligands for receptors governing production of inflammatory mediators thereby affecting intestinal inflammation. Indole-3-lactic acid, a tryptophan metabolite produced by a number of genera including *Bifidobacterium* and *Lactobacillus*, has been noted for its anti-inflammatory property³⁸. The molecular interaction occurs through ILA binding to the aryl hydrocarbon receptor (AhR) on T cells and colonocytes, affecting expression of anti- and pro-inflammatory cytokines³⁹. Despite that both *B. infantis* and *L. reuteri* are capable of producing ILA^{38,40}, we observed significantly lower concentrations of fecal ILA in infants supplemented with *L. reuteri*. This may be attributed to a lower relative abundance of lactobacilli and a greater relative proportion of bifidobacteria that enables increased turnover of tryptophan to ILA. We have

previously shown that HMO consumption by *B. infantis* increased ILA production compared with the same strain grown on glucose¹⁵, providing a rationale for increased fecal ILA in infants given *B. infantis*. Measuring gut inflammation by fecal calprotectin, a marker of neutrophil infiltrate, revealed lower inflammation in infants given *B. infantis* relative to *L. reuteri*. Moreover, levels of calprotectin had a modest inverse correlation with fecal ILA supporting the relationship between ILA and immune function. There are many factors that contribute to the overall inflammatory state and future experimental studies are needed to investigate the mechanisms through which gut microbes mediate this response.

In summary, we find that the premature infant gut is amenable to probiotic supplementation resulting in increased proportions of these probiotic taxa whilst altering fecal metabolite profiles and intestinal inflammation. Due to its functional capacity to degrade HMOs, select bifidobacteria are enriched with human milk feeding favoring a shift toward a saccharolytic gut microbiota. Increases in microbial metabolites produced by bifidobacteria such as ILA are associated with reduced intestinal inflammation as was observed in this cohort; however, there are many factors that contribute to the overall inflammatory status. This observational cohort study compares the use of two widely administered probiotics, providing evidence for the therapeutic value of supplementing preterm infants with *B. infantis* and providing MOM or donor milk to support gut microbial development and metabolism at this critical stage of life.

Strengths of this study include the collection of samples from a single NICU that reduces confounding that may occur from multiple sites. Additionally, we were able to obtain matched samples of milk and feces for a majority of infants at each time of sample collection, providing an accurate relationship between microbiota development and milk HMO content. The use of corrected gestational ages for sample collection provides an assessment of microbiome

development at specific time points. There are limitations and caveats to this study that should be acknowledged when making inferences from the analysis. This is a prospective cohort study and not a randomized control trial. There is no reference group that had not received a probiotic to compare with *B. infantis* and *L. reuteri* supplemented infants as the study was conducted at a single NICU where probiotic treatment is administered as routine standard of care for infants with birth weight < 1500 grams and gestational age less than 32 weeks to decrease risk of death and necrotizing enterocolitis. Additionally, as recommended by the manufacturers, the *B. infantis* probiotic was provided at higher dose (8×10^9 microbes) compared to the *L. reuteri* probiotic (1×10^8 microbes).

5. Methods

Ethical approval and sample collection

This ongoing prospective cohort study of preterm infants was approved by the Institutional Review Board of the University of California Davis in April 2016 and was registered at clinicaltrials.gov (NCT03717584). Parents of infants born at less than 33 weeks gestation were approached for consent either prior to birth or in the first week of life. Collection of specimens began at birth or the time of parental consent and included twice weekly stool samples from a soiled diaper, once weekly samples of milk being fed to the infant (either mother's own milk (MOM) or pasteurized donor human milk (PDHM), and leftover plasma from routine clinical laboratory analyses. Clinical data were collected from the infant's electronic medical record.

Human study design

Prior to initiation of the cohort study, the UC Davis NICU initiated a standardized feeding protocol which included feeding of mother's colostrum as soon as it was available, a prescribed method of advancement of feeding volume, early fortification with bovine-based human milk fortifier (when

the infant was tolerating feeding volumes of 40 mL/kg/d), provision of PDHM when MOM volume was not sufficient (beginning at 48 hours of life), and provision of probiotic microbes in an attempt to decrease risk of death and necrotizing enterocolitis (NEC). When infants reached postmenstrual age of 34 weeks, the probiotic product was stopped and if the volume of MOM was insufficient, infants received preterm infant formula rather than PDHM. At the onset of the cohort study, the probiotic product *Lactobacillus reuteri* DSM 17938 (Biogaia Protectis with Vitamin D) was provided at a daily dose of 5 drops (100 million organisms). This product was continued until our routine prophylactic probiotic product was changed to *Bifidobacterium longum* subspecies *infantis* EVC001 (Evolve BioSystems, Evivo) at a daily dose of 8 billion microbes. Both products doses were administered per manufacturer instructions. The change was based on a clinical consensus among the attending neonatologists established from clinical data from our institution demonstrating effective colonization in preterm infants and prevention of NEC in a preclinical model with a similar strain (*B. infantis* ATCC 15697)^{17,41}.

This prospective cohort study included 45 low birth weight (< 2500 g) premature infants with gestational age (GA) ranging from 23 to 32 weeks. This study is not a randomized clinical trial. All of the infants with birth weight < 1500 grams and less than 33 weeks GA received both human milk and one of the two probiotic products. In order to avoid cross-contamination, enrollment in the cohort study was stopped in Mar 2018 (two months prior to the change in probiotic products) and then restarted in late July 2018 (two months after the change in probiotic). None of the enrolled infants received both products. One infant received *L. reuteri* during the *B. infantis* time period at the preference of the attending neonatologist on service. From the 80 infants enrolled in the cohort study from its onset to September 2019, data from all infants treated with a probiotic for whom we had samples of stool (plus or minus milk or plasma) available at 30 and/or 32 weeks

postmenstrual age (N=45) were analyzed. We also analyzed stool samples from 5 infants born with birth weight > 1500 grams for whom probiotic supplementation was not clinically indicated.

Sample processing

Fecal samples were weighed, combined with 500 μ L of ice-cold PBS and vortexed for 4 minutes at maximum setting. Samples were then centrifuged at 6k RCF, at 4 °C for 15 minutes and the supernatant transferred to a new 1.5 mL tube. The fecal pellet was freeze dried to obtain dry weight. The supernatant was spun down at 14k RCF, at 4°C for 10 minutes and transferred to a 3 kDa filter to remove microbes and proteins (14k RCF, 4°C for 45 minutes). Filtrate was spiked with internal standard containing 5 mM 3-(trimethylsilyl)-1-propanesulfonic acid-d₆ (DSS-d₆), NaN₃, and D₂O at 10% final volume and adjusted to pH 6.8 \pm 0.1 using HCl and/or NaOH. Milk samples were centrifuged at 11k RCF, at 4°C for 5 minutes to separate the aqueous layer from other milk components. An aliquot of aqueous milk was transferred to a 3 kDa filter and centrifuged at 14k RCF, at 4 °C for 45 minutes. The resulting filtrate was spiked with the DSS-d₆ internal standard at 10% final volume and adjusted to pH 6.8 \pm 0.1 using HCl and/or NaOH. Plasma samples were thawed and transferred to a 3 kDa filter and centrifuged for 60 minutes at 14k RCF and 4 °C. 207 μ L of filtrate was transferred to a clean 1.5 mL tube and combined with 23 μ L of the DSS-d₆ internal standard. Sample pH was adjusted to 6.8 \pm 0.1 using HCl and/or NaOH.

¹H NMR Spectroscopy

Metabolite concentrations from feces, plasma, and milk samples were determined using ¹H nuclear magnetic resonance (NMR) spectroscopy using a NOESY ¹H pre-saturation experiment on a Bruker Avance 600 MHz spectrometer (Bruker BioSpin, Germany) as previously described in He et al.⁴².

