Infant Feeding Practices and the Intestinal Microbiome in the First Year of Life

By

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmacology and Toxicology

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

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2022

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Abstract

A healthy intestinal microbiome is critical for lifelong health; however, recent studies have reported a dysfunctional intestinal microbiome in breastfed infants. Probiotics have been used in an attempt to restore the intestinal microbiome, but colonization has been transient, inconsistent among individuals, or has not positively impacted the host's gut. Additionally, studies have shown that dietary interventions can alter the intestinal microbiome in animal models and adults; these changes have not been observed in infants. The weaning period provides a unique opportunity to observe how specific food change the intestinal microbiome composition and microbial metabolism and allows us to investigate specific microbe-food interactions that would not be possible in adults with a diverse diet. We found that in breastfed, term, newborn-infants, probiotic supplementation with *B. infantis* within the first month postnatal, in combination with breast milk, resulted in stable colonization that persisted until at least 1 year postnatal. In healthy breastfed infants 2-4 months old, probiotic supplementation with *B. infantis* for 28 days resulted in stable colonization that persisted until at least one-month post-supplementation. Lastly, the introduction of different solid foods, pear and sweet potato, to 6–8-month-old, breastfed infants led to changes in microbial metabolism as evidenced by changes in fecal organic acid and glycan content. Thus, probiotic and dietary interventions in the first year of life are able to change both intestinal microbial community structure but also metabolic function which may have implications for long-term health.

Introduction

In infancy, the intestinal microbiome is highly dynamic with high inter- and intra-individual variability. During the first 2 years of life, the intestinal microbiome shifts and gradually becomes more adult-like as demonstrated by an increase in α-diversity and decrease in β-diversity(1). This shift coincides with the introduction of solid foods and the cessation of breastfeeding between 6 months to 2 years of life.

While recent studies utilizing molecular techniques have demonstrated the potential for in-utero microbial colonization, the first major intestinal colonization event occurs at birth upon exposure to maternal vaginal, skin, and fecal microbiota (2-4). Major differences in intestinal microbiota have been observed between infants born via vaginal delivery or cesarean section (C-section). Infants born vaginally are initially enriched with *Escherichia coli*, *Staphylococcus*, *Streptococcus, Lactobacillus,* and *Prevotella*) while infants born via C-section are colonized by skin bacteria such as *Staphylococcus*, *Corynebacterium,* and *Propionibacterium* spp. and have lower abundances of *Bifidobacterium* and *Bacteroides* (5-7). At approximately 6 weeks of life, differences in microbial communities between vaginally and C-section delivered infants largely disappear (8).

A second shift in the intestinal microbiome occurs within the first few weeks of life driven largely by mode of feeding. It is well documented that exclusively breastfed infants have lower diversity but higher taxa from the protective bacterial class Actinobacteria compared with formula-fed infants who have higher microbial intestinal diversity but also higher levels of the pro-inflammatory bacterial class γ-Proteobacteria (1, 9-11).

Breast milk contains a diversity and large quantities (~10-20 g/L) of human milk oligosaccharides (HMOs) that are non-digestible by the infant but selectively support the competitive growth of protective *Bifidobacterium* strains within the intestine of the breastfed infant (12-16). In particular, a subspecies of *Bifidobacterium*, *Bifidobacterium longum* subsp. *infantis* (*B. infantis*), has unique characteristics that differ from other bifidobacterial species in its genetic capabilities to bind, transport, and ferment HMOs into short chain fatty acids (17, 18). As a result of HMO fermentation, intestinal pH is decreased which is undesirable environment for potential pathogens (19) and preventing the infiltration of toxic molecules produced by pathogenic bacteria by upregulating intestinal barrier function and inhibiting pro-inflammatory and apoptotic responses (20).

Historically, the gut of the breastfed infant was dominated by these protective *Bifidobacterium* strains until the cessation of breastfeeding (1, 21-23). However, the dominance of fecal *Bifidobacterium* and *B. infantis* has declined in recent decades in resource-rich countries as demonstrated by an increase in fecal pH and higher levels of fecal enteropathogens over the past 100 years regardless of breastfeeding status (24, 25). It is hypothesized that this reduction in *Bifidobacterium*, increase in potential pathogens, and the resulting dysbiosis in the infant intestinal microbiome is a result of increased antibiotic usage, infant formula feeding (26) and cesarean section deliveries (27). The microbiome plays a significant role in the development of the immune system in early life; intestinal dysbiosis in infancy is of concern because perturbations in the infant microbiome have been associated with increased risk for metabolic, allergic, and auto-immune diseases later in life (28-30). Recently, Henrick et al., showed that indole-3 lactic acid produced by *B. infantis* upregulated immunoregulatory galectin-1 in intestinal T helper 2 (Th2) and Th17 cells during polarization, providing a functional link between beneficial intestinal microorganisms

and immunoregulation during the first months of life (31). Thus, the early colonization and establishment of a healthy microbiome in infancy is critical for establishing life-long health.

Because infancy is a time of great instability within the intestinal microbiome, it is an optimal time to provide beneficial microbes in the form of probiotics. Probiotics may be a viable tool to combat infant intestinal dysbiosis, thereby facilitating proper immune system and intestinal development. It has recently been demonstrated that supplementation with *B. infantis* probiotics in breastfed newborns leads to the stable colonization of *B. infantis* in the infants' gut up to one year post-natal (32-34). Additionally, *B. infantis* supplementation significantly increased fecal short-chain fatty acids and decreased fecal pH and fecal HMO content, which suggests a higher consumption of HMOs by *B. infantis* and changes in intestinal fermentation and biochemistry (33).

A third major shift in the intestinal microbiome occurs during the weaning period upon the introduction of solid foods. Bifidobacterial species remain dominant throughout the early weaning period when breast milk is still the sole source of nutrition (1, 35). As more solids foods are introduced and breastfeeding ceases, the intestinal microbiota becomes more adult-like (36), shifting towards the bacterial phyla Bacteriodetes and Firmicutes (37).

Infancy is a time of low microbial diversity and stability that could particularly be responsive to introductions of solid foods. These first foods can either help foster the development of a healthy intestinal microbiome or disrupt the bacterial composition and function in the intestine leading to dysbiosis and unknown long-term health consequences. The transition from breastfeeding to solid food represents an easy demarcation from which to compare the effects of specific solid foods on the microbiome and, as with the utilization of probiotics in early infancy, a potential mechanism to combat intestinal dysbiosis and promote the development of a healthy intestinal microbiome. The introduction of plant-derived complex carbohydrates is a particularly

attractive mechanism to alter the intestinal microbiome as they cannot be broken down by humans and are thus uniquely suited to influence the development of the microbiome (38-40). Numerous studies have shown that the microbiota adopted during weaning can persist into adult life and; thus, these early microbiota-food interactions are tremendously important in setting the stage for life-long health (41, 42). Additionally, as it is recommended that solid foods be introduced one at a time, the weaning period can offer unique insights into glycan-microbe relationships that are more difficult to observe in adult populations (43, 44).

Chapter 1

Early Probiotic Supplementation with *B. infantis* in Breastfed Infants Leads to Persistent Colonization at One Year

Introduction

Breast milk delivers a wide spectrum of biologically active molecules that aid in the development and maturation of the gut and the innate and adaptive immune systems and support the growth of protective intestinal microbiota. Specifically, human milk oligosaccharides (HMOs), the third most abundant component in human milk (~10–20 g/L) (45, 46), are a group of complex sugars that are non-digestible by the human infant and support the competitive growth of protective bifidobacterial strains within the intestine (18, 47). In particular, the natural colonization of a protective subspecies of *Bifidobacterium*, *Bifidobacterium longum* subsp. *infantis* (*B. infantis*), unlike other bifidobacterial species in breastfed infants, is based on its genetic capabilities to bind, transport, and ferment HMOs into lactate and acetate (17, 18). These fermentative products maintain a lower pH of the intestinal milieu, support the transport of these compounds into the intestinal epithelium for use by the host (48), create an undesirable environment for potential pathogens (19) and prevent the infiltration of toxic molecules produced by pathogenic bacteria by upregulating intestinal barrier function and inhibiting pro-inflammatory and apoptotic responses (20).

Historically, the gut of the breastfed infant was dominated by a near-monoculture of *Bifidobacterium* until the cessation of breastfeeding (49). However, findings from Henrick et al. (2017) reported a generational loss of *Bifidobacterium* in breastfed infants from resource-rich nations within the past 100 years accompanied by higher levels of enteropathogens and higher fecal pH (50). The reduction in *Bifidobacterium* and increase in potential pathogens in the infant

gut microbiome are likely a result of the unintended consequences of antibiotic use, infant formula feeding (26) and cesarean section deliveries (27), all of which have been implicated in the increased risk for allergic and autoimmune diseases prevalent in resource-rich nations (28-30). Colonization of a dysfunctional gut microbiome in early infancy during the critical window of immune system development is reported to increase the risk for the development of immune disease later in life (51).

We previously published findings from the IMPRINT Study in which healthy, term, breastfed infants supplemented with 1.8 x 10^{10} CFU of *B. infantis* EVC001 per day for 21 consecutive days starting on day 7 postnatal, demonstrated persistent colonization of fecal *B. infantis* one month post-supplementation. Given the diversity among *B. infantis* strains (52, 53), we selected *B. infantis* EVC001 because we knew this strain had the full cassette of genes needed to completely digest all HMOs from human milk. Supplementation with *B. infantis* EVC001 was well-tolerated (32) and increased fecal *Bifidobacteriaceae* by 79% and reduced enteropathogens by 80%, decreased fecal HMOs by 10-fold (consistent with increased HMO consumption by gut microbes) and increased fecal lactate and acetate by 2-fold, resulting in a decrease in fecal pH by 1 log unit (33). Intestinal colonization of *B. infantis* persisted one month post-supplementation. These results are unprecedented as probiotics have only been found to transiently exist in the gut during supplementation in infants, without showing persistent colonization in most individuals or altering the gut microbiome composition in adults (54). In the follow-up study reported herein, infants who completed the IMPRINT Study at 2 months of age were followed up at 4, 6, 8, 10, 12, 18, and 24 months postnatal. The aims of this follow-up study were to determine if *B. infantis* colonization persisted up to one year postnatal and identify differences in reported health outcomes between *B. infantis* EVC001 supplemented and un-supplemented infants.

Methods

Subjects and Design

The details of the main 2-month long IMPRINT Study are reported elsewhere (32). Briefly, mother-infant dyads were recruited in the Davis and Sacramento metropolitan region of Northern California. Mothers received either lactation support, or lactation support and 1.8 x 10^{10} CFU of *B. infantis* EVC001 (ATCC SD-7035; manufactured by Evolve BioSystems, Inc.) to feed their infants daily from day 7 to day 27 postnatal. *B. infantis* EVC001 was delivered as 156 mg of live bacteria (1.8 x 10^{10} CFU) diluted in 469 mg of lactose as an excipient. Mothers were trained by lactation consultants to mix the *B. infantis* EVC001 powder with 5 mL of expressed breast milk and feed the mixture to their infant using a feeding syringe. The probiotic was stored at –20°C by the mothers during the study. Upon completing the parent trial when their infants were about 2 months of age, participants were offered the opportunity to enroll in two independent follow-up studies: Follow-up #1 which was designed to determine if *B. infantis* persisted up to one year postnatal; and Follow-up #2 which was designed to determine if *B. infantis* supplementation early in life was protective against the development of health conditions at 18 and 24 months postnatal. In the Follow-up #1 study, mothers completed a paper questionnaire about their infants' health and diet at 4, 6, 8, 10 and 12 months postnatal and collected one matching infant fecal sample at each time point. In the Follow-up #2 study, mothers completed an online questionnaire about their infants' health and diet at 18 and 24 months postnatal. The study and methods were approved by the UC Davis Institutional Review Board, and the study was registered at Clinicaltrials.gov (NCT02457338). All mothers provided written informed consent to participate in every aspect of the study.

Questionnaires

Follow-up studies

Mothers who completed the parent IMPRINT study were invited to enroll in two different follow-up studies: Follow-up #1 and Follow-up #2. In Follow-up #1, upon providing written informed consent, mothers completed up to five paper questionnaires that coincided with the collection of their infants' stool at 4, 6, 8, 10 and 12 months postnatal (**Supplemental File 1.1**). In Follow-up #2, upon providing email informed consent, mothers completed up to two questionnaires (without stool collection) at 18 and 24 months postnatal (**Supplemental File 1.2**). The questionnaires that were used in both follow-up studies prompted mothers to answer questions about their infants' health and diet. Specifically, in Follow-up #1, mothers were prompted to report on their infants' health and diet over the past two months (either since completing the parent IMPRINT Study or since completing the previous questionnaire). In Follow-up #2, mothers were prompted to report their infants' health and diet over the past six months at 18 months and 24 months postnatal. In both the Follow-up #1 and #2 studies, mothers were asked questions about their infants' dietary patterns (intake of breast milk, infant formula and solid foods), use of medications, supplements and vitamins, illnesses, sick doctor visits, hospitalizations, antibiotic (oral/IV) usage, and probiotic intake.

In Follow-up #1 only, mothers were asked if their infants were ever diagnosed with common infant health illnesses and conditions by a healthcare professional and infant age at diagnosis. The answer options were "diagnosed" or "not diagnosed". Mothers were asked to report the frequency of common infant conditions and illnesses. The answer options were "never", "sometimes", "often", "very often", "unsure", and "refuse". When mothers answered

"sometimes", "often", or "very often", they were prompted to rate the severity of the gastrointestinal symptoms from 1 to 10, with 1 as the least severe and 10 as the most severe.

In Follow-up #2, mothers were asked if their infants had experienced and were diagnosed with any allergies, wheezing, asthma, eczema, gastroesophageal reflux disease and lactose intolerance. The answer options were "yes", "no", "unsure", and "refuse". When mothers answered "yes", they were prompted to report the number of times their infants had experienced and if they had been diagnosed with common infant illnesses and conditions.

Samples

Follow-up #1

Fecal samples were collected at home at 4, 6, 8, 10 and 12 months postnatal. Fecal samples were stored in participants' home freezers and transferred on dry ice to a –80°C freezer for storage prior to DNA extraction. All individuals who processed and analyzed the samples were blinded to treatment allocation.