16S rRNA gene sequencing

Fecal microbiota were characterized by amplification and sequencing of the V4 region of the 16S rRNA gene as described in Huda et al.⁴³. The resulting reads were processed using the DADA2 pipeline in QIIME2 as previously described^{44,45}. Samples with less than 1500 reads were removed from the amplicon sequence variant (ASV) table before continuing with the analysis.

Fecal calprotectin

Quantitation of fecal calprotectin was accomplished using BÜHLMANN fCAL® ELISA (BÜHLMANN Laboratories AG, Switzerland), according to the manufacturer's instructions. Absorbance was measured at a wavelength of 450 nm and blanked at 620 nm using a Tecan Sunrise (Tecan Trading AG, Switzerland) plate reader. Samples were measured in duplicate and the average OD was used for analysis.

Statistical analysis

All statistical analyses and graphics were generated using R (v4.0.2) unless otherwise stated. 16S rRNA gene sequencing data was analyzed with the vegan package (2.5-6) in R. Non-metric multidimensional scaling (NMDS) plots were generated (vegan::metaMDS, k = 3, distance = "bray") to visualize microbial community structure. Compositional differences in fecal microbiota between probiotic groups were assessed for β -diversity through PERMANOVA with default parameters of vegan::adonis2 after checking for differences in group dispersion with vegan::betadisper. Differences in proportions of family level taxa were compared using the Wilcoxon-rank sum test. For metabolite data, normality was assessed using the Shapiro-Wilk test in addition to observing deviations in the residuals of Quantile-Quantile plots. Generalized log transformations ($\log(y + \sqrt{y^2 + 1})$) were performed to approximate normality for non-normally distributed metabolite data. Non-parametric tests were applied in cases where metabolite data did not approximate normality after transformation.

For fecal calprotectin measurement, a sigmoidal standard curve was created using a four point logistic regression model in GraphPad Prism v 8.4.3 for interpolation of fecal calprotectin concentrations. Log concentrations were input into the model and back transformed for visualization. Samples were measured in duplicate and the average OD used. Three data points were unable to be fit to the standard curve based on either extremely high or low values (outliers) and therefore omitted in the analysis. Spearman correlations were used to measure the association in heteroscedastic data for metabolites, calprotectin and milk FHMOs. All p -values were corrected for multiple testing by FDR correction with significance assessed as $p < 0.05$ and statistical trends considered as $p < 0.1$.

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Author contributions

Conceptualization, C.M.S, M.A.U. and J.A.L.; Methodology, C.M.S, J.A.L. and D.H.T.; Formal analysis, J.A.L.; Investigation, J.A.L. and D.H.T.; Resources, C.M.S., K.K., K.I., S.I., M.A.U., D.A.M., Writing – Original Draft, J.A.L and C.M.S.; Writing – Review & Editing, J.A.L, C.M.S, M.A.U., D.A.M., and K.K.; Visualization, J.A.L., Funding Acquisition, C.M.S and D.A.M.; Supervision, C.M.S., M.A.U. and D.A.M.

Declaration of interests

D.A.M. is a co-founder of Evolve Biosystems, a company focused on diet-based manipulation of the gut microbiota and BCD Biosciences, a company advancing novel bioactive glycans. Neither Evolve Biosystems nor BCD Biosciences had a role in the conceptualization, design, data collection, analysis, or preparation of this manuscript.

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7. Tables

Table 3. Characteristics of infants who received probiotics

	<i>L. reuteri</i> (N=16)	<i>B. infantis</i> (N=29)	p-value
Birth weight (g), mean (SD)	1078 (334)	1165 (334)	0.41
Gestational age (weeks), mean (SD)	28 (2.5)	28.5 (2.1)	0.38
Male N (%)	10 (63)	13 (45)	0.35
Cesarean N (%)	9 (56)	20 (69)	0.51
One minute Apgar, median (IQR)	3 (2,4)	6 (4,7)	0.023 ^a
Five minute Apgar, median (IQR)	7 (6,8.5)	7 (6,8)	0.88
DOL first feeding, median (IQR)	1 (1,2)	2 (1,2)	0.077
DOL full enteral feeding, median (IQR)	14.5 (10,25)	13 (9,17)	0.27
Total days NPO, median (IQR)	1 (1,3.3)	2 (1,3)	0.66
First feeding MOM, N (%)	12 (75)	20 (69)	0.74
Feeding mode; only MOM, N (%)	7 (44)	19 (70)	0.19
Feeding mode; mostly (>50%) MOM, N (%)	6 (38)	4 (15)	0.19
Feeding mode; mostly (>50%) PDHM, N (%)	3 (19)	4 (15)	0.19
Antibiotic days, mean (SD)	8.6 (13)	5.1 (9.1)	0.31
NEC stage 2 or 3, N (%)	0 (0)	2 (7)	0.53
SIP, N (%)	0 (0)	2 (7)	0.53
BPD, N (%)	7 (44)	10 (37) ^b	0.75
Culture positive sepsis, N (%)	3 (19)	3 (10)	0.65
Death, N (%)	1 (6)	1 (4) ^b	1.0
Length of hospital stay (days), mean (SD)	85 (47)	72 (36)	0.31
DOL first probiotic dose, median (IQR)	1 (1,1)	3 (2,5)	< 0.001 ^a
DOL final probiotic dose, median (IQR)	38 (26,58)	37 (26,49)	0.48

N=number, SD=standard deviation, IQR=interquartile range, DOL=day of life, MOM=mother's own milk, NEC=necrotizing enterocolitis, SIP=spontaneous intestinal perforation, BPD=bronchopulmonary dysplasia.

^aNo significant differences between groups except for one minute Apgar score (p<0.05) and DOL first probiotic dose (p<0.01).

^bone set of twins was transferred to another NICU at 33 weeks postmenstrual age and is not included in the calculations for BPD or death.

8. Figures

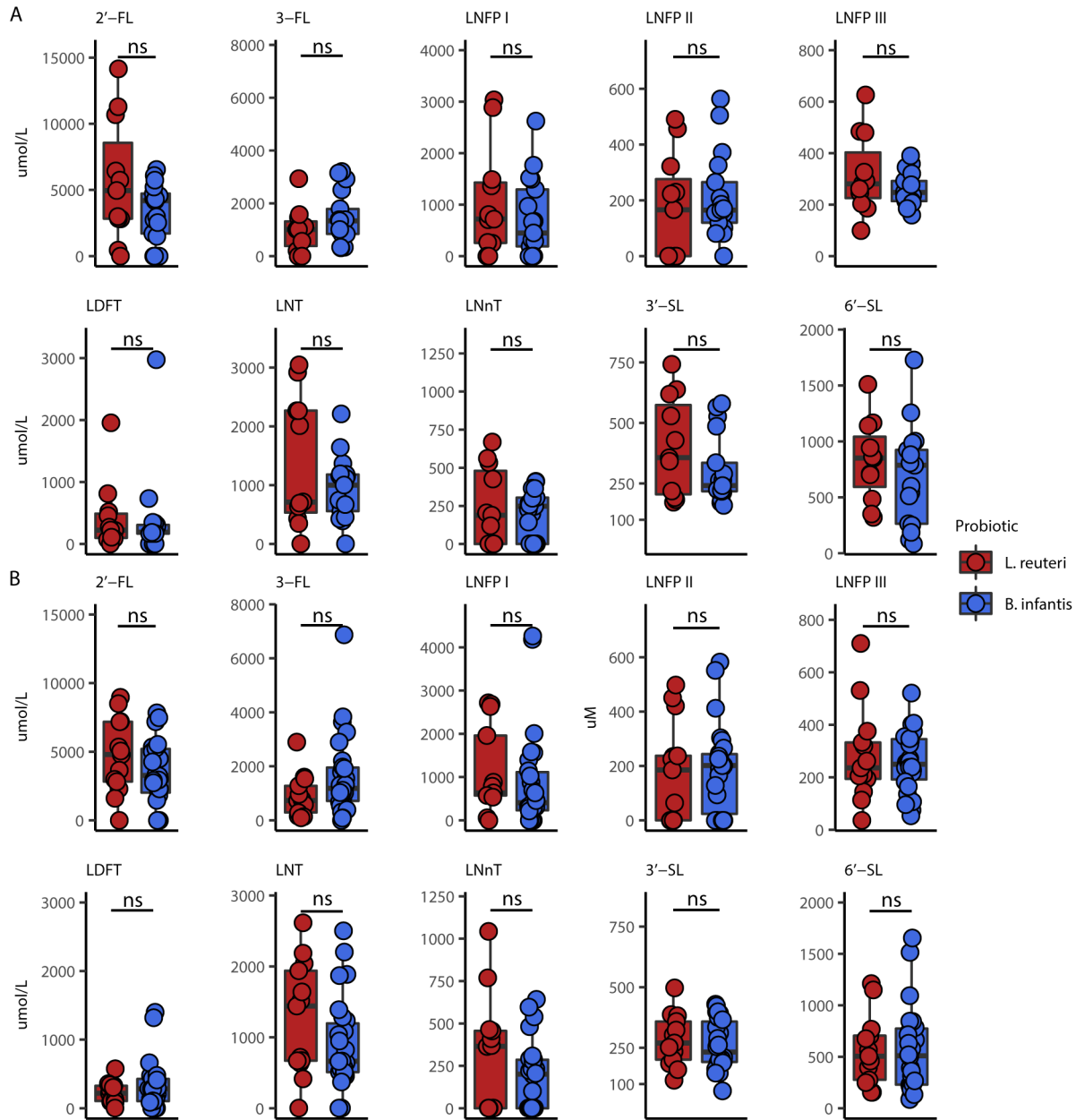


Figure 10. Concentrations of HMOs from milk samples consumed by infants in *L. reuteri* and *B. infantis* groups collected at 30 and 32 weeks CGA. (A) HMO concentrations from milk provided at 30 weeks CGA. (B) HMO concentrations from milk provided at 32 weeks CGA. Boxplots represent medians and interquartile range (IQR) with whisker end points equal to the maximum and minimum values below or above the median at 1.5 times the IQR. Between group differences were evaluated by Wilcoxon rank-sum test with FDR correction for multiple comparisons. ns; non-significant. Abbreviations: 2'-FL, 2'-fucosyllactose; 3-FL, 3-fucosyllactose; LNFP I, Lacto-N-fucopentaose I; LNFP II, Lacto-N-fucopentaose II; LNFP III, Lacto-N-fucopentaose III; LDFT, Lactodifucotetraose; LNT, Lacto-N-tetraose; LNnT, Lacto-N-neotetraose; 3'-SL, 3'-sialyllactose; 6'-SL, 6'-sialyllactose.

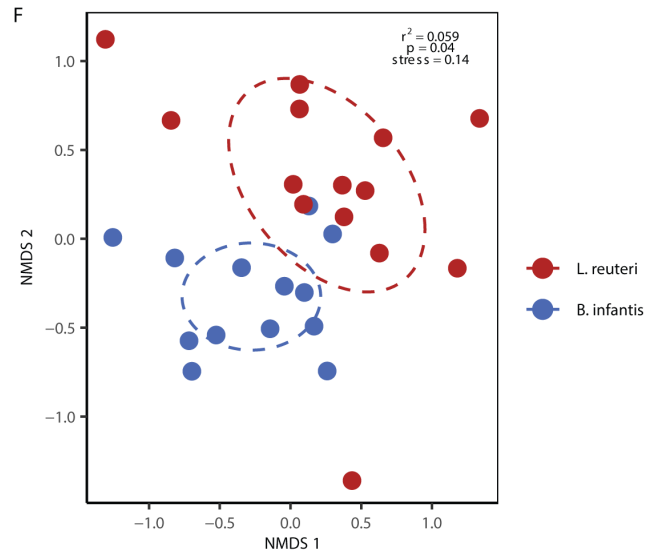
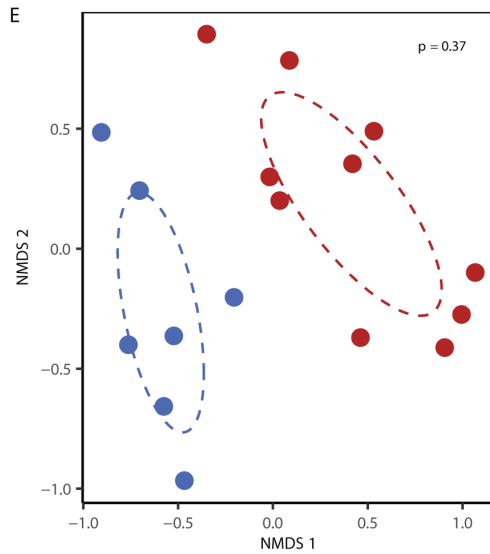
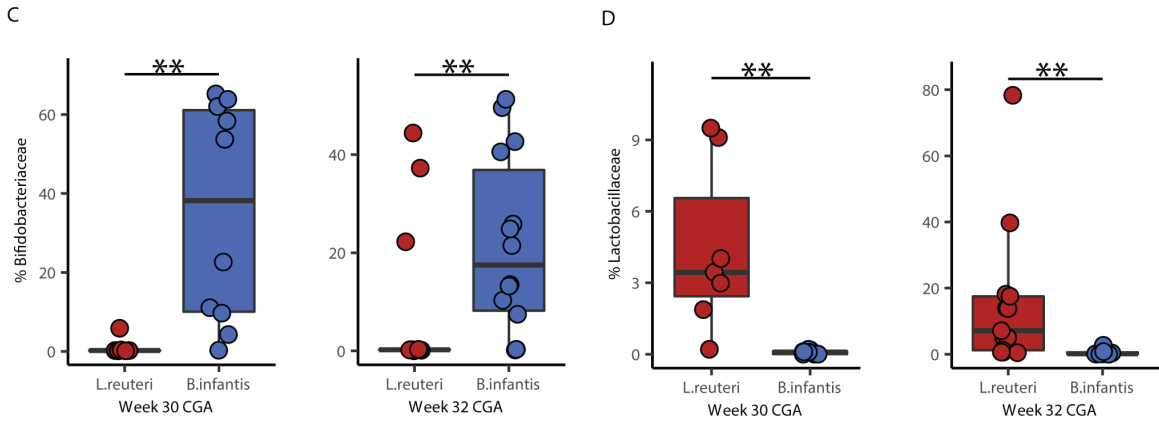
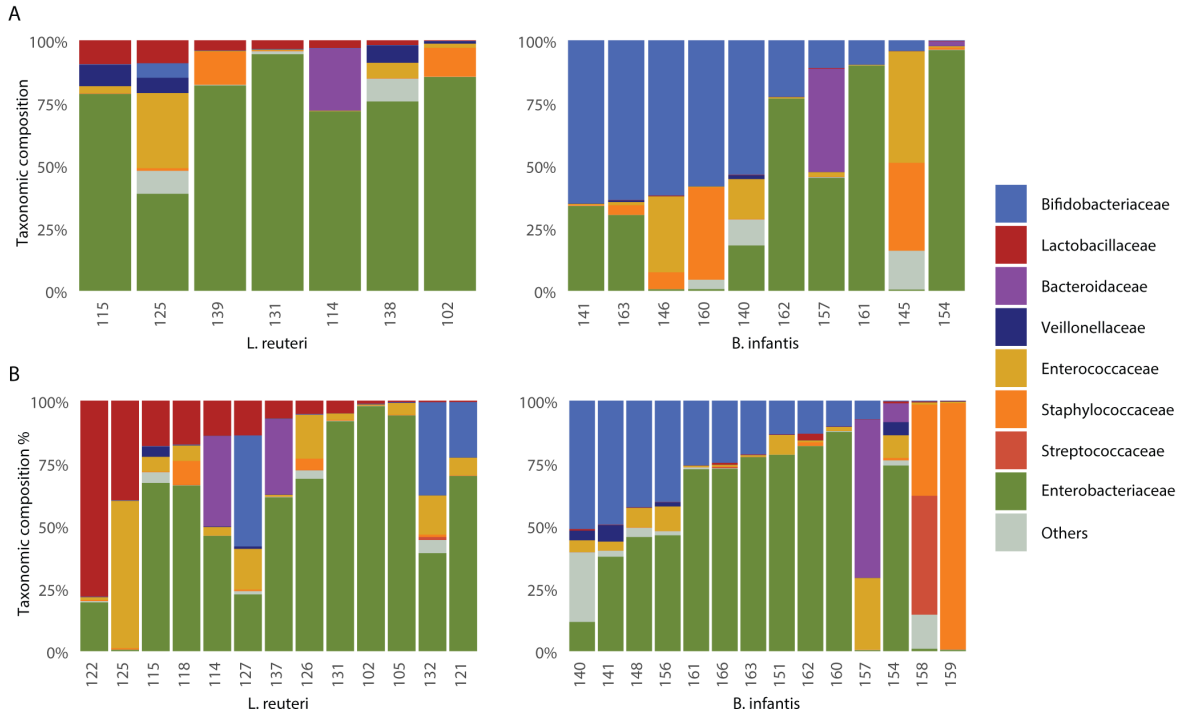