Molecular Methods and Analyses

As previously described (33), total DNA was extracted from approximately 100 mg of feces, using the Zymo Fecal DNA Miniprep kit according to the manufacturer's instructions (Zymo Research, Irvine, CA). Negative controls to detect kit contamination were included and failed to produce visible PCR bands in an agarose gel but were analyzed as quality controls. Samples were subjected to 16S rRNA gene sequencing as previously described (33). Quantification of the total *B. infantis* was performed by quantitative real-time PCR using Blon_2348 sialidase gene primers Inf2348F (5'- ATA CAG CAG AAC CTT GGC CT -3'), Inf2348_R (5'- GCG ATC ACA TGG ACG AGA AC -3'), and Inf2348_P (5'- /56-FAM/TTT CAC GGA /ZEN/TCA CCG GAC CAT ACG /3lABkFQ/-3'). The Blon_2348 gene is found in all *B. infantis* strains including

EVC001. The primer and probe sequence specificity has been previously described (55). Each reaction contained 10μL of 2× TaqMan Universal Master Mix II with UNG master mix (ThermoFisher Scientific, Waltham, MA), 0.9 μM of each primer, 0.25 μM probe and 5 μL of template DNA. Thermal cycling was performed on a QuantStudio 3 Real-Time PCR System (ThermoFisher Scientific, Waltham, MA) and consisted of an initial UNG activation step of 2 minutes at 50°C followed by a 10 minute denaturation at 95°C, succeeded by 40 cycles of 15 s at 95°C and 1 min at 60°C. All samples were run in duplicate with a standard curve on each plate. Quantification of *B. infantis* was determined (CFU/g stool) using a standard curve of genomic DNA derived from a pure culture of *B. infantis* EVC001 using colony forming units (CFU) counts and normalized for input stool wet weight (56). Standard curve genomic DNA was extracted from a 1mL aliquots of *B. infantis* EVC001 grown anaerobically at 37°C for 16 hours in deMann Rogosa Sharpe (MRS) medium (BD Biosciences, San Jose, CA) supplemented with 0.05% L-cysteine HCl. CFU counts of the 16-hour *B. infantis* EVC001 culture were determined by serial dilution in 0.9% NaCl on MRS agar plates containing 0.05% L-Cysteine HCl. Plates were incubated anaerobically at 37°C for 48 hours then counted and the CFU/ml value was calculated.

16S rRNA bioinformatics analysis

Sequences were analyzed using QIIME 1.9.1 (doi:10.1038/nmeth.f.303). Open-reference operational taxonomical unit (OTU) picking was performed using UCLUST at 97% identity against the Greengenes database (v.13_8) (10.1128/AEM.03006-05), and chimera filtering was checked as part of the QIIME pipeline using USEARCH 6.1 (57).

A representative set of sequences was taken for each OTU and a taxonomic classification was performed using uclust consensus taxonomy in QIIME. Representative sequences were then

aligned using PyNAST (https://biocore.github.io/pynast/) to the Greengenes core reference alignment and a phylogenetic tree was built using FastTree (58). After quality filtering, a mean of 26,354 (±8,830 [SD]) and a median of 27,646 reads were obtained per sample. Several multivariate linear modeling analyses (https://huttenhower.sph.harvard.edu/maaslin/) were computed to compare groups of samples at the family and genus levels, using subject as random effect to account for time and other clinical metadata including treatment status, delivery mode and feeding as fixed effects. MaAsLin2 was run with a false-discovery rate of 0.05, a minimum of 0.0001 for feature relative abundance filtering, and a minimum of 0.01 for feature prevalence filtering. Fixed effects used in the MaAsLin2 model include any use of the following by the infant: antibiotics, probiotics, probiotics containing *B. infantis*, infant formula, solid food, and breast milk. Additionally, the model included delivery mode, supplementation allocation. Subject ID was used as a random affect and time was used as a continuous variable. P-values were adjusted via FDR (Q-values) and considered significant if Q-value < 0.25. Raw data is accessible at the following link: https://www.ncbi.nlm.nih.gov/sra, under accession number PRJNA670448.

Diversity analysis

Rarefaction curves were computed to estimate the distribution of the identified OTUs at a depth of 1,538 sequences/sample. Alpha diversity was computed using the Shannon diversity index in QIIME. A nonparametric two-sample t-test was used to compare alpha diversity according to treatment status using Monte Carlo permutations (n = 999). Beta-diversity was computed using UniFrac distances and a dissimilarity matrix was constructed to estimate the global OTUs differences among samples and visualized via a Principal Coordinate Analysis (PCoA). A Permutational Multivariate Analysis of Variance Using Distance Matrices (adonis) was used to assess OTUs differences between treatments and the effect-size (R2) of colonization by EVC001.

P-values for the PCoA panel was computed using F-tests based on sequential sums of squares from permutations of the raw data.

Statistics

The Mann-Whitney U Test was used to compare mean ranks for fecal *B. infantis* between EVC001 supplemented (EVC) and un-supplemented (UNS) groups at each time point during the first follow-up period (4, 6, 8, 10 and 12 months postnatal). The Mann-Whitney U Test was performed on 1) all infants, 2) infants who had not used any infant formula, antibiotics or probiotics since completing the parent IMPRINT Study (2 months postnatal), and 3) infants who used infant formula or antibiotics at 6 months postnatal (or time-point closest to 6 months). Because solid foods are commonly introduced to infants by 6 months of age, solid food consumption was not excluded in any of the analyses. Infant weight was measured at each study visit using a Pediatric Tanita digital scale and mean ranks for infant weight were compared between EVC and UNS groups using Mann-Whitney U. The significance level for all Mann-Whitney U analyses was set at an alpha 0.05 with a Bonferroni adjustment using the 2-tailed exact test statistic which is appropriate for small, unbalanced, or poorly distributed data. Mean ranks for frequency ordinal data and for severity continuous data were compared between EVC and UNS groups using Mann-Whitney U. Categorical data that resulted in the answers “yes”, “no”, “unsure”, or “refuse” were analyzed using a 2-sided Fisher’s Exact Test with an alpha 0.05 with a Bonferroni adjustment, whereby “unsure” and “refuse” responses were excluded from the analysis. SPSS version 25 was used for these analyses.

Results

Follow-up #1

Of the sixty-eight mothers enrolled in the parent IMPRINT Study, forty-eight mothers enrolled in the Follow-up #1 Study. Of these forty-eight mother-infant dyads, n = 22 had received the UNS treatment and n = 26 had received the EVC treatment. There was a significantly higher number of primiparous women in the EVC group than the UNS group ($P < 0.01$) (**Table 1.1**). There were no other differences in demographic, labor, delivery, and health history characteristics between the two groups. Infants enrolled in the EVC group were born at a younger gestational age than infants enrolled in the UNS group ($P < 0.05$) (**Table 1.2**); however, all infants were full term at birth. A detailed description of infants' diet, intake of antibiotics and probiotics, and exposure to other infants via daycare are reported in **Table 1.3**. There were no differences in the number of infants who consumed breast milk; breast milk and infant formula; infant formula without breast milk; solid foods; used antibiotics or probiotics; or were enrolled in daycare at any time point (**Table 1.3**). There was no difference in weight between groups across time (**Figure 1.1**).

Table 1.1. Maternal demographics, labor, delivery, and health history

Characteristics	UNS (n = 22)		EVC (n = 26)	
	Mean	SD	Mean	SD
Maternal Age at Enrollment (yr)	31.0	3.4	33	4.7
Pre-Pregnancy BMI	24.5	3.1	26.2	3.5
Pregnancy Weight Gain (kg)	31.1	7.7	33.7	11.8
Hours in Labor (hr)	22.0	26.0	11.3	12.6
Ruptured Membranes Prior to Birth (hr)	12.4	19.2	7.1	12.0
Number of Pregnancies	2.0	1.5	2.8	1.7*
Number of Live Births	1.5	1.0	2.2	1.1**
Parity, % (n)				
Primiparous	77.3% (17)		34.6% (9)**	
Multiparous	22.7% (5)		65.4% (17)	
Mode of Delivery, % (n)				
Vaginal	63.6% (14)		69.2% (18)	
Vaginal Water Birth	18.2% (4)		0% (0)	
C-section, Emergent	13.6% (3)		15.4% (4)	
C-section, Elective	4.5% (1)		15.4% (4)	
Ethnicity, % (n)				
Not Hispanic	90.9% (20)		76.9% (20)	
Hispanic	9.1% (2)		23.1% (6)	
Race, % (n)				
Asian	4.5% (1)		0% (0)	
Black	4.5% (1)		0% (0)	
White	81.8% (18)		73.1% (19)	
Other	0% (0)		7.7% (2)	
2 or More Races	9.1% (2)		19.2% (5)	
Education, % (n)				
Some College, No Degree; or AA Degree	13.6% (3)		19.2% (5)	
Bachelor's Degree (BA or BS)	36.4% (8)		34.6% (9)	
Master's, Professional, or Doctorate Degree	50% (11)		46.2% (12)	
Antibiotic Use During Labor, % (n)				
Yes	18.2% (4)		26.9% (7)	
No	81.8% (18)		73.1% (19)	
Gestational Diabetes Mellitus Positive Diagnosis, % (n)				

	Yes	9.1% (2)	7.7% (2)
	No	90.9% (20)	92.3% (24)
Group B *Streptococcus* (GBS) Colonization Positive Diagnosis, % (n)			
	Yes	22.7% (5)	30.8% (8)
	No	77.3% (17)	69.2% (18)
Any Allergy Diagnosis in Past 10 Years, % (n)			
	Yes	36.4% (8)	26.9% (7)
	No	63.6% (14)	73.1% (19)
Asthma Diagnosis in Past 10 Years, % (n)			
	Yes	22.7% (5)	7.7% (2)
	No	77.3% (17)	92.3% (24)
Hay Fever Diagnosis in Past 10 Years, % (n)			
	Yes	0% (0)	7.7% (2)
	No	100% (22)	92.3% (24)
Autoimmune Disease Diagnosis in Past 10 Years, % (n)			
	Yes	0% (0)	15.4% (4)
	No	100% (22)	84.6% (22)
Impaired Glucose Tolerance in Past 10 Years, % (n)			
	Yes	0% (0)	0% (0)
	No	100% (22)	100% (26)

*P < 0.05, **P < 0.01 for differences between treatment groups.

Table 1.2. Infant characteristics

Infant Characteristics	UNS (n = 22)		EVC (n = 26)	
	Mean	SD	Mean	SD
Gestational Age at Birth (wk)	40.2	1.0	39.5	1.3*
Birth Weight (g)	3669.2	587.8	3448.8	396.3
Birth Length (cm)	51.1	2.4	50.5	2.2
Gender, % (n)				
Male	40.9% (9)		65.4% (17)	
Female	59.1% (13)		34.6% (9)	

*P < 0.05 for differences between treatment groups.

Table 1.3. Infant diet and environment[1]

Feeding and Environment	Month									
	4		6		8		10		12	
	UNS	EVC	UNS	EVC	UNS	EVC	UNS	EVC	UNS	EVC
Total (n)	11	7	16	13	18	16	19	23	21	26
Breast milk[1] (n)	8	6	12	9	10	10	9	13	10	12
Breast milk and infant formula[1] (n)	2	1	1	4	3	4	2	5	4	5
Infant formula[1] (n)	0	0	1	0	1	1	2	1	3	3
Solids (n)	1	0	14	9	18	16	19	23	21	26
Antibiotics (n)	0	0	1	0	2	1	4	4	3	4
Probiotics[2] (n)	1	0	1	0	3	1	4	2	1	3
Daycare (n)	27%	0%	50%	23%	50%	25%	47%	35%	57%	50%

[1]Excludes infants who took antibiotics and/or probiotics.

[2]n =4 infants in the EVC and n = 5 in the UNS consumed five different probiotic supplement products at various times during the follow-up study. Participants in the study were able to recall the product names for four of the five probiotic supplements they fed to their infants. The four probiotic products recalled contained the following microorganisms: 1) *Lactobacillus acidophilus* and *Lactobacillus helveticus* (unspecified strains), 2) *Bifidobacterium longum* and *Bifidobacterium infantis* (unspecified strains), 3) proprietary probiotic

blend containing five *Lactobacillus* and five *Bifidobacterium* species, and 4) *B. infantis* EVC001 (one participant enrolled in the study found one sachet of the study probiotic in her freezer and fed it to her infant at 11 months postnatal).

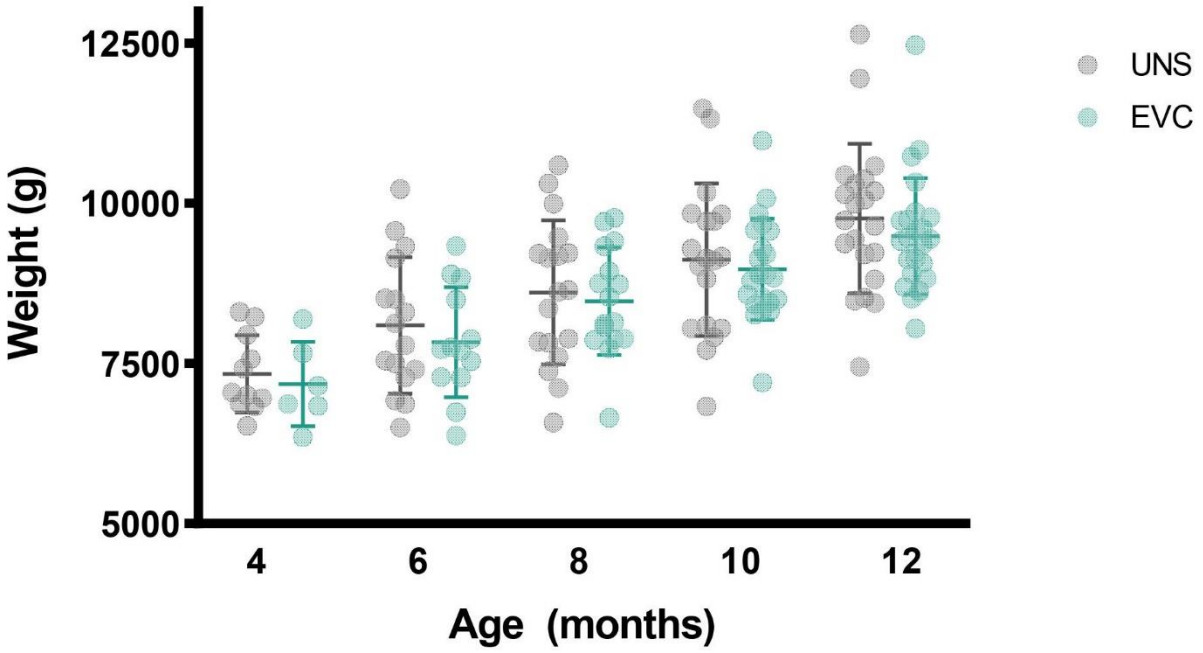


Figure 1.1. Infant weight across time and between treatment groups for all infants. Sample size is not consistent with Table 3 due to missed weights for EVC: Day 120, n=6; Day 300, n=22; Day 365, n=25.