Figure 11. Composition and diversity of infant fecal microbiota communities differ by probiotic supplementation (A) Taxonomic composition at 30 weeks CGA for each infant receiving *B. infantis* or *L. reuteri*. (B) Taxonomic composition at 32 weeks CGA for each infant receiving *B. infantis* or *L. reuteri*. (C) Relative abundance of *Bifidobacteriaceae* in fecal microbiota at 30 and 32 weeks CGA in infants receiving *B. infantis* or *L. reuteri*. (D) *Lactobacillaceae* of fecal microbiota at 30 and 32 weeks CGA in infants receiving *B. infantis* or *L. reuteri*. (E) β -diversity for fecal microbiota of infants at 30 weeks CGA (F) β -diversity for fecal microbiota of infants at 32 weeks CGA. Taxa classified as unknown or with mean proportions of <1% are grouped in “Others”. Boxplots represent medians and interquartile range (IQR) with whisker end points equal to the maximum and minimum values below or above the median at 1.5 times the IQR. ** $p < 0.01$; Wilcoxon rank-sum test with FDR correction for multiple comparisons. Differences in β -diversity were compared using PERMANOVA for Bray-Curtis dissimilarity.

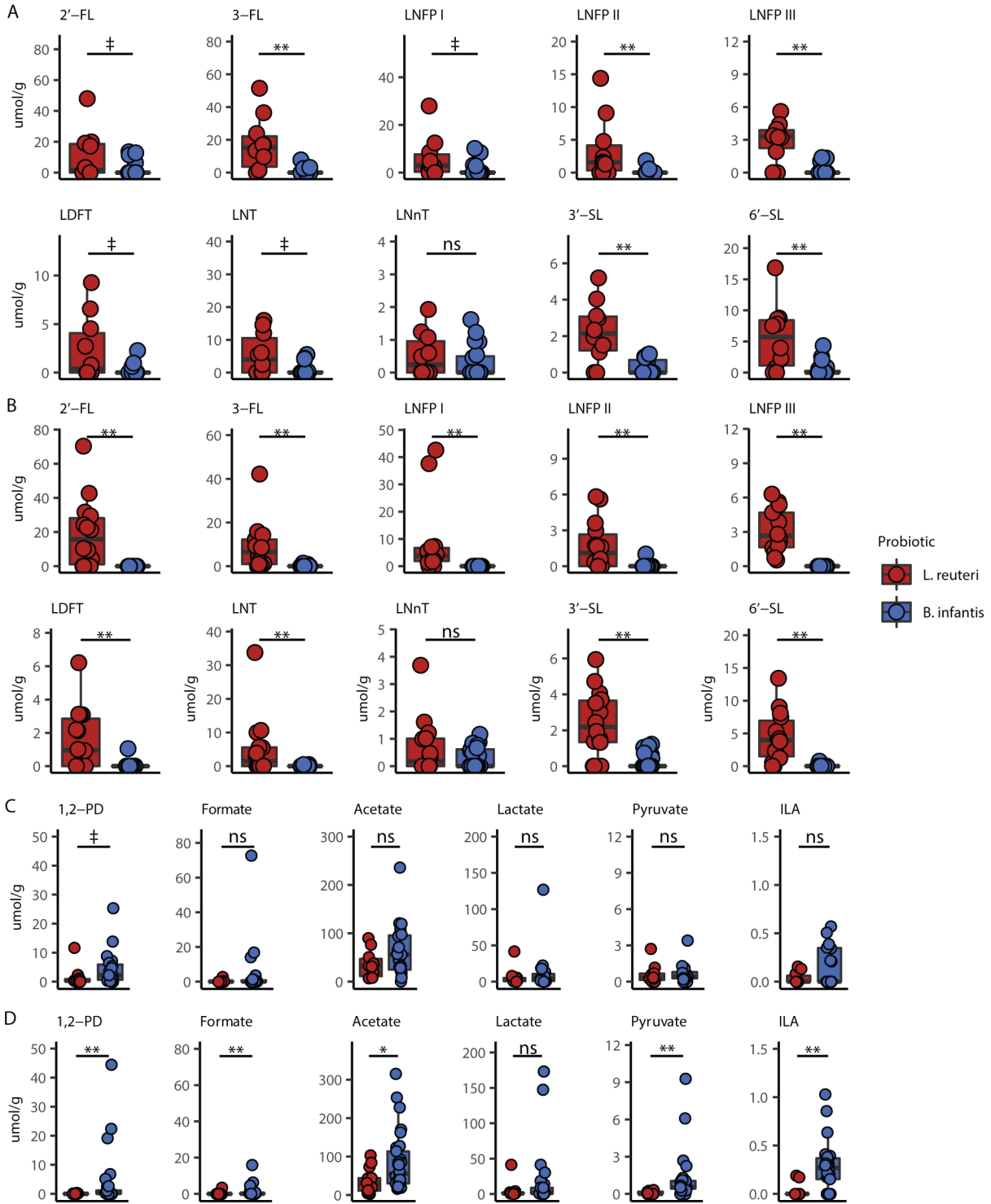


Figure 12. Probiotic supplementation is associated with differences in fecal HMO and metabolite profiles. (A) HMOs concentrations in fecal samples from infants at 30 weeks CGA. (B) HMOs concentrations in fecal samples from infants at 32 weeks CGA. (C) Fecal metabolites related to lactic acid bacteria in infants at 30 weeks CGA (D) Fecal metabolites related to lactic acid bacteria in infants at 32 weeks CGA. Boxplots represent medians and interquartile range (IQR) with whisker end points equal to the maximum and minimum values below or above the median at 1.5 times the IQR. ‡ $p < 0.10$, * $p < 0.05$, ** $p < 0.01$; Wilcoxon rank-sum test with FDR correction for multiple comparisons.

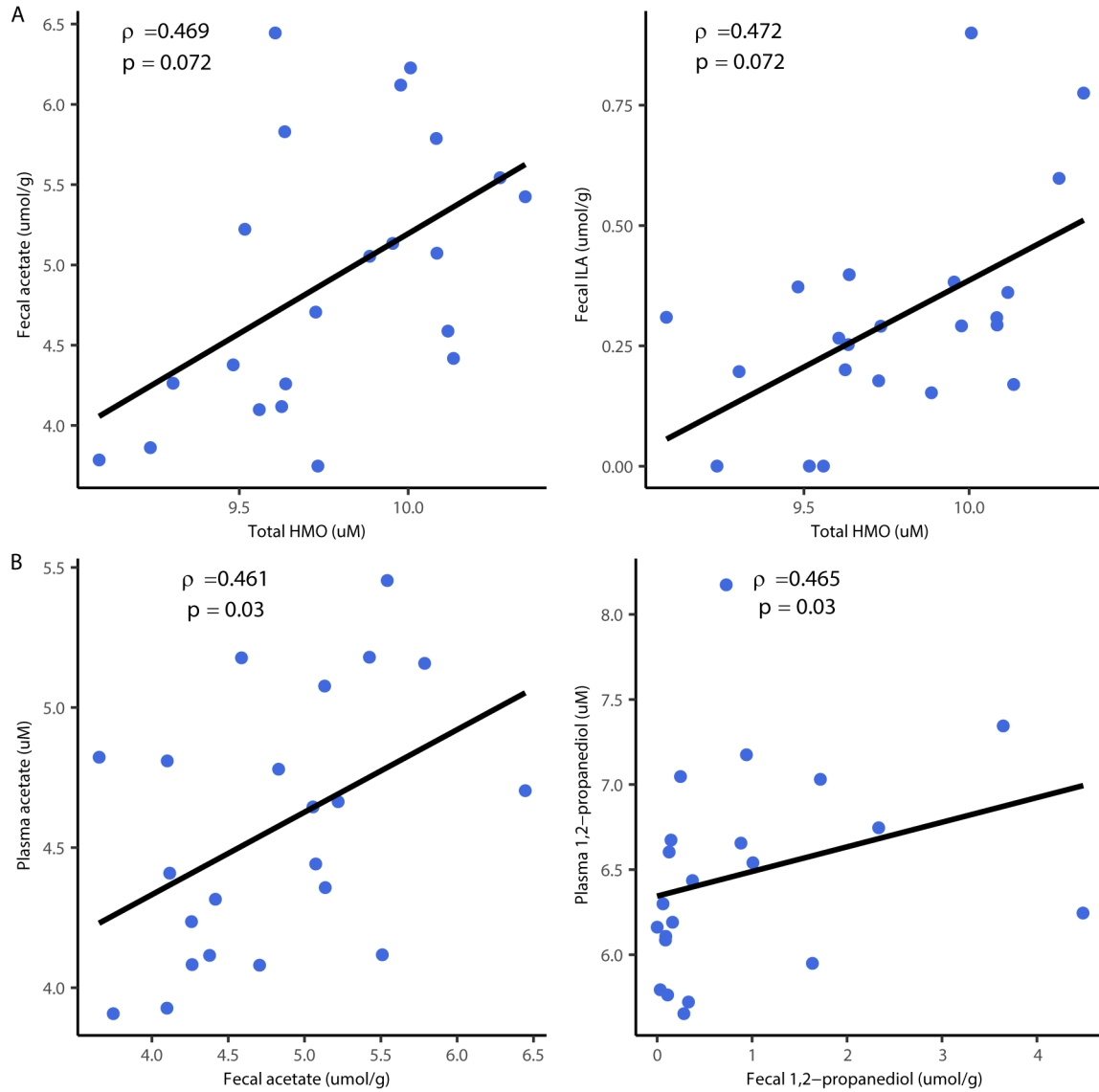


Figure 13. Fecal metabolites are associated with total milk oligosaccharides and their concentrations in plasma for infants receiving *B. infantis* at 32 weeks CGA. (A) Spearman's correlations between fecal acetate, ILA and total HMOs. (B) Spearman's correlations between fecal and plasma acetate or 1,2-propanediol. Non-secretor milk samples ($2\text{'-FL} < 100 \text{ uM}$) were removed before analysis. Data shown are generalized log transformed ($\log(y + \sqrt{y^2 + 1})$) and FDR corrected for multiple comparisons. Total HMO is the sum of 2'-fucosyllactose, 3-fucosyllactose, Lacto-N-fucopentaose I, Lacto-N-fucopentaose II, Lacto-N-fucopentaose III, Lactodifucotetraose, Lacto-N-tetraose, Lacto-N-neotetraose, 3'-sialyllactose and 6'-sialyllactose