With the inclusion of all infants, fecal *B. infantis* was 2.5 to 3.5 logs higher at 6 months through 12 months in the EVC group compared with the UNS group ($P < 0.01$) (**Figure 1.2a**). In a subgroup of infants who did not receive infant formula, antibiotics or probiotics, fecal *B. infantis* was 3.6 to 5.2 logs higher at 6 months through 12 months in the EVC group compared with the UNS group ($P < 0.001$) (**Figure 1.2b**). To further focus on these confounding variables, we conducted Mann-Whitney U testing on three sub-groups: infants breast milk-fed without intake of infant formula, antibiotics or probiotics (BM); infants mixed-fed with breast milk and infant formula without intake of antibiotics or probiotics (BM+FF); and infants exposed to antibiotics

and/or additional probiotics (all feeding types) (ABX). We selected one time point, as close to 6 months as possible, when breast milk volume intake would be the highest and the introduction of solid foods would be minimal. For this analysis, for the BM sub-group, fecal *B. infantis* was 3.3 logs higher in infants in the EVC group compared to the UNS group ($P < 0.0005$). However, for both the BM + FF and ABX sub-groups, fecal *B. infantis* was not different between EVC and UNS groups (**Figure 1.3**). To further investigate how *B. infantis* supplementation influences the gut microbial composition across all time points, we used Multivariate Association with Linear Models 2 (MaAsLin2), to determine if treatment altered gut microbial taxa. Infants in the EVC group had significantly higher *Bifidobacteriaceae* (R = 0.24, FDR-adjusted Q-value <0.01), *Lactobacillales* unclassified family I (R = 0.01, FDR-adjusted Q-value = 0.05), *Lactobacillales* unclassified family II (R = 0.003, FDR-adjusted Q-value = 0.12), *Enterococcaceae* (R = 0.02, FDR-adjusted Q-value = 0.14), and *Bacillales* unclassified family (R = 0.002, FDR-adjusted Q-value = 0.17) and significantly lower *Lachnospiraceae* (R = 0.14, FDR-adjusted Q-value <0.01), *Erysipelotrichaceae* (R = 0.04, FDR-adjusted Q-value < 0.05), Bacteroidaceae (R = 0.12, FDR-adjusted Q-value = 0.09) and *Pasteurellaceae* (R = 0.008, FDR-adjusted Q-value = 0.17) compared with the UNS group (**Figure 1.4a**) even after adjustments for infant formula, antibiotics, probiotics, delivery mode, postnatal age and subject as a random variable. Of the taxa that were significantly different between treatments according to MaAsLin2, Mann-Whitney U Test was used to compare differences for taxa between treatments for each time point and confirmed statistical differences for only fecal *Bifidobacteriaceae* and *Lachnospiraceae* at 6, 8, 10 and 12 months postnatal ($P < 0.05$) and *Bacteroidaceae* at 12 months postnatal ($P < 0.01$) (**Table 1.4**). The same MaAsLin2 modeling used on a family-level showed higher correlation coefficients between supplementation and gut microbial composition on a genus-level (**Figure 1.4b**). The

genera that were significantly different between treatments according to MaAsLin2 modeling were compared statistically at each time point using Mann-Whitney U Test. Infants in the EVC group had significantly higher fecal *Bifidobacterium* at 6, 8, 10 and 12 months postnatal, and *Enterococcus* at 6 months postnatal and lower *Lachnospiraceae* (unclassified genus) at 6, 8, and 10 months postnatal; *Ruminococcus* at 8 months postnatal, and *Erysipelotrichaceae* (unclassified genus) at 6 and 8 months postnatal (**Supplemental Table S1.1**)

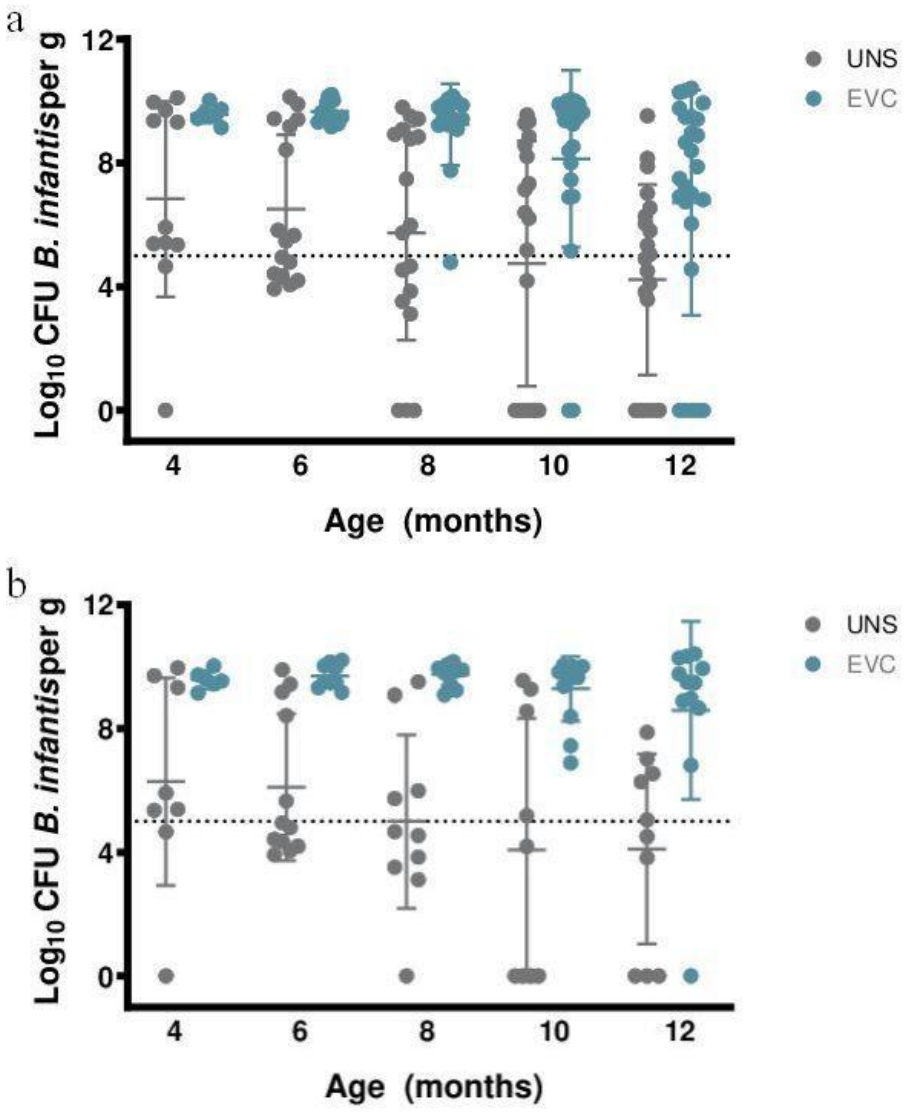


Figure 1.2. Infant fecal *B. infantis* across time and between treatment groups. a) Inclusion of all infants. b) In a subgroup of infants who did not receive infant formula, antibiotics, or probiotics. *P < 0.01, **P < 0.001, ^ P<0.0005 for differences between treatment groups.

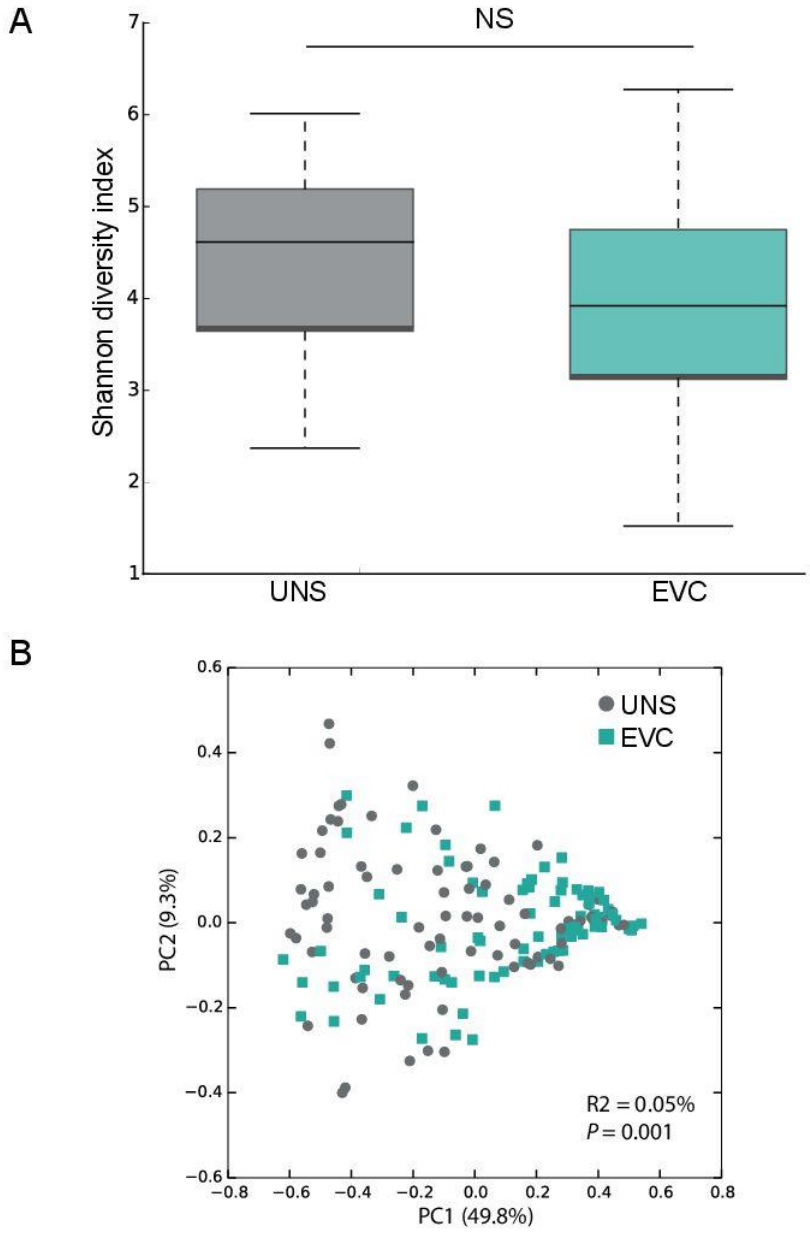
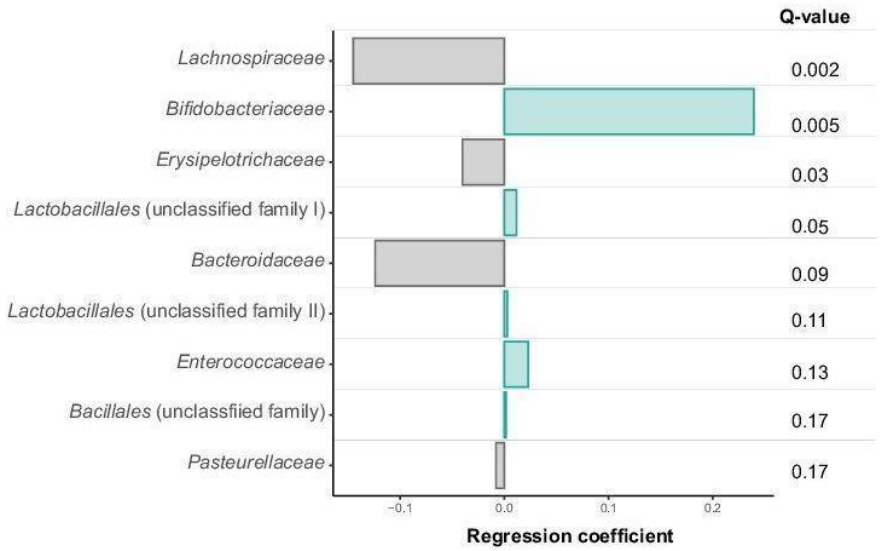


Figure 1.3. Infant fecal *B. infantis* at 6 months postnatal among three subgroups of infants based on diet and exposure to antibiotics. Breast milk (including solids), BM; breast milk and formula-

fed (including solids), BM + FF; antibiotic use, ABX. ^ P<0.0005 for differences between treatment groups.

a



b

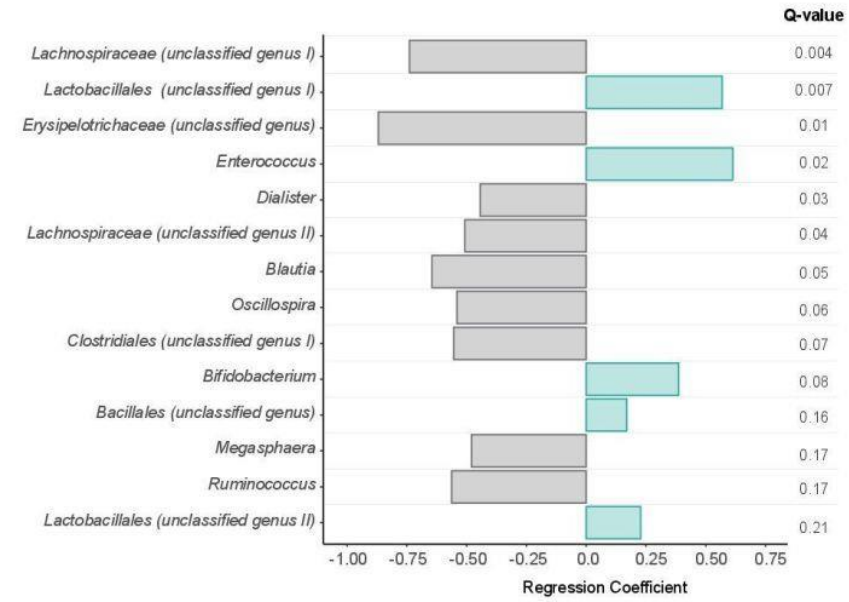


Figure 1.4. Relationships between infant fecal microbial families and treatment groups based on MaAsLin2 for all infants. a) Family-level. b) Genus-level. P-values were adjusted via FDR (Q-values) and considered significant if Q-value < 0.25.

Table 1.4. Infant fecal microbial families measured by 16s rRNA amplicon sequencing

Postnatal month	Family	% Mean relative abundance (SD)	
		UNS	EVC
4		*n* = 11	*n* = 7
	Bifidobacteriaceae	59.8 (27.0)	81.9 (9.72)
	Coriobacteriaceae	1.87 (5.62)	0.248 (0.479)
	Bacteroidaceae	7.19 (9.96)	4.45 (5.05)
	Prevotellaceae	0.001 (0.004)	0 (0.001)
	Enterococcaceae	0.476 (0.438)	1.02 (1.05)
	Lactobacillaceae	0.532 (0.711)	0.061 (0.159)
	Clostridiaceae	2.12 (5.77)	1.30 (1.99)
	Lachnospiraceae	8.67 (9.68)	1.81 (3.21)
	Streptococcaceae	1.19 (1.32)	2.41 (2.53)
	Ruminococcaceae	0.111 (0.257)	0.008 (0.009)
	Veillonellaceae	2.01 (3.50)	0.251 (0.340)
	Erysipelotrichaceae	3.72 (5.73)	0.161 (0.423)
	Enterobacteriaceae	8.31 (8.96)	4.77 (4.64)
	Other bacteria	4.03 (4.02)	1.62 (1.04)
6		*n* = 16	*n* = 13
	Bifidobacteriaceae	48.8 (26.9)	73.4 (16.2) **
	Coriobacteriaceae	1.68 (4.05)	0.232 (0.368)
	Bacteroidaceae	10.8 (13.7)	5.51 (6.65)
	Prevotellaceae	0.387 (1.52)	0.002 (0.003)
	Enterococcaceae	0.494 (0.759)	1.01 (0.867)
	Lactobacillaceae	2.49 (8.49)	0.693 (1.51)
	Clostridiaceae	1.17 (1.59)	1.26 (2.13)
	Lachnospiraceae	9.98 (11.3)	3.42 (7.95)*
	Streptococcaceae	1.19 (1.41)	1.79 (2.76)
	Ruminococcaceae	0.342 (0.534)	0.066 (0.169)
	Veillonellaceae	5.65 (6.60)	3.18 (5.30)

	Erysipelotrichaceae	2.57 (3.41)	0.248 (0.699)
	Enterobacteriaceae	9.18 (8.56)	6.34 (6.97)
	Other bacteria	5.19 (8.88)	2.81 (2.84)
		n = 18	*n* = 16
	Bifidobacteriaceae	38.2 (24.6)	61.2 (23.6) *
	Coriobacteriaceae	1.49 (4.78)	0.349 (0.552)
	Bacteroidaceae	17.4 (19.6)	8.60 (10.8)
	Prevotellaceae	0.965 (3.58)	0.004 (0.008)
	Enterococcaceae	0.678 (0.855)	0.903 (0.938)
	Lactobacillaceae	0.942 (1.60)	1.09 (1.57)
	Clostridiaceae	2.30 (2.66)	2.62 (3.41)
8	*Lachnospiraceae*	14.7 (15.3)	7.39 (13.2) *
	Streptococcaceae	1.00 (1.58)	1.20 (2.44)
	Ruminococcaceae	1.19 (2.32)	0.920 (3.00)
	Veillonellaceae	6.88 (4.24)	4.05 (4.27)
	Erysipelotrichaceae	2.07 (2.87)	0.872 (1.75)
	Enterobacteriaceae	7.94 (8.37)	7.21 (6.28)
	Other bacteria	4.25 (5.90)	3.64 (5.64)
		n = 19	*n* = 23
	Bifidobacteriaceae	31.3 (20.7)	48.2 (24.7)*
	Coriobacteriaceae	1.39 (2.61)	0.733 (0.881)
	Bacteroidaceae	15.2 (17.2)	11.4 (12.5)
	Prevotellaceae	2.06 (5.88)	0.216 (0.744)
	Enterococcaceae	0.483 (0.540)	3.44 (13.1)
	Lactobacillaceae	1.11 (1.95)	0.656 (1.44)
	Clostridiaceae	1.53 (1.65)	1.62 (1.94)
10	*Lachnospiraceae*	19.5 (15.4)	10.9 (13.5)*
	Streptococcaceae	0.776 (1.05)	1.09 (1.13)
	Ruminococcaceae	3.75 (8.52)	2.91 (5.50)
	Veillonellaceae	7.49 (7.99)	5.72 (6.28)
	Erysipelotrichaceae	2.69 (4.17)	0.699 (0.685)
	Enterobacteriaceae	6.76 (8.24)	4.58 (4.83)
	Other bacteria	5.90 (8.97)	7.80 (9.55)
		n = 21	*n* = 26
	Bifidobacteriaceae	15.4 (14.0)	33.1 (22.4) **
	Coriobacteriaceae	0.930 (2.06)	0.779 (1.05)
12	*Bacteroidaceae*	28.2 (19.1)	11.8 (11.1)**
	Prevotellaceae	2.18 (8.73)	2.16 (7.88)

Enterococcaceae	0.169 (0.479)	0.657 (1.20)
Lactobacillaceae	0.405 (1.17)	0.960 (2.04)
Clostridiaceae	0.951 (1.12)	1.38 (1.31)
Lachnospiraceae	24.6 (11.5)	17.4 (12.2)*
Streptococcaceae	1.64 (1.99)	5.76 (14.7)
Ruminococcaceae	10.2 (10.1)	7.49 (9.06)
Veillonellaceae	5.55 (9.60)	7.15 (8.07)
Erysipelotrichaceae	1.13 (1.31)	0.786 (1.08)
Enterobacteriaceae	3.34 (5.29)	3.49 (4.40)
Other bacteria	5.33 (4.46)	7.09 (5.92)

*P < 0.05, **P < 0.01 for differences between treatments groups.

To investigate if supplementation with *B. infantis* resulted in differences in gut related symptoms, mothers were asked how often infants experienced symptoms (never =0, sometimes =1, often = 2, very often =3, unsure = 4 and refuse = 5, whereby unsure and refuse responses were excluded from the statistical analysis) and to rate the severity of these symptoms. The mean frequencies for GI symptoms were not statistically significant between treatments at any study time point. Reported severity for infant constipation was 83% higher in the UNS vs. EVC group ($P < 0.001$), however neither value was considered severe (**Supplemental Table S1.2**). The frequency for illnesses, sick doctor visits, hospitalizations, ear infections, respiratory tract infections, other infections, thrush, allergy, wheezing, asthma, eczema, and other conditions were not significantly different between treatments across time (**Supplemental Table S1.3**). There was also no difference in reported use of antibiotics, anti-gas medication, gripe water, probiotics with or without *B. infantis*, prescribed medications, or over-the-counter medications (**Supplemental Table S1.4**).

Diversity analysis

Rarefaction curves were computed to assess differences in alpha diversity composition as measured by the Shannon diversity index based on treatment status. No statistical difference was observed between groups (**Supplemental Figure 1.3A**). Beta diversity analysis was performed using UniFrac distances and the effect size of probiotic feeding was calculated, resulting in a significant (P = 0.001; adonis) though weak effect size (R2 = 0.05%; adonis) (**Supplemental figure 1.3B**).

Follow-up #2

Of the sixty-eight mothers enrolled in the parent IMPRINT Study, fifty-one mothers enrolled in the Follow-up #2 Study. Of these participants, n = 19 in the UNS and n = 17 in the EVC group completed the 18-month health questionnaire and n = 21 in the UNS and n = 20 in the EVC group completed the 24-month health questionnaire. There were no treatment differences in the number of children who experienced or were diagnosed with any common infant conditions or experiences (**Supplemental Table S1.5**). There were no significant differences in the mean number of experiences or diagnoses of common infant conditions (**Supplemental Table S1.6**).

Discussion

The dominance of fecal *Bifidobacterium* and, specifically, *B. infantis* in the gut of breastfed infants has declined in recent decades in resource-rich nations resulting in an increase in potential gut pathogens and immune dysfunction (50, 59-63). Probiotic supplementation with *B. infantis* EVC001 in 7-day old breastfed infants for 21 consecutive days resulted in a 7-log increase in fecal *B. infantis*, an increase in fecal *Bifidobacteriaceae* by 79%, a decrease in enteropathogens by 80%; an increase in fecal lactate and acetate by 2-fold, a decrease in fecal pH by 1-log (33), a decrease

in antibiotic resistance genes, a sign of reduced enteropathogens known to harbor these genes (64), a reduction in mucin degradation (65) and reduced enteric inflammatory markers by several-fold (61) during and one-month post-supplementation. These data demonstrate that the combination of breast milk and *B. infantis* EVC001 successfully restores the gut microbiome and biochemistry to historical norms observed a century ago (49).

The infant gut microbiome is influenced by several maternal, dietary, and environmental factors including delivery mode, feeding status (i.e., breast milk, infant formula, solid foods), and use of antibiotics. The current study showed that fecal *B. infantis* was 2.5 – 3.5 logs higher in infants in the EVC group compared with the UNS group at 6, 8, 10, and 12 months despite feeding status, use of antibiotics or probiotics. The greatest difference in fecal *B. infantis* was observed in the earlier time points (6 and 8 months) when breast milk was the most abundant food source. The smallest difference in fecal *B. infantis* was observed at 12 months when infants' diets were much more diverse and breast milk was less abundant. After excluding infants with confounding variables that impact the gut microbiome such as infant formula, antibiotics and probiotics, fecal *B. infantis* was 3.6 – 5.2 logs higher in infants in the EVC group compared with the UNS group at 6, 8, 10, and 12 months. We were unable to determine if probiotic intake during the one year follow-up period independently influenced fecal *B. infantis* abundance because six of the nine infants who consumed probiotics also received antibiotics. Taken together, these data suggest that a lack of HMOs, the preferred carbon source for *B. infantis,* and the use of antibiotics impact fecal *B. infantis* levels.

When infants were grouped by feeding type and exposures (breast milk-fed without intake of infant formula, antibiotics or probiotics, mixed-fed with breast milk and infant formula without intake of antibiotics or probiotics, and intake of antibiotics (all feeding types and probiotics), we

found that fecal *B. infantis* was significantly higher in infants in the breast milk-fed group who were supplemented with *B. infantis* EVC001compared with the UNS group. These findings further support the observation that breast milk is critical in supporting the colonization of *B. infantis*.

The UNS group had significantly higher *Lachnospiraceae,* including the genera, *Ruminococcus*, and *Blautia*, *Bacteroidaceae*, and lower *Bifidobacteriaceae* levels compared with the EVC group. These taxa differ in their preferences for carbohydrate substrates, metabolism of their preferred substrates into end-products and their consequent biochemical effects in the gut and on infant health. For example, members of the family *Lachnospiraceae* consist of spore-forming, anerobic bacteria that ferment complex plant polysaccharides into short chain fatty acids (SCFAs) such as acetate, butyrate, and propionate (66). While gut microbes that produce SCFAs that lower luminal pH are considered beneficial, health outcomes associated with this family are mixed and likely vary with the genus or species and with host factors (e.g., infant vs. adult). For example, some members of this family that are commonly found in the human gut microbiome have been associated with a number of adverse health outcomes in adults (e.g. bloating, irritable bowel disease, metabolic disorders) (67-69). In a prospective cohort study, the abundance of the family *Lachnospiraceae* at 3-4 months was higher in the gut of formula-fed infants compared to breastfed infants in a dose-dependent manner and associated with an 89% increase in risk of overweight by 12 months (70). Emerging evidence suggests the species, *Ruminococcus gnavus*, which belongs to the family *Lachnospiraceae* (71), may play a key role in allergy and immune development in infants (72) and inflammation in the gut of adult patients with Crohn's disease (73).

In this study we also found higher levels of the family *Bacteroidaceae* in the UNS group at 12 months postnatal. *Bacteroidaceae*, is a family of gram-negative, obligate anaerobic, nonsporulating bacilli, that is commonly found in the healthy human adult colon. While most

members of this family are considered commensals, some species such as *Bacteroides fragilis*, include pathogenic strains (74). Additionally, members of the *Bacteroidaceae* family contain an expanded set of genes encoded in polysaccharide utilization loci (PULs), allowing for the consumption of both dietary polysaccharides, as well as host-derived glycans (75). Specifically, *Bacteroides thetaiotaomicron* and *Bacteroides fragilis*, common members of the neonate gut, utilize a large set of mucin degradation PULs to catabolize HMOs (76). Previous studies in gnotobiotic mice have shown that downstream products derived from *Bacteroides*-driven HMO catabolism confer a growth advantage to potentially pathogenic *Enterobacteriaceae*, specifically *E. coli*. This cross-feeding event was found to drive the *E. coli* bloom in a dextran sodium sulfate -induced colitis mouse model, thereby compounding the inflammatory response (77). On the other hand, the subspecies of *B. longum*, *B. infantis* and specific strains of *B. infantis* (17) such as EVC001 preferentially consume human milk oligosaccharides, which are fermented into acetate and lactate via the "bifid shunt" (12, 17). These end-products maintain a lower pH of the intestinal milieu, supporting the transport of these compounds into the intestinal epithelium for use by the host (48) and creating an undesirable environment for potential pathogens (19). Acetate also blocks the infiltration of toxic molecules produced by pathogenic bacteria by enhancing intestinal barrier function and inhibiting pro-inflammatory and apoptotic responses (20). The clinical importance of infant fecal pH has been highlighted recently as a risk indicator for childhood stunting (78), and is also reflected in the updated reference range for infants provided by national diagnostic labs. The gut of infants enriched with the genus *Bifidobacterium* and low levels of potential pathogens decreases the risk of autoimmune diseases (30, 51) supporting that supplementation with *B. infantis* EVC001in early life may help protect infants from developing autoimmune diseases. Alpha diversity was not different between groups, however we found significant yet weak

differences in beta diversity between the two groups suggesting that only a few OTUs were contributing to the overall beta diversity in response to treatment status.

MaAsLin 2 modeling also discovered that EVC supplementation was positively correlated with *Enterococcaceae* and *Enterococcus*. Confirmation of these data with statistical analyses at each time point found that *Enterococcaceae* was not different, however, *Enterococcus* was significantly higher by 0.5% in the EVC group compared with UNS at 6 months postnatal. Species that belong to the genus *Enterococcus* exert a range of functions in the gut as commensals to nosocomial pathogens that possess antibiotic resistance genes (79). In this study, *Enterococcus* represented a mean of 1% of the gut microbiome across both treatments and all time points, yet the variation was high ranging from 0.16% to 3.4% of the gut microbiome. For example, this genus represented 63% of the gut microbiome in one infant in the EVC group after the intake of antibiotics but was reduced to 0% in this same infant two months later. We have previously reported that EVC supplementation reduced antibiotic resistance genes (64) and that this taxon was not associated with enteric inflammation (61).