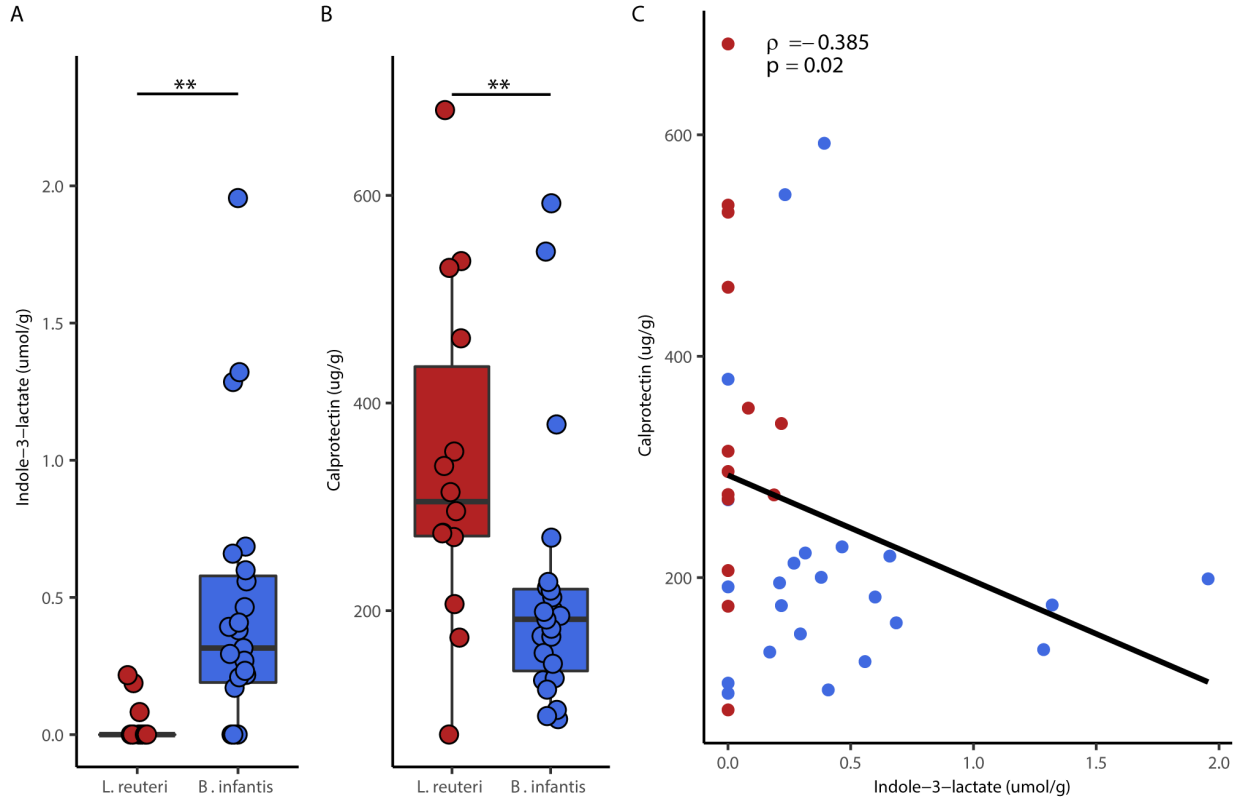


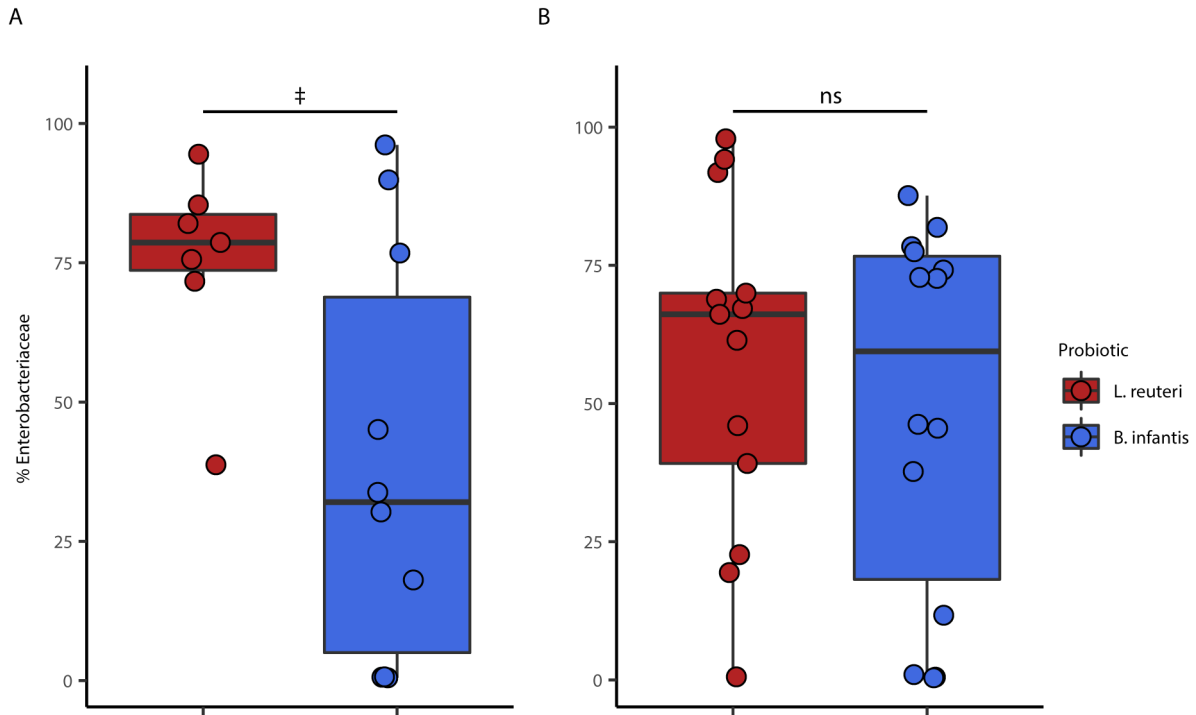
Figure 14. Bifidobacterium treatment is associated with higher ILA and reduced intestinal inflammation. (A) Fecal ILA concentrations for each probiotic group (B) Fecal calprotectin for each probiotic group (C) Partial spearman correlation between fecal ILA and calprotectin adjusted for gestational age at birth. Boxplots represent medians and interquartile range (IQR) with whisker end points equal to the maximum and minimum values below or above the median at 1.5 times the IQR. Correlation includes independent samples from infants at 30, 31 and 32 weeks CGA. ** $p < 0.01$; Wilcoxon rank-sum test with FDR correction for multiple comparisons.

9. Supplementary information

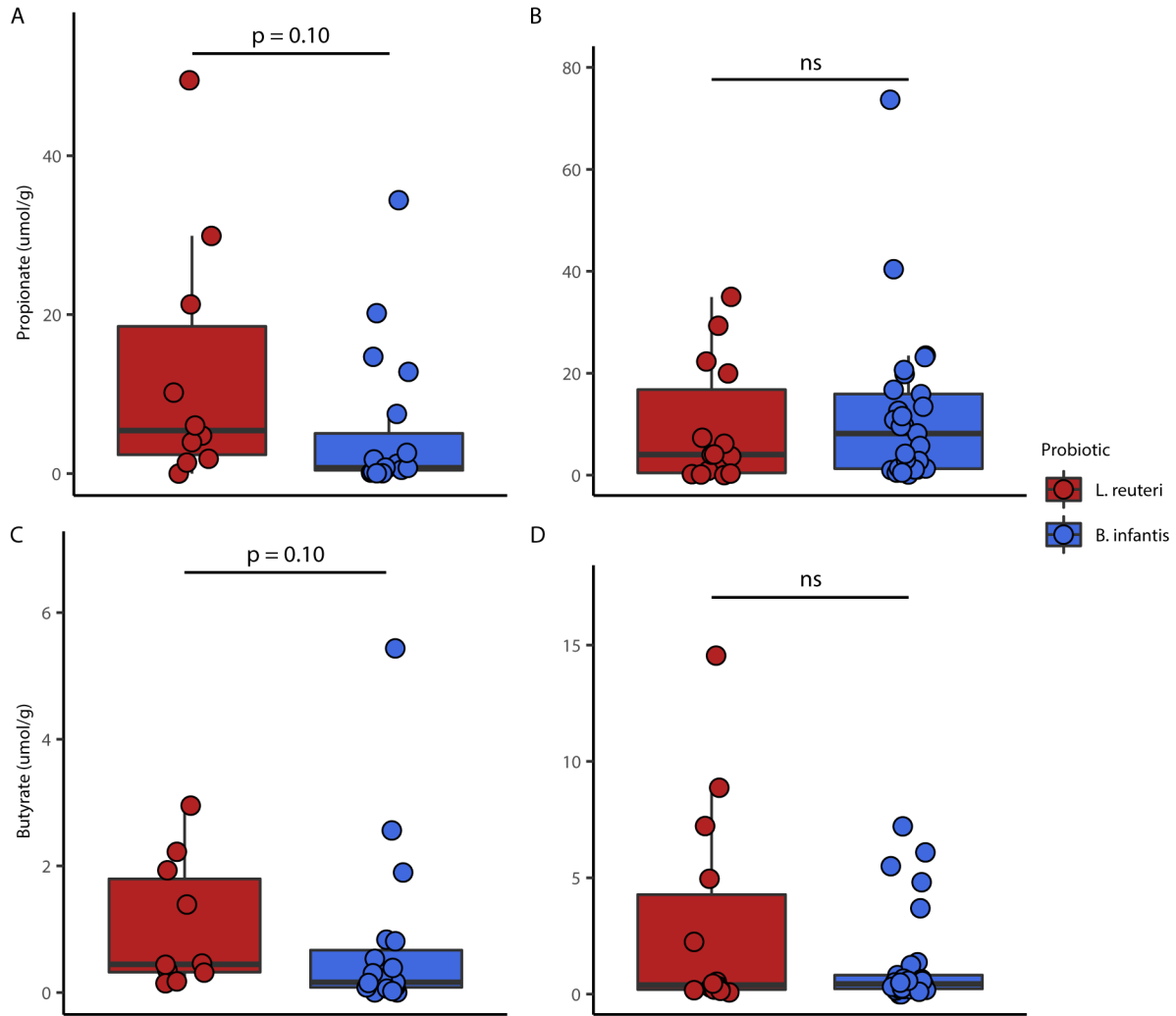
Supplementary Table 2. Characteristics of infants who had not received probiotics.