In the Follow-up #1 Study, there was no difference in the frequency of illnesses, doctor visits, hospitalization, or health conditions between the EVC and UNS groups. Compared with the EVC group, participants in the UNS group reported a significantly higher score for the severity of their infants' constipation (2.9 vs. 1.2), yet this value is not considered moderately or highly severe. In the Follow-up #2 Study, there were no differences between the EVC and UNS groups for the number of infants who were diagnosed with or experienced any common health conditions. While several larger studies have reported that probiotics can influence health conditions such as eczema (80, 81), it is likely that the sample sizes in both Follow-up #1 and Follow-up #2 (n = 48 and n =

51, respectively) were too small to detect any significant differences in health outcomes or differences that may arise later in life.

One limitation of this study is that primers specific to the full genomic sequence for EVC001 were not used in this study. Based on the literature, *B. infantis* is an uncommon bifidobacterial subspecies found in infants who reside in Northern California (33, 59). In the parent study published in Frese et al. (2017) (33), fecal *B. infantis* was on average 8 logs higher in infants supplemented with *B. infantis* EVC001 compared with unsupplemented infants. Thus, we are confident that the several-fold difference in fecal *B. infantis* found in infants during the follow-up period is due to supplementation with EVC001 and not a random effect. Another limitation is that following completion of the parent study, factors that have confounding effects on the gut microbiome were not controlled. Although there weren't significant differences in the number of infants among the different subgroups: breast milk; breast milk and infant formula; infant formula without breast milk; solid foods, or used antibiotics, probiotics or were enrollees in daycare at any time point, given the small number in each subgroup it is possible that some of these factors had an impact on the gut microbiome. Second, different individuals participated in Follow-up #1 and Follow-up #2, limiting our ability to make direct comparisons between the gut microbiome results of Follow-up #1 and the health outcomes measured in Follow-up #2. Although Follow-up #1 and #2 are independent of one another, both sets of participants stemmed from the parent study allowing us to make direct comparisons between treatment groups. Third, for both Follow-up #1 and #2, not every participant provided a stool sample and questionnaire at every time point. As such, it was not possible to use paired data to compare the gut microbiome and health outcomes across time and, therefore, our statistical analyses were limited to treatment group comparisons at each time point. Lastly, the parent study was originally designed to determine differences in the

gut microbiome composition and fecal biochemistry at one month post *B. infantis* EVC001 feeding and was not designed or powered to identify differences in health outcomes between treatment groups. Neither Follow-up #1 nor #2 were designed or powered to detect differences in health outcomes between treatment groups. For example, previous longitudinal studies that have investigated the relationships between the early infant gut microbiome and atopic wheezing, and asthma have included both control and at-risk groups with sample sizes between 100-300 infants (82, 83).

Long-term colonization of a probiotic after cessation of its consumption has not been previously been demonstrated. These findings support the importance of matching a specific microorganism with a carbohydrate source that it selectively consumes thereby providing an open ecological niche for the microbe to occupy. We found that feeding breastfed infants a specific strain of *B. infantis* (EVC001) that efficiently utilizes all HMO structures in human milk for a brief period resulted in sustained colonization one year post-supplementation. The gut microbiome in early infancy plays a critical role in immune system development and metabolic programming that has lifelong health impacts. Changes in the composition of the gut microbiome with lower protective microbes and higher potential pathogens associated with a Western lifestyle appear to increase the risks of developing allergic, inflammatory, and autoimmune diseases. Based on our findings, large clinical trials are warranted to determine whether *B. infantis* EVC001 supplementation early in life prevents the development of these diseases in child- through adulthood.

Chapter 2

Probiotic Supplementation with *B. infantis* in Two- to Four-Month-Old Breastfed Infants Leads to Persistent Colonization One Month Post-Supplementation

Introduction

Breast milk plays a critical role in the development of a healthy infant intestinal microbiome in the weeks and months after birth, providing both nutrition and protection to support the development of the vulnerable neonate. It delivers a wide spectrum of biologically active molecules that aid in the development and maturation of the gut and the innate and acquired immune systems, and support the growth of protective intestinal microbiota, namely, a protective subspecies of *Bifidobacterium*, (*B. infantis*) (84, 85). Specifically, human milk oligosaccharides (HMO), an abundant component of human milk that is not digestible by the infant, act as prebiotics that selectively enrich the growth of *B. infantis*. Unlike other bacteria found in the infant gut, *B. infantis* has uniquely adapted to utlize HMOs, resulting in the production of organic acids such as acetate and lactate. These byproducts decrease intestinal pH which have been shown to inhibit the growth of potential pathogenic bacteria and increase intestinal barrier function (18, 20, 84-86).

Historically, the gut of breastfed infants have been dominated by strains of *Bifidobacterium* from birth until cessation of breastfeeding (1, 59, 87). However, in recent decades, the dominance of fecal *Bifidobacterium* and *B. infantis* has declined in developed countries, as evidenced by an increase in fecal pH from 5.0 to 6.5 over the past 100 years (50). Delivery mode, feeding practices, and medications all have profound impacts on the developing microbiome with cesarean section delivery, formula feeding, and antibiotic use disrupting normal development. The loss of *Bifidobacterium* has resulted in an increase in intestinal dysbiosis in infants born in resource-rich

countries (59, 60). This disruption during early childhood has been associated with alterations in metabolic, immunologic, and neurologic function with consequences ranging from allergies, asthma, diabetes, and obesity (72, 88-91). Thus, establishing a healthy intestinal microbiome early in life is critical for establishing life-long health.

Intestinal dysbiosis due to loss of *Bifidobacterium* may be combated with supplementation of a probiotic containing *B. infantis*. Administration of *B. infantis* resulted in increased relative abundance of fecal bifidobacteria in both formula-fed and breastfed pre-term infants (92). Results from a phase 1 clinical trial have demonstrated that *B. infantis* supplementation of newborns leads to the stable colonization of *B. infantis* in the infants' gut at one month post-natal (32, 33). Additionally, supplementation significantly increased fecal short-chain fatty acids and decreased fecal pH and fecal HMO content, which suggests a higher consumption of HMOs by *B. infantis* and changes in intestinal fermentation (33). A dominance of fecal *Bifidobacterium* in response to *B. infantis* supplementation also reduced the abundance antibiotic resistance bacteria (64) and also resulted in reduced enteric inflammation (61). Follow-up studies have demonstrated that this colonization persists up to one year post-natal (34). Recently, Henrick et al., showed that indole-3 lactic acid produced by *B. infantis* upregulated immunoregulatory galectin-1 in intestinal T helper 2 (Th2) and Th17 cells during polarization, providing a functional link between beneficial intestinal microbes and immunoregulation during the first months of life (31). Thus, *B. infantis* probiotic supplements may be a viable tool to combat infant intestinal dysbiosis, thereby facilitating proper immune system and intestinal development.

No studies to date have determined the effectiveness of a probiotic supplement in colonizing the intestinal tract in older infants. Because intestinal microbial communities become more stable and complex over time, it is unknown if probiotic supplementation with *B. infantis* in

older infants results in similar effects on microbial colonization compared with newborn infants (1, 93). Thus, it is important to identify the window of opportunity when *B. infantis* administration can successfully establish colonization of healthy microbial species and ensure their persistence.

To address this question, we conducted a randomized, double-blind, placebo-controlled study to (1) determine if *B. infantis* supplementation in older (2-4-month-old) exclusively breastfed infants results in the stable colonization of *B. infantis* in the gut and (2) determine the minimally effective dose of *B. infantis* supplementation needed to increase fecal *B. infantis* to levels similar to exclusively breastfed newborns.

Methods

Study Population

Between April 2018 and March 2019, healthy women who had recently delivered healthy full-term infants and lived within the Davis and Sacramento metropolitan region of Northern California (USA) were recruited to enroll in this study. Inclusion criteria for study participants were as follows: healthy women 21 years of age or older; healthy infants born full-term (greater than 37 weeks gestation) without medical complications who are 60-125 days old at time of enrollment; infants exclusively breastfed with maternal intent to continue exclusive breastfeeding for at least 9 additional weeks following study enrollment; mothers who are willing to refrain from feeding their infants infant formula, solid foods, and iron or non-study supplements before the end of the study period. Exclusion criteria for study participants were as follows: infants born in a multiple birth; infants who have taken antibiotics, iron supplements or consumed infant formula or *Bifidobacterium*-containing probiotics within 4 weeks of enrollment or during the Baseline period; infants who have consumed any probiotics containing *B. infantis* since birth; infants who

consumed any solid food since birth; mothers who consumed probiotics containing *B. infantis* during the 3rd trimester of pregnancy and gave birth vaginally; and mothers who smoked cigarettes during pregnancy, currently smoke, or who planned to resume smoking during the study period.

Study Design

This was a randomized, double-blind, placebo controlled clinical trial which was approved by the University of California Davis Institutional Review Board (IRB #: 1166403) and registered on clinicaltrials.gov (Identifier: NCT03476447).

The study duration was 9-weeks and consisted of a one-week Baseline period (Days 1-7), 28-day Intervention period (Days 8-35) and a 28-day Post-Intervention period (Days 36-65). After meeting study criteria, participants provided written, informed consent. On study day 7, participants underwent final screening for the consumption of infant formula, antibiotics, probiotics, iron supplements, solid foods, or beverages other than breast milk and water and were randomized into one of four treatment groups.

Randomization was generated through Statistics & Data Corporation (SDC) (Tempe, Arizona) utilizing Interactive Response Technology built into a clinical data management system (iMedNet™) and study personnel were blinded to the treatment allocation. Because delivery mode has been shown to influence the infant intestinal microbiome, participants were stratified to one of two randomization schemes based on mode of delivery—vaginal or cesarean section (1, 94, 95). Randomization to supplementation of 0 CFU/day *B. infantis* EVC001 (pharmaceutical-grade lactose placebo), 4.0 x 10^9 CFU/day *B. infantis* EVC001 (low dose), 8.0 x 10^9 CFU/day *B. infantis* EVC001 (medium dose), or 1.8x 10^{10} CFU/day *B. infantis* EVC001 (high dose) was in a 1:1:1:1 ratio for both randomization schemes. The placebo and *B. infantis* EVC001 supplements were provided by Evolve BioSystems Inc. (Davis, CA). Each *B. infantis* EVC001 supplement was made up of a blend

of the probiotic and pharmaceutical-grade lactose. Each supplement sachet contained 625 mg of the blended powder.

A total of 41 participants were enrolled in this study, however only Ten infants were randomized into each group. One participant withdrew from the study prior to randomization and one participant from the 8.0 x 10^9 CFU/day (medium dose) *B. infantis* group withdrew from the study during the Intervention period; thus, only nine subjects received the 8.0 x 10^9 CFU/day (medium) *B. infantis* supplement.

This study is powered based on the minimum effective dose to increase fecal *B. infantis* compared with a placebo-control in exclusively breastfed infants. Based on Day 21-30 infant fecal *B. infantis* levels from the IMPRINT study (33), we needed to enroll 4 infants in each dosing group to identify a 9.7-log difference, with an $\alpha = 0.01$ (to account for multiple testing within each family of hypotheses), and power =90%.

Infants received one daily serving of the study supplement (lactose placebo, low, medium, or high doses) for 21 consecutive days beginning on Day 8 and continuing through Day 35. During the Day 7 randomization visit, mothers were trained by study personnel to mix the contents of each supplement sachet with approximately 5 mL of their breast milk in a plastic medicine cup, and to syringe feed the mixture to their infants. The product was stored in a freezer at −20 °C at the UC Davis campus until distributed to study participants. Mothers received 21 sachets, plus four extra sachets to be used in the event of damage or misplacement. All sachets were kept frozen in the mothers' kitchen freezers until time of use. Participants were instructed to keep all used and unused sachets provided. Compliance was assessed on Day 37 by recording the number of used and unused *B. infantis* sachets. Compliance was defined as at least 21 doses (75% of the scheduled

supplementation) of the randomized study supplement. % compliance was calculated as follows: (# of actual supplements received / 28) *100.

Participants were followed from Day 36 to Day 65 to assess persistence of *B.infantis* one month post-supplementation. Following completing of the study on Day 65, all participants were offered a 28-day supply of Evolve Biosystems's commercially available *B. infantis* probiotic product (Evivo®). Infant weight was measured by study personnel with a digital infant scale (Tanita) on Days 7, 37, and 65.

Questionnaires

At the enrollment visit on Day 0, mothers completed questionnaires regarding their pregnancy, labor, and delivery experience, reproductive health, and their infant's health and diet since birth. At the subsequent three study visits, mothers completed questionnaires about their and their infant's health and diet since the previous study visit. On each day throughout the entire study, participants were asked to keep prospective, daily logs regarding their infant's general health, stool patterns (stool number, size, and consistency (96)), diet, and medication usage. Participants were asked to record all periods of sleep, crying and fussing (minutes) for two 24-hour periods (the two 24-hour periods did not have to be consecutive) during Baseline (Days 1-6) and the Intervention period (Days 21-28) if they lasted for 5 minutes or longer.

Samples

Fecal samples were collected at home from their infant's diapers before study Day 7 (Baseline), and on Study Days 10, 14, 21, 28, 35, 42 and 63 using PurFlock Ultra Flocked Swabs (Puritan, Guilford, ME) and DNA/RNA Shield Lysis and Collection Tubes (Zymo Research, Irvine, CA). Additional fecal samples were collected before study Day 7 and on Days 14, 21, 28 and 63 using a Burkle SteriPlast micro spatula (VWR, cat # 75876-080) and a disposable cosmetic

spatula (Pana Brand) and were placed into a 5mL Eppendorf tube (VWR, cat # 89429-310). Fecal samples were stored in participants' home freezers and transferred in cooler bags on ice packs provided by study personnel to a −80 °C freezer for storage prior to DNA extraction. Upon arrival at the University of California Davis, samples were visually inspected for thawing by study personnel and, if any thawing was noted, participants were asked to recollect samples. All individuals who processed and analyzed the samples were blinded to treatment allocation.