	<i>No probiotic</i> (N=5)
Birth weight (g), mean (SD)	1958 (145)
Gestational age (weeks), mean (SD)	31.6 (0.5)
Male N (%)	5 (100)
Cesarean N (%)	4 (80)
One minute Apgar, median (IQR)	5 (1,7)
Five minute Apgar, median (IQR)	8 (5,8)
DOL first feeding, median (IQR)	1 (1,3)
DOL full enteral feeding, median (IQR)	9 (7,9)
Total days NPO, median (IQR)	1 (0,3)
First feeding MOM, N (%)	5 (100)
Antibiotic days, mean (SD)	2 (0)
NEC stage 2 or 3, N (%)	0 (0)
SIP, N (%)	0 (0)
BPD, N (%)	1 (20)
Culture positive sepsis, N (%)	0 (0)
Death, N (%)	0 (0)
Length of hospital stay (days), mean (SD)	38 (18)

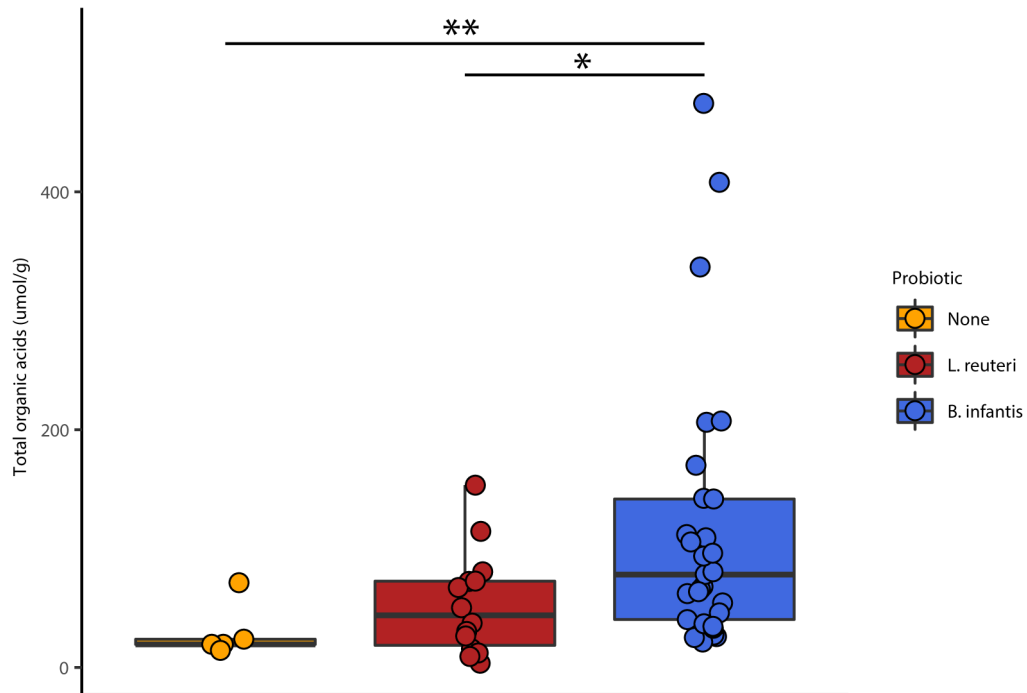
N=number, SD=standard deviation, IQR=interquartile range, DOL=day of life, MOM=mother's own milk, NEC=necrotizing enterocolitis, SIP=spontaneous intestinal perforation, BPD=bronchopulmonary dysplasia



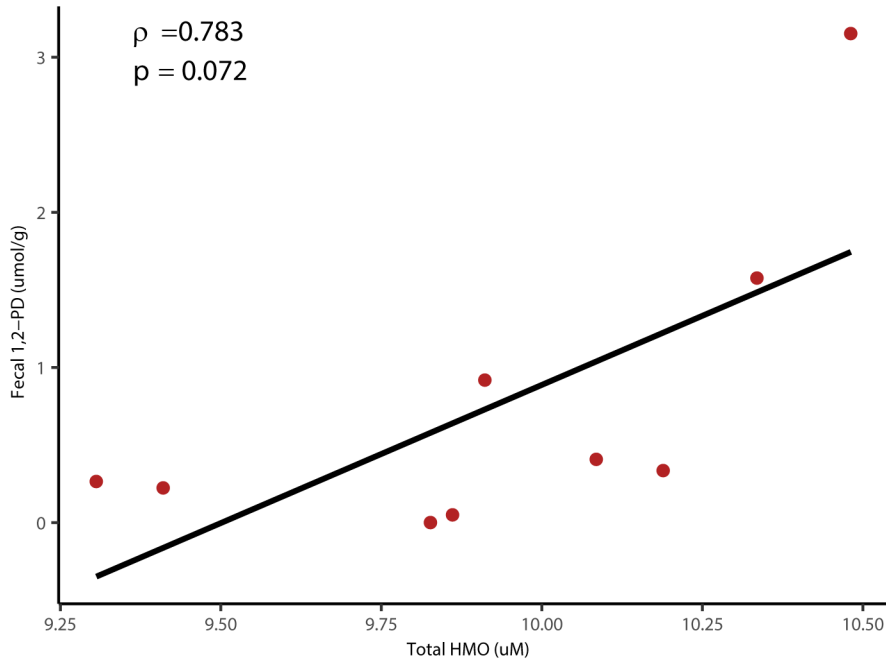
Supplementary Figure 3. Relative abundance of *Enterobacteriaceae* in preterm infant fecal microbiota at (A) 30 and (B) 32 weeks CGA in infants receiving *L. reuteri* or *B. infantis*. Boxplots represent medians and interquartile range (IQR) with whisker end points equal to the maximum and minimum values below or above the median at 1.5 times the IQR. ‡ $p < 0.10$, ns; non-significant. Wilcoxon rank-sum test with FDR correction for multiple comparisons.



Supplementary Figure 4. Fecal propionate in preterm infants at (A) 30 weeks and (B) 32 weeks and butyrate at (C) 30 weeks and (D) 32 weeks CGA. Boxplots represent medians and interquartile range (IQR) with whisker end points equal to the maximum and minimum values below or above the median at 1.5 times the IQR. ns; non-significant; Wilcoxon rank-sum test with FDR correction for multiple comparisons.



Supplementary Figure 5. Total organic acids in fecal samples at 32 weeks CGA from infants receiving no probiotic (“None”), *L. reuteri* or *B. infantis*. Total organic acids are the sum of formate, acetate, propionate, butyrate, lactate and pyruvate. Boxplots represent medians and interquartile range (IQR) with whisker end points equal to the maximum and minimum values below or above the median at 1.5 times the IQR. * $p < 0.05$, ** $p < 0.01$; Kruskal-Wallis H-test with Dunn’s post hoc test corrected for multiple comparisons by setting FDR to 0.05.



Supplementary Figure 6. Spearman's correlation between 1,2-PD with total HMO content in milk *L. reuteri* group at week 30 CGA. Data shown are generalized log transformed ($\log(y + \sqrt{y^2 + 1})$). Total HMO is the sum of 2'-fucosyllactose, 3-fucosyllactose, Lacto-N-fucopentaose I, Lacto-N-fucopentaose II, Lacto-N-fucopentaose III, Lactodifucotetraose, Lacto-N-tetraose, Lacto-N-neotetraose, 3'-sialyllactose and 6'-sialyllactose.

Chapter 4: Concluding Remarks

Selective pressures guiding the coevolution of mammals and microbes have fostered a mutualistic relationship whereby host physiological processes develop optimally in the presence of commensal gut microbiota. Members of the gut microbial community perform a range of biochemical functions that influence host physiology by modulating metabolic, endocrine, and immunologic pathways. The ability of infant associated *Bifidobacterium* to utilize complex glycans found in human milk is the underlying basis for their enrichment in the gut as described in **Chapter 1**. Moreover, the cellular components involved in the acquisition and processing of carbohydrates by bifidobacteria are interspecies or strain dependent and determine *Bifidobacterium* species distributions in the gut¹⁻³. Human milk oligosaccharide (HMO) metabolism by bifidobacteria is followed by the secretion of metabolites in the gut lumen that are capable of modulating metabolism and gut barrier function as demonstrated in preclinical animal models⁴. Therefore, investigating how probiotic or synbiotic formulations of bifidobacteria shift the microbiome and its metabolic output contribute to understanding their role in promoting health of the developing infant.