Molecular Methods and Analysis

As previously described (33), total DNA was extracted from ~100 mg of feces, using the Zymo Fecal DNA Miniprep Kit according to the manufacturer's instructions (Zymo Research, Irvine, CA). Negative controls to detect kit contamination were included and failed to produce visible PCR bands in an agarose gel but were analyzed as quality controls. Samples were subjected to 16S ribosomal RNA (rRNA) gene sequencing as previously described (33). Quantification of the total *B. infantis* was performed by quantitative real-time PCR using Blon_2348 sialidase gene primers Inf2348F (5′-ATA CAG CAG AAC CTT GGC CT-3′), Inf2348_R (5′-GCG ATC ACA TGG ACG AGA AC-3′), and Inf2348_P (5′-/56-FAM/TTT CAC GGA /ZEN/TCA CCG GAC CAT ACG/3lABkFQ/-3′). The *Blon_2348* gene is found in all *B. infantis* strains including EVC001. The primer and probe sequence specificity has been previously described (55). Each reaction contained 10 µL of 2× TaqMan Universal Master Mix II with UNG master mix (Thermo Fisher Scientific, Waltham, MA), 0.9 µM of each primer, 0.25 µM probe, and 5 µL of template DNA. Thermal cycling was performed on a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA) and consisted of an initial UNG activation step of 2 min at 50 °C, followed by a 10-min denaturation at 95 °C, succeeded by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. All samples were run in duplicate with a standard curve on each plate. Quantification of *B.*

infantis was determined (CFU/g stool) using a standard curve of genomic DNA derived from a pure culture of *B. infantis* EVC001 using CFU counts and normalized for input stool wet weight (56). Standard curve genomic DNA was extracted from 1 mL aliquots of *B. infantis* EVC001 grown anaerobically at 37 °C for 16 h in deMann Rogosa Sharpe (MRS) medium (BD Biosciences, San Jose, CA) supplemented with 0.05% L-cysteine HCl. CFU counts of the 16-h *B. infantis* EVC001 culture were determined by serial dilution in 0.9% NaCl on MRS agar plates containing 0.05% L-cysteine HCl. Plates were incubated anaerobically at 37 °C for 48 h, then counted, and the CFU/mL value was calculated.

16S rRNA Bioinformatics Analysis

Sequences were analyzed using QIIME 1.9.1 (https://doi.org/10.1038/nmeth.f.303). Open-reference operational taxonomical unit (OTU) picking was performed using UCLUST at 97% identity against the Greengenes database (v.13_8) (10.1128/AEM.03006-05), and chimera filtering was checked as part of the QIIME pipeline using USEARCH 6.1 (57).

A representative set of sequences was taken for each OTU and taxonomic classification was performed using UCLUST consensus taxonomy in QIIME. Representative sequences were then aligned using PyNAST (https://biocore.github.io/pynast/) to the Greengenes core reference alignment and a phylogenetic tree was built using FastTree (58). After quality filtering, a mean of 26,354 (±8830 [SD]) and a median of 27,646 reads were obtained per sample. Several multivariate linear modeling analyses (https://huttenhower.sph.harvard.edu/maaslin/) were computed to compare groups of samples at the family and genus levels, using the subject as a random effect to account for time and other clinical metadata, including treatment status, delivery mode, and feeding as fixed effects.

Multivariate Association with Linear Models 2 (MaAsLin2) was run with a false-discovery rate (FDR) of 0.05, a minimum of 0.0001 for feature relative abundance filtering, and a minimum of 0.01 for feature prevalence filtering. Fixed effects used in the MaAsLin2 model included the treatment (placebo, low, medium and high *B. infantis* doses). Participant ID and study target day (Baseline, Day 28 and Day 63) were used as random variables. P values were adjusted via FDR (Q values) and considered significant if Q value < 0.25. Raw data are accessible under the accession number TBD. For confirmation, Wilcoxson rank sum test was performed on the families that were considered significant by the MaAsLin2 results to determine which groups were significantly different from placebo.

Taxa present in at least 50% of participants across all time points are summarized and presented herein. Wilcoxson rank sum test was used to compare microbial family abundances at each time point for each EVC group (low, medium, and high doses, and EVC) to placebo. A category "Other bacteria" was calculated by adding all additional families that were present in less than 50% of participants across all timepoints.

Statistics

The primary endpoint was analyzed using intent-to-treat population which includes all participants who were randomized. All other secondary analyses were conducted using the per-protocol population which includes all randomized participants who did not have major protocol deviations and considered a supportive population for the primary and key secondary efficacy analyses. Levels of infant fecal *B. infantis* and total fecal *Bifidobacterium* were $\log_{10}$ transformed prior to analyses. All hypothesis testing for primary and secondary analyses were two-sided, and the family-wise type I error rate (α) was maintained at 0.05. For some statistical analyses, the low, medium and high dose groups were combined, and this group is referred to as EVC throughout.

Primary Analysis

The primary analysis utilized Kruskal-Wallis test to compare levels of infant fecal *B. infantis* (as measured by *B. infantis* qPCR) at Day 28 in treatment groups (all doses) compared with placebo. If the p-value for the test of treatment (all doses) was statistically significant at the $\alpha = 0.05$ level, the sequential Holm's step-down testing strategy was employed to determine which dose(s) of *B. infantis* were statistically significantly more effective than placebo for increasing levels of infant fecal *B. infantis* following 28-days of supplementation. Pairwise comparisons of each dose of *B. infantis* versus placebo was conducted using the nonparametric Wilcoxon Rank Sum test.

Secondary Analyses

The difference between the three *B. infantis* doses on levels of infant fecal *B. infantis* on Day 28 was the key secondary efficacy analysis. All hypothesis testing was two-sided and the family-wise type I error rate (α) for the key secondary efficacy analysis was maintained at 0.05 through application of the Holm's procedure. All three doses of *B. infantis* were required to be statistically superior to placebo in order to formally test all pairwise comparisons of *B. infantis* doses. Pairwise comparisons of each dose of *B. infantis* were conducted using the Wilcoxon Rank Sum test. Holm's procedure was applied to control the family-wise type I error rate (α) for multiple treatment comparisons and p values were adjusted based on three comparisons ($\alpha/3$, $\alpha/2$, and α) for placebo vs. each dose and each dose vs. another dose.

Differences in fecal *B. infantis* levels between treatment groups at Baseline were determined using Kruskal-Wallis. Differences in fecal *B. infantis* levels at all post-Baseline timepoints (other than Day 28) and total *Bifidobacterium* levels at all post-Baseline time points (Days 10, 14, 21, 28, 35, 42, 63) between treatment groups were determined using a one-way

ANOVA model using the ranked normal score of fecal *B. infantis* and total *Bifidobacterium* levels as the dependent variables. Pairwise comparisons of each *B. infantis* dose versus placebo were conducted using Wilcoxson rank sum test; unadjusted and Dunnett adjusted p-values were generated.

As a sensitivity analysis, estimates of treatment effect at Day 28 and across all post-Baseline timepoints based on missing at random assumption (MAR) for missing values were obtained from a mixed model for repeated measurements (MMRM), inclusive of values from all post-Baseline sampling time points and with Baseline as a covariate and factors for treatment, time and interaction of treatment by time. Due to concerns with the ability to satisfy the normality assumption, MMRM modeled the van der Waerden normal score transformed ranks of infant fecal *B. infantis* levels rather than actual values. Specifically, ranks were determined separately for all time points included, including Baseline, followed by van der Waerden normal score derived from the rank.

Differences in infant and maternal Baseline characteristics; infant weight (Baseline, Days 35, 63); sleep and crying hours at Baseline, during the Intervention period, and the change between the Baseline and Intervention period; and stool number, size, and consistency (Baseline, Days 35, 63) between all four treatment groups (placebo, low, medium, and high) were determined using Kruskal-Wallis and Wilcoxson rank sum test (placebo vs. all *B. infantis* doses combined). Pairwise comparisons were conducted using Wilcoxson rank sum test. Differences in the number of infant stools in each treatment group: placebo, low, medium, high, and EVC (all doses) across time were determined using the Friedman test.

Comparisons of the proportion of days infants in the placebo versus EVC experienced any of the following symptoms or conditions: cold, runny nose, or cough; fever at or above 103°C

blood in stool, prolonged abdominal bloating or straining, vomiting, and diaper rash were conducted using Wilcoxson rank sum test for the Intervention and Post-Intervention period. These data were binned across the three study time periods Baseline, Intervention, and Post-Intervention, means and proportions were calculated for continuous and categorical variables across each study period. Proportions for binary categorical variables were calculated as the number of days reported/total number of days in each study period. The calculated values were multiplied by 100 to generate percentages.

Results

Study Participation

Sixty-one mothers were screened for eligibility to participate in the study. Forty-one women met initial study criteria and were enrolled in the study, of which forty women were randomly assigned into one of four treatment groups as one participant withdrew from the study prior to randomization (**Figure 2.1**). Data for all randomized participants in each group (n = 10 per group) are reported except for the Intervention and Post-Intervention period for one participant who was enrolled into the medium dose (8.0 x 10^9 CFU/day) group and withdrew during the Intervention period. One participant in the high dose group reported giving her infant antibiotics during the Intervention period and was thus not included in the primary analysis. The overall attrition rate for this study was 5%, consistent with our previous probiotic study in healthy, breastfed, term infants (32). 97.5% of randomized participants consumed at least 21 once-daily servings of *B. infantis*. No infants consumed more than one dose per day.

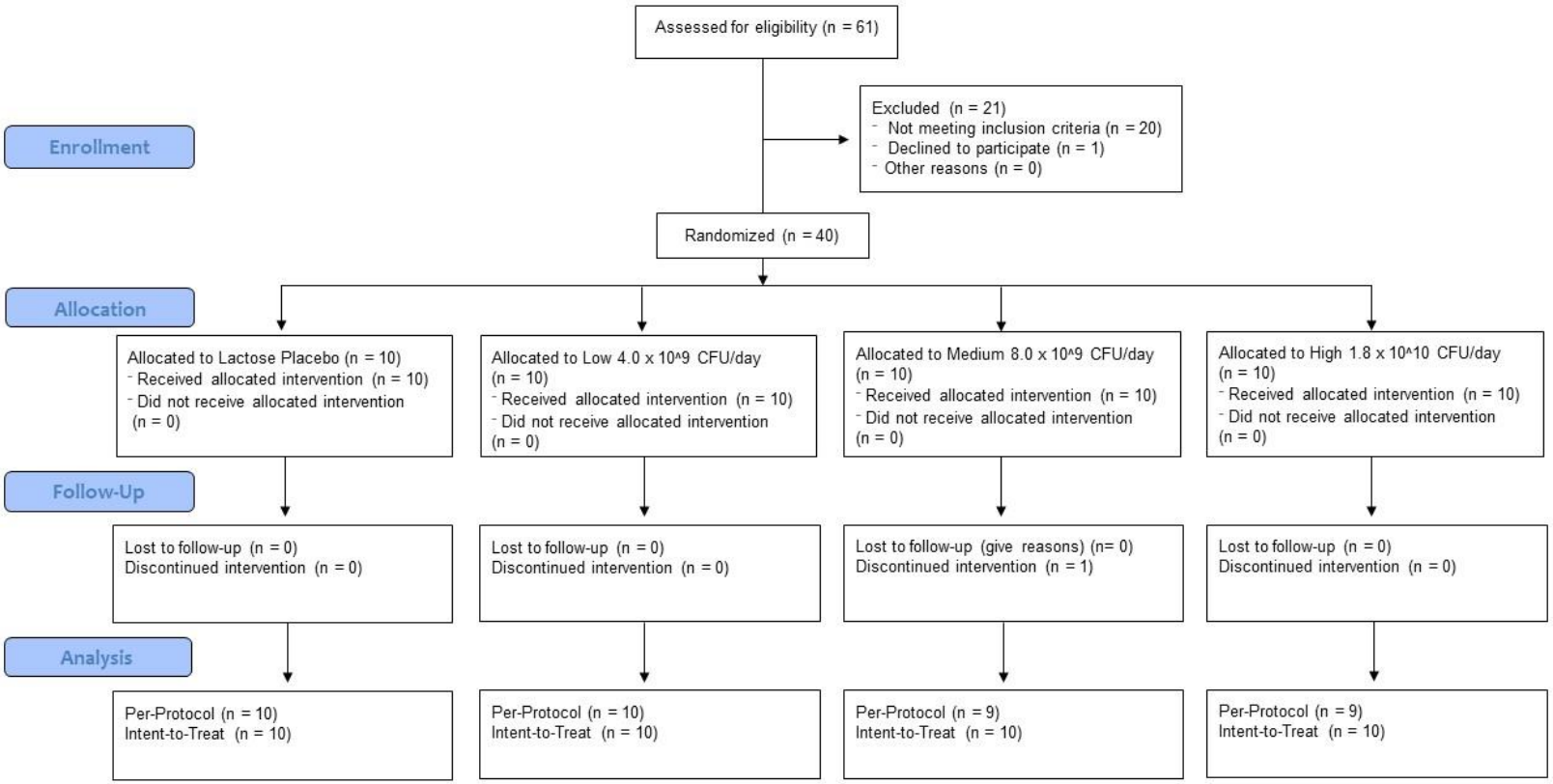


Figure 2.1. Consort diagram describing the number of participants who were screened, enrolled, randomized, or discontinued throughout the study period and the number of participants included in the Per-Protocol and Intent-to-Treat analyses.

Maternal Characteristics

Maternal age at enrollment, pre-pregnancy BMI, weight gain during pregnancy, number of times pregnant, number of live births, number of children living in the home, and number of hours in labor (**Table 2.1**) were not significantly different between treatment groups. Additional maternal Baseline characteristics can be found in **Supplemental Table 2.1**.

Table 2.1. Maternal Baseline Characteristics.

	0 CFU/day (*n* = 10)		4B CFU/day (*n* = 10)		8B CFU/day (*n* = 10)		18B CFU/day (*n* = 10)		EVC (*n* = 30)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Age at Enrollment (yr)	33.2	1.9	33.0	4.7	34.3	3.5	32.1	4.7	33.1	4.3
Ethnicity, *n* (%)										
Not Hispanic	9 (90%)		9 (90%)		10 (100%)		9 (90%)		28 (93.3%)	
Hispanic	1 (10%)		1 (10%)		0 (0%)		0 (0%)		1 (3.3%)	
Unsure	0 (0%)		0 (0%)		0 (0%)		1 (10%)		1 (3.3%)	
Race, *n* (%)										
Asian	1 (10%)		1 (10%)		2 (20%)		0 (0%)		3 (10%)	
Black or African American	0 (0%)		0 (0%)		0 (0%)		2 (20%)		2 (6.7%)	
White (including Middle Eastern)	8 (80%)		8 (80%)		6 (60%)		8 (80%)		22 (73.3%)	
2 or More Races	1 (10%)		1 (10%)		2 (20%)		0 (0%)		3 (10%)	
Education, *n* (%)										
Some College, No Degree	0 (0%)		1 (10%)		0 (0%)		2 (20%)		3 (10%)	
Associate degree	1 (10%)		0 (0%)		0 (0%)		1 (10%)		1 (3.3%)	
Bachelor's Degree (BA, BS, etc.)	5 (50%)		4 (40%)		4 (40%)		5 (50%)		13 (43.3%)	
Secondary Degree (MA, MS, MEng, MSW, etc.)	2 (20%)		5 (50%)		4 (40%)		0 (0%)		9 (30%)	
Professional or Doctorate (MD, DDS, JD, PhD, EdD, etc)	2 (20%)		0 (0%)		2 (20%)		2 (20%)		4 (13.3%)	
Marital Status, *n* (%)										

Married/ Couple	10 (100%)		9 (90%)		10 (100%)		10 (100%)		29 (96.7%)	
Never Married	0 (0%)		1 (10%)		0 (0%)		0 (0%)		1 (3.3%)	
Pre-Pregnancy BMI	26.3	4.4	25.1	4.8	24.9	5.1	26.7	5.0	25.6	4.8
Pregnancy Weight Gain (kg)	29.8	8.8	27.4	16.7	26.1	9.2	26.0	12.5	26.5	12.7
Hours in Labor (hr)	15.2	15.0	13.2	13.2	16.6	8.0	14.5	11.2	14.8	10.7
Number of Pregnancies	1.6	1.3	1.2	1.4	1.3	1.8	1.7	1.4	1.4	1.5
Number of Live Births	1.6	0.7	1.2	0.4	1.0	0.0	1.3	1.0	1.2	0.6
Number of Children	1.3	0.9	0.8	0.6	0.6	0.5	0.9	1.0	0.8	0.7
Parity, *n* (%)										
Primiparous	2 (20%)		4 (40%)		4 (40%)		3 (30%)		11 (36.7%)	
Multiparous	8 (80%)		6 (60%)		6 (60%)		7 (70%)		19 (63.3%)	
Delivery Location, *n* (%)										
Hospital or Birthing Center	8 (80%)		10 (100%)		10 (100%)		10 (100%)		30 (100%)	
Home Birth	2 (20%)		0 (0%)		0 (0%)		0 (0%)		0 (0%)	
Mode of Delivery, *n* (%)										
Vaginal	6 (60%)		7 (70%)		6 (60%)		8 (80%)		21 (70%)	
Vaginal Water Birth	2 (20%)		1 (10%)		2 (20%)		0 (0%)		3 (10%)	
C-section, Elective	0 (0%)		2 (20%)		2 (20%)		2 (20%)		6 (20%)	
C-section, Emergent	2 (20%)		0 (0%)		0 (0%)		0 (0%)		0 (0%)	

Infant Characteristics

Infant age at enrollment, birth weight, birth length and gender were not significantly different between treatment groups (**Table 2.2**). Infant gestational age at birth was significantly different between the placebo, low, medium and high dose groups (P <0.05) and between placebo and the EVC group (P <0.05)). Pairwise comparisons showed that there was a significant difference between the placebo (mean = 40.2 weeks) and low dose groups (mean = 38.9 weeks) (P <0.05). Differences in gestational age at birth are not biologically significant as all infants in this study were full-term. Infant weight was not significantly different between treatment groups at Study Days 7, 37 or 65 (**Supplemental Figure 2.1**). Additional infant Baseline characteristics can be found in **Supplemental Table 2.2**.

Table 2. Infant Baseline Characteristics.

Infant Baseline Characteristics	0 CFU/day (*n* = 10)		4B CFU/day (*n* = 10)		8B CFU/day (*n* = 10)		18B CFU/day (*n* = 10)		EVC (*n* = 30)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Gestational Age (wk)	40.2*	1.1	38.9	1.0	39.8	0.6	39.9	1.2	39.5	1.0
Birth Weight (g)	3454.4	410.0	3477.1	536.2	3468.6	394.7	3647.2	356.4	3530.9	428.9
Infant Birth Length (cm)	50.1 [3]	2.9	51.2	1.6	50.4	1.8	50.7	3.2	50.8	2.2
Age at Enrollment (day)	95.8	18.5	87.7	23.5	95.5	19.3	101.1	13.0	94.8	19.2
Infant Gender, *n* (%)										
Male	3 (30%)		4 (40%)		5 (50%)		3 (30%)		12 (40%)	
Female	7 (70%)		6 (60%)		5 (50%)		7 (70%)		18 (60%)	
Ethnicity, *n* (%)										
Not Hispanic	9 (90%)		9 (90%)		9 (90%)		8 (80%)		26 (86.7%)	
Hispanic	1 (10%)		1 (10%)		1 (10%)		1 (10%)		3 (10%)	
Unsure	0 (0%)		0 (0%)		0 (0%)		1 (10%)		1 (3.3%)	
Race, *n* (%)										
Asian	0 (0%)		1 (10%)		2 (20%)		0 (0%)		3 (10%)	
Black or African American	0 (0%)		0 (0%)		0 (0%)		0 (0%)		0 (0%)	
White (including Middle Eastern)	9 (90%)		7 (70%)		6 (60%)		6 (60%)		19 (63.3%)	
2 or More Races	1 (10%)		2 (20%)		2 (20%)		3 (30%)		7 (23.3%)	
Refuse	0 (0%)		0 (0%)		0 (0%)		1 (10%)		1 (3.3%)	

[1] *n* = 9, one participant did not report birth length

* Significant differences between placebo, low, medium, and high doses, and between placebo and EVC, $P < 0.05$ (Kruskal-Wallis). Pairwise comparisons showed a significant difference between the placebo and low dose, $P < 0.05$ (Wilcoxson rank sum).

Infant Diet

All women reported feeding their infant breast milk (at the breast or by bottle) and no one reported feeding their infant any amount of infant formula or non-study probiotics throughout the duration of the study (**Supplemental Table 2.3**). Two women in the high dose group reported feeding their infants solid food during the Post-Intervention period. One mother in the high dose group reported feeding her infant antibiotics during the Intervention and Post-Intervention periods.

Infant Gastrointestinal Health and Tolerability

The number of infant stools were not significantly different between treatment groups during Baseline, Intervention, and Post-Intervention. The number of infant stools was significantly different within the low dose group only across time (P <0.001) between the Baseline and Intervention (P <0.05) and the Baseline and Post-Intervention (P <0.01), however, after Bonferroni correction, only Baseline and Post-Intervention were statistically different from each other within the low dose group (P <0.01). The number of infant stools was significantly different within the EVC group across time (P <0.0005) between the Baseline and Intervention (P <0.0005), Baseline and Post-Intervention timepoints (P <0.0005), and Intervention and Post-Intervention timepoints (P <0.0005). After Bonferroni correction, only the Baseline and Intervention (P <0.001) and Baseline and Post-Intervention timepoints (P <0.0005) were significantly different from each other within the EVC group (**Figure 2.2A, Figure 2.2B, Supplemental Table 2.3**).

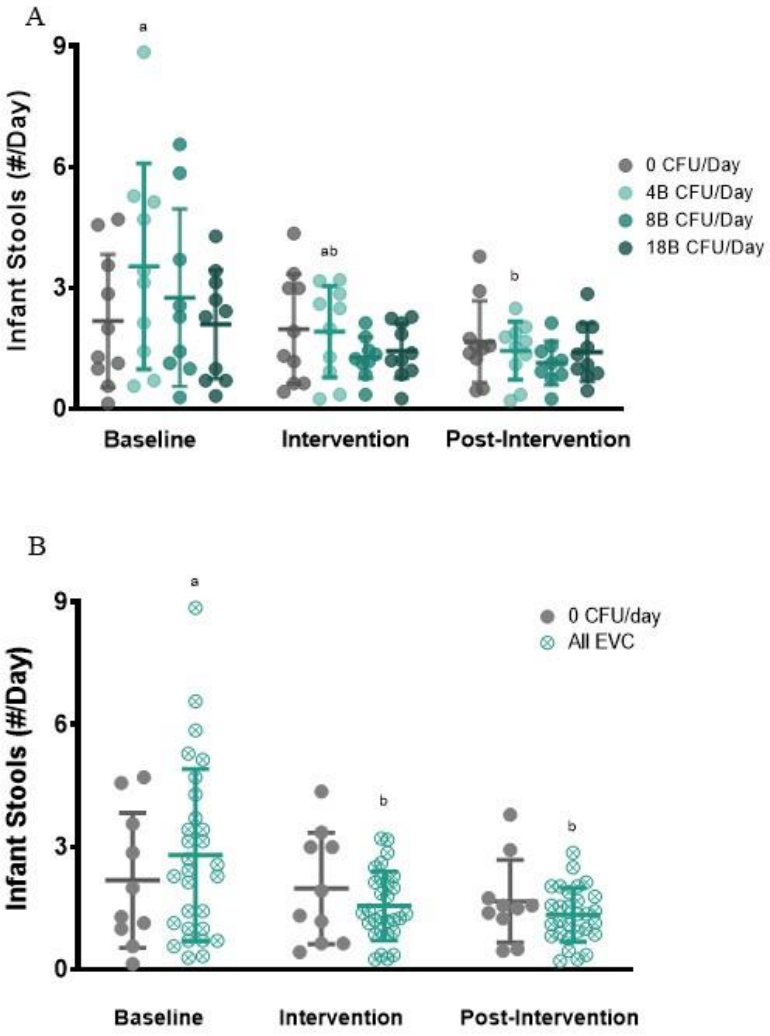


Figure 2.2. Infant stool number. A: Across time and inclusive of all dose groups, Bonferroni adjusted $P < 0.01$ for differences between Baseline and Post-Intervention within the low dose (4B CFU/day) group (Friedman test). B: Across time and among placebo and EVC (all doses combined). Bonferroni adjusted $P < 0.001$ for differences between Baseline and Intervention and Bonferroni adjusted $P < 0.0005$ for differences between Baseline and Post-Intervention within the EVC groups (Friedman test).

Maternal reports of stool consistency as the proportion of watery, formed, soft or hard stools during the Baseline, Intervention, and Post-Intervention periods were not significantly different between treatment groups (**Supplemental Figure 2.2, Supplemental Table 2.3**). Maternal reports for the size of stools as the proportion of stools that were a smear on the infant's diaper, measured up to 25% of the diaper, 25-50% of the diaper, or >50% of the diaper during the Baseline, Intervention, and Post-Intervention periods were not significantly different between treatment groups (**Supplemental Figure 2.3, Supplemental Table 2.3**).

The proportion of infants who experienced a cold, runny nose, or cough, fever at or above 103°C blood in stool, prolonged abdominal bloating or straining, vomiting, and diaper rash were not significantly different between the placebo and EVC group during the Intervention or Post-Intervention periods (**Supplemental Table 2.3**).

Infant Sleep and Crying

Maternal reports for the hours their infant spent sleeping or crying/fussing were not significantly different between treatment groups at Baseline or during the Intervention period. The change in hours spent sleeping or crying/fussing from Baseline was not significantly different between treatment groups (**Supplemental Table 2.4**).

Changes in the Fecal Microbiome

There was no significant difference between fecal *B. infantis* levels between treatment groups at Baseline.

There was a significant difference in fecal *B. infantis* levels at Day 28 between treatment groups (all doses) and placebo (P <0.01) (**Figure 2.3**). Fecal *B. infantis* levels in the low, medium, and high dose groups were significantly different from placebo at Day 28 (Holm's adjusted: Low: P <0.01, Medium P <0.01, High P <0.01). There were no differences in fecal *B. infantis* levels at

Day 28 between *B. infantis* EVC001 doses. The MMRM confirmed these findings (*P* 0.001-0.01). There were significant differences in the ranks of fecal *B. infantis* levels between treatment groups (all doses) and placebo at Day 10 (*P* <0.0005), Day 14 (*P* <0.0005), Day 21 (*P* <0.0005), Day 35 (*P* <0.0005), Day 42 (*P* <0.0005) and Day 63 (*P* <0.0005) (**Figure 2.4**). Pairwise comparisons identified significant differences in fecal *B. infantis* levels after Dunnett adjustment between the low, medium, and high doses compared with placebo at all post-Baseline timepoints (Low: *P* <0.0005, Medium <0.0005, High *P* <0.0005). The MMRM confirmed these findings (*P* < 0.0001).

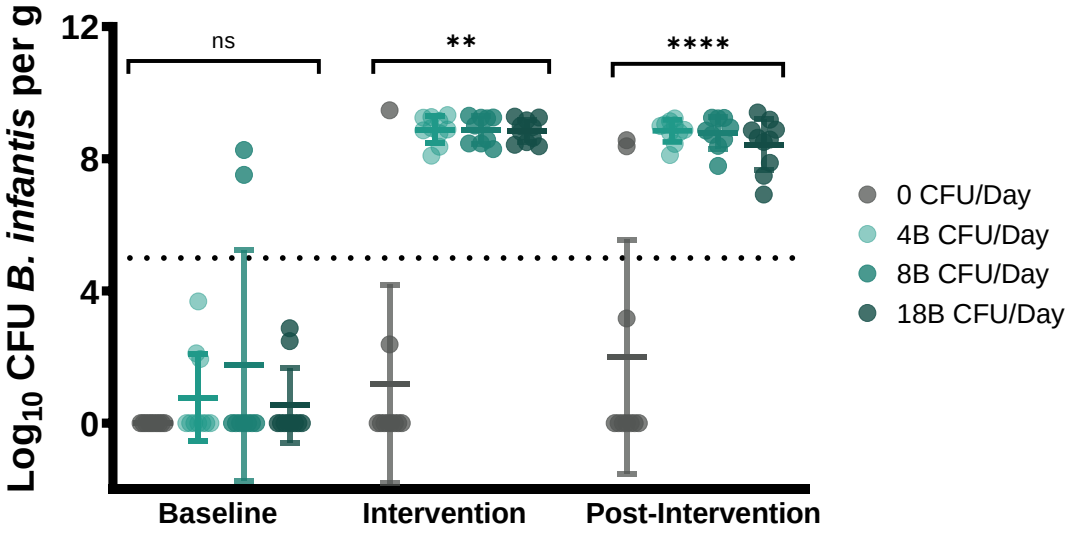


Figure 2.3. Infant fecal *B. infantis* at Baseline, Intervention, and Post-Intervention periods between treatment groups. ns = not significant, ** *P* < 0.01, **** *P* < 0.0005 for differences between treatment groups at Baseline (Day 3), Intervention (Day 28) and Post-Intervention (Day 63). Differences at Day 3 and Day 28 were determined using Kruskal-Wallis and pairwise comparisons at Day 28 showed that each dose was significantly different from placebo (*P* <

0.01). Differences at Day 63 were determined by an ANOVA model and pairwise comparisons showed that each dose was significantly different from placebo ($P < 0.0005$).