To investigate the co-metabolic interactions between *Bifidobacterium* and the host, a mouse model was developed using 2'-fucosyllactose (2'-FL) for selective enrichment of an infant gut bifidobacteria isolate; *Bifidobacterium pseudocatenulatum* MP80 (*B. p.* MP80) (**Chapter 2**). The approach was to assess the importance of modeling persistent, rather than transient, populations of *Bifidobacterium* in the gut along with corresponding changes in concentrations of microbial metabolites. Persistence of bifidobacteria was only achieved when co-administered with 2'-FL rather than probiotic supplementation alone. This suggests that colonization resistance from the indigenous microbiota can be attenuated by supplementation of a prebiotic utilized by

Bifidobacterium and relatively inaccessible to other community members. However, the relative abundance of bifidobacteria in the synbiotic treated mice had a heterogeneous distribution at the final day of sample collection. While there were no observed differences in α - or β -diversity at baseline between mice with high and low persistence, a putative explanation for variable persistence may be attributed to trophic interactions taking place between microbes in the gut. Indeed, the spatiotemporal nature of these microbe-microbe interactions in the gut contribute to the dynamic competition for resources and their metabolic outputs⁵.

The heterogeneous response in bifidobacterial persistence was associated with discrete metabolite profiles in the gut. Products of human milk oligosaccharide (HMO) fermentation by *Bifidobacterium* including lactate, formate and 1,2-propanediol were significantly higher in mice with high bifidobacteria persistence compared to those with low persistence. Conversely, mice with low persistence of bifidobacteria had increased colonic acetate, propionate and butyrate. Differential abundance testing revealed a significantly increased log ratio of *Bacteroidaceae* to *Bifidobacteriaceae* across median gut propionate concentrations whereas the log ratio of *Bifidobacteriaceae* to *Lachnospiraceae* and *Ruminococcaceae* was significantly increased in mice with greater than median concentrations of 1,2-PD. A significantly higher log ratio of *Lachnospiraceae* and *Ruminococcaceae* to *Bifidobacteriaceae* was detected in mice with above median colonic butyrate concentrations. These results indicate the metabolic output of the microbiota is linked to the dominant taxa in the gut which can be modulated by prebiotic or synbiotic intervention.

Beyond the gut environment, serum metabolite profiles were similar across groups with the exception of the fucose metabolite, 1,2-propanediol (1,2-PD), in the synbiotic treated mice. Moreover, mice with high persistence of bifidobacteria had significantly higher concentrations of

1,2-PD compared to those with low count of bifidobacteria, suggesting that persistence of the *Bifidobacterium* probiotic is responsible for higher circulating levels of this microbial metabolite. Metabolite profiling of liver and brain showed that 1,2-PD was only detectable in mice provided the synbiotic with high persistence compared to mice without prebiotic or probiotic supplementation. Interestingly, a previous study using ¹³C labeled 2'-FL provided to mice found enrichment in several organs including liver and brain⁶. Although the authors did not specifically identify the molecules related to enrichment it can be inferred that 1,2-PD is a possible candidate given the results of our study.

In summary, the synbiotic pairing of 2'-FL and *B. p.* MP80 facilitated persistence (> median; 50.5% *Bifidobacteriaceae*) in four of twelve mice at the end of the study, one week following the final oral gavage. Probiotic treatment alone did not increase the proportion of bifidobacteria during the study indicating our infant derived strain is not adapted to colonize the murine gut without the assistance of a prebiotic. Mice categorized as having high persistence of bifidobacteria had distinct metabolic profiles associated with a bifidobacteria metabolites showing increased levels of 1,2-PD, lactate and formate. Moreover, 1,2-PD was significantly increased in the serum and detected in the liver and brain of mice with high persistence of *Bifidobacteriaceae*. The enrichment of microbial metabolites in the gut, serum and organs may have important effects on host metabolism that require additional investigation.

The breastfed infant gut microbiome is associated with high bifidobacteria counts in part due to the prebiotic nature of HMOs. However, infants born preterm have delayed microbial community development due to factors such as increased antibiotic exposure that disrupt the process of gut colonization^{7,8}. In **Chapter 3** we compared how the supplementation of two probiotic strains; *Bifidobacterium longum* subsp. *infantis* EVC001 (*B. infantis*) or *Lactobacillus*

reuteri DSM 17938 (*L. reuteri*) could affect the gut microbial community, their metabolic activities and inflammation in the gut. While each probiotic was associated with an increase in the respective taxa in the gut, infants provided *B. infantis* had a greater abundance of *Bifidobacteriaceae* compared to the proportion of *Lactobacillaceae* in infants supplemented with *L. reuteri*. It is tantalizing to speculate that this difference is based on the several HMO gene clusters encoded in the genome of *B. infantis*, whereas *L. reuteri* lacks the ability to consume many prominent HMOs⁹⁻¹². Additionally, we quantified HMOs in the feces of these infants and found that nine of the ten fecal HMOs were significantly higher in infants provided *L. reuteri* compared to *B. infantis* supporting the inference that the bifidobacteria probiotic can utilize this substrate to increase its presence in the infant gut.

Differences were also found in the fecal metabolites of *B. infantis* and *L. reuteri* supplemented infants. We profiled several metabolites related to lactic acid bacteria (which include bifidobacteria and lactobacilli) and found that relative to *L. reuteri*, infants provided *B. infantis* had significantly increased acetate, pyruvate, formate, 1,2-PD and the tryptophan metabolite indole-3-lactic acid (ILA) by 32 weeks corrected gestational age. The increased concentration of lactic acid bacterial metabolites and along with lower fecal HMOs indicate greater metabolic activity from *B. infantis* which may have benefits to the host. To investigate this, we quantified fecal calprotectin, a marker of gut inflammation and found lower calprotectin in infants treated with *B. infantis* relative to *L. reuteri*. A modest, negative correlation between ILA and calprotectin across both probiotic groups also indicated a relationship between this microbial metabolite and the inflammatory marker. Given the myriad molecular interactions in the gut, it can be expected that many effectors such as other metabolites and peptides are collectively contributing to the overall inflammatory state within the gut and that ILA is working in concert with these other

stimuli. The findings of this observational study support that supplementing an HMO-consuming bifidobacteria probiotic in human milk fed preterm infants is associated with reduced enteric inflammation. This process may be mediated through increased metabolic activity in the gut relative to a non-HMO-consuming lactobacillus probiotic.

Both of the studies in **Chapters 2 and 3** focus on the enrichment of a *Bifidobacterium* probiotic in the gut microbiome and the potential to affect changes in metabolite concentrations in the host. The results of this work provide evidence that (1) HMOs are a key driver in selective enrichment of bifidobacteria in the gut and (2) the secreted products of bifidobacterial metabolism in the gut corresponds with their enrichment. The preclinical mouse model further highlights that persistent colonization (one week post inoculum) occurs with a synbiotic pairing rather than the probiotic alone. However, the heterogeneity of *Bifidobacterium* enrichment in mice receiving the synbiotic suggests colonization resistance is not equal among conventionally colonized C57BL/6J mice. This exemplifies the complexity of the gut microbiome and that a degree of personalization that exists even in laboratory mice that are bred expressly for reproducible results. In the human preterm infant, antibiotic exposures and other perturbations delay assembly of the gut microbiome and the related benefits of metabolic and immune maturation. Therefore, the underlying goal of probiotic therapies are to rectify this state of disrepair in an effort to restore functionality, i.e. the production of bioactive molecules that may interact with the host to elicit a favorable physiologic response. Moreover, it is imperative to recognize that the fitness and function of a probiotic depends on the availability of resources and indigenous microbes within the gut. While the human milk fed infant gut is an environment conducive to colonization of *B. infantis* based on its capacity to utilize HMOs, other synergies involving different pairings of *Bifidobacterium* species and non-digestible carbohydrates can promote gastrointestinal health in later life stages. Future work will

be needed to investigate these interactions to devise effective and commercially viable synbiotic formulations.

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