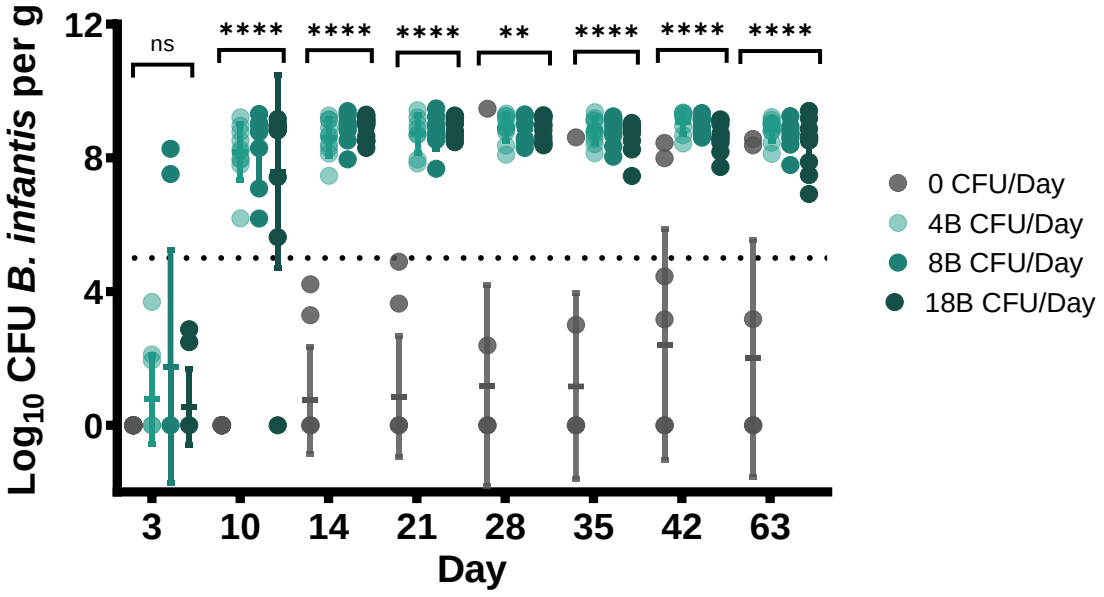


Figure 2.4. Infant fecal *B. infantis* across time and between treatment groups. ns = not significant, ** $P < 0.01$, **** $P < 0.0005$ for differences between treatment groups at each time point. Differences among all post-baseline timepoints (other than Day 28) were determined by an ANOVA model and pairwise comparisons showed that each dose was significantly different from placebo ($P < 0.0005$). Differences at Day 3 and Day 28 were determined using Kruskal-Wallis and pairwise comparisons at Day 28 showed that each dose was significantly different from placebo ($P < 0.01$).

There were significant differences in the ranks of fecal *Bifidobacterium* levels between treatment groups at Day 10 (P <0.01), Day 14 (P <0.01), Day 21 (P <0.05), Day 28 (P <0.05), Day 35 (P <0.001), and Day 42 (P <0.0005) (**Figure 2.5**). After Dunnett adjustment, pairwise comparisons showed that at Day 63, there was no significant difference in fecal *Bifidobacterium*

levels between treatment groups. At Day 10, there were significant differences between the low dose (P<0.01) and the high dose (P<0.01) when compared with placebo. At Day 14, there were significant differences between the low (P<0.01), medium (P<0.01), and high (P<0.01) doses when compared with placebo. At Day 21, there were significant differences between the medium (P<0.05) and high (P<0.05) doses when compared with placebo. At Day 28, there was a significant difference between the medium (P<0.05) dose and placebo. At Day 35, there were significant differences between the low (P<0.05), medium (P<0.001), and high (P<0.01) doses when compared with placebo. At Day 42, there were significant differences between the low (P<0.0005) and medium (P<0.01) doses when compared with placebo. The MMRM confirmed these findings (P 0.0001-0.01).

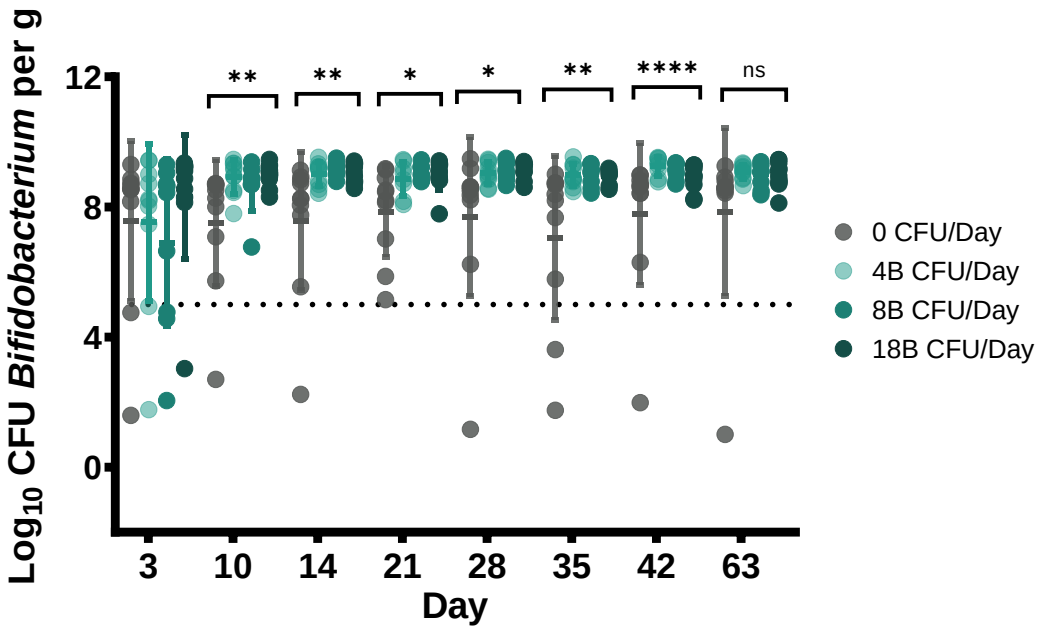


Figure 2.5. Total fecal *Bifidobacterium* across time and among treatment groups. * P < 0.05, ** P < 0.01, **** P < 0.0005, ns = not significant for differences in the ranks between treatment groups at each time point as determined by an ANOVA model and pairwise comparisons showed

significant differences between placebo and EVC doses ($P < 0.0005$ - .05) at Days 10, 14, 21, 28, 35, and 42).

To further investigate how *B. infantis* supplementation influences the intestinal microbial composition at each time point, we used MaAsLin2, to determine if treatment altered intestinal microbial taxa. Infants in the combined EVC group had significantly higher *Bifidobacteriaceae* at Day 28 (R = 0.15, FDR-adjusted Q value = 0.02) and Day 63 (R = 0.13, FDR-adjusted Q value = 0.03), *Enterococcaceae* at Day 28 (R = 0.08, FDR-adjusted Q value = 0.16) and Day 63 (R = 0.06, FDR-adjusted Q value = 0.20), and *Coriobacteriaceae* at Day 63 (R = 0.03, FDR-adjusted Q value = 0.20) and significantly lower *Ruminococcaceae* at Day 3 (R = -0.07, FDR-adjusted Q value = 0.20) and Day 28 (R = -0.06, FDR-adjusted Q value = 0.21) and *Erysipelotrichaceae* at Day 28 (R = -0.09, FDR-adjusted Q value = 0.20) and 63 (R = -0.11, FDR-adjusted Q value = 0.11) compared to placebo.

Supplementation resulted in increased levels of fecal *Bifidobacteriaceae* and *Entercoccaceae,* and decreased *Ruminococcaceae* in the intervention period (Day 28) and increased *Bifidobacteriaceae* and decreased *Erysipelotrichaceae* in the post-intervention period (Day 63) compared to baseline **Table 2.3**.

Table 2.3. Infant fecal microbial families measured by 16s rRNA amplicon sequencing.

		% mean relative abundance (SD) in study group				
Study Day	Family	Placebo	Low	Medium	High	EVC
3	*Bifidobacteriaceae*	20.98 (17.23)	27.37 (29.85)	21.47 (29.21)	34.57 (22.70)	28.02 (26.93)
	Bacteroidaceae	34.77 (24.63)	20.24 (26.54)	16.08 (18.96)	18.21 (22.17)	18.25 (22.14)
	Clostridiaceae	2.52 (3.69)	4.89 (6.33)	10.86 (21.18)	3.11 (7.48)	6.13 (13.04)
	Enterobacteriaceae	20.43 (18.27)	25.14 (13.20)	27.51 (22.43)	21.22 (13.32)	24.52 (16.24)
	Enterococcaceae	0.23 (0.36)	0.20 (0.34)	0.27 (0.52)	1.20 (2.14)	0.57 (1.35)
	Lachnospiraceae	4.92 (6.06)	4.36 (6.19)	3.84 (5.93)	5.47 (8.59)	4.58 (6.82)
	Streptococcaceae	0.34 (0.59)	0.73 (1.23)	2.90 (5.28)	0.50 (1.05)	1.32 (3.16)
	Veillonellaceae	2.07 (2.69)	5.83 (10.63)	1.61 (1.53)	4.62 (6.83)	4.10 (7.43)
	Other bacteria	13.96 (14.59)	11.44 (15.60)	15.75 (18.28)	12.29 (9.38)	13.07 (14.33)
10	*Bifidobacteriaceae*	19.01 (17.54)	43.99 (25.53)	40.97 (23.35)	47.08 (18.42)	44.12 (21.92)
	Bacteroidaceae	30.04 (21.67)	15.27 (20.63)	19.51 (21.17)	17.87 (17.05)	17.48 (19.01)
	Clostridiaceae	11.30 (21.48)	3.88 (8.10)	0.62 (1.30)	0.67 (1.47)	1.76 (4.97)
	Enterobacteriaceae	19.62 (15.26)	19.13 (14.25)	24.44 (24.84)	16.25 (15.83)	19.79 (18.27)
	Enterococcaceae	0.29 (0.52)	0.59 (0.91)	0.88 (1.48)	1.22 (1.78)	0.90 (1.41)
	Lachnospiraceae	5.28 (6.19)	2.39 (4.29)	1.85 (2.37)	2.34 (3.29)	2.21 (3.33)
	Streptococcaceae	0.32 (0.58)	2.87 (7.58)	0.84 (0.80)	1.76 (2.94)	1.86 (4.70)
	Veillonellaceae	1.23 (1.44)	4.44 (8.53)	4.48 (10.49)	4.31 (7.27)	4.41 (8.47)
	Other bacteria	12.92 (16.11)	7.43 (7.73)	6.41 (11.64)	8.50 (8.08)	7.48 (8.93)
14	*Bifidobacteriaceae*	20.54 (15.31)	54.42 (19.51)	63.80 (18.25)	51.13 (21.04)	55.93 (19.71)
	Bacteroidaceae	32.84 (21.27)	14.85 (24.01)	12.94 (14.19)	23.66 (24.57)	17.45 (21.64)
	Clostridiaceae	10.05 (18.36)	1.46 (2.53)	0.40 (0.76)	0.15 (0.28)	0.69 (1.63)
	Enterobacteriaceae	19.01 (13.30)	15.26 (8.89)	9.97 (5.55)	11.16 (7.11)	12.28 (7.52)

	Enterococcaceae	0.06 (0.11)	0.66 (1.26)	1.21 (1.27)	1.28 (1.05)	1.04 (1.18)
	Lachnospiraceae	4.43 (7.98)	1.58 (2.58)	1.96 (3.02)	1.11 (2.07)	1.52 (2.48)
	Streptococcaceae	0.83 (1.35)	1.91 (2.78)	1.00 (1.06)	1.34 (2.34)	1.45 (2.20)
	Veillonellaceae	0.68 (0.72)	2.89 (5.05)	4.01 (5.89)	2.68 (2.57)	3.14 (4.47)
	Other bacteria	11.56 (14.19)	6.96 (7.53)	4.70 (6.82)	7.49 (8.10)	6.50 (7.36)
	Bifidobacteriaceae	22.44 (18.80)	51.61 (25.09)	58.44 (19.38)	54.26 (19.49)	54.64 (20.97)
	Bacteroidaceae	32.84 (22.82)	13.72 (20.89)	14.12 (17.42)	19.48 (17.57)	15.83 (18.26)
	Clostridiaceae	10.04 (21.76)	1.52 (2.62)	0.44 (0.94)	0.89 (2.00)	0.97 (1.99)
	Enterobacteriaceae	16.11 (7.64)	18.85 (16.75)	12.21 (6.49)	11.07 (5.25)	14.11 (11.12)
21	*Enterococcaceae*	0.57 (1.23)	0.57 (1.00)	2.72 (3.53)	1.39 (1.34)	1.52 (2.29)
	Lachnospiraceae	5.92 (7.82)	1.56 (2.69)	1.13 (1.79)	1.56 (2.17)	1.43 (2.19)
	Streptococcaceae	1.79 (2.87)	0.89 (1.65)	1.13 (1.26)	1.91 (2.40)	1.31 (1.84)
	Veillonellaceae	0.55 (0.68)	4.11 (6.07)	4.56 (6.31)	2.19 (1.86)	3.59 (5.04)
60	Other bacteria	9.75 (12.18)	7.17 (8.27)	5.25 (10.21)	7.23 (8.78)	6.59 (8.80)
	Bifidobacteriaceae	23.55 (28.41)	52.99 (20.49)**	58.43 (21.69)**	46.93 (17.07)**	52.59 (19.64) ****
	Bacteroidaceae	29.49 (23.02)	14.86 (21.31)	13.00 (20.02)	18.51 (20.58)	15.54 (20.05)
	Clostridiaceae	4.79 (7.94)	2.05 (3.61)	1.09 (2.08)	4.43 (7.10)	2.58 (4.86)
	Enterobacteriaceae	21.40 (18.52)	16.09 (13.00)	12.57 (7.31)	11.51 (4.03)	13.42 (8.88)
28	*Enterococcaceae*	0.18 (0.28)	1.61 (1.71)*	1.44 (1.46)*	2.28 (4.07)*	1.79 (2.65)**
	Lachnospiraceae	5.80 (8.75)	1.37 (2.44)	1.99 (2.76)	1.77 (2.85)	1.70 (2.60)
	Streptococcaceae	0.25 (0.62)	1.17 (1.53)	1.06 (1.08)	0.52 (0.74)	0.91 (1.16)
	Veillonellaceae	1.46 (2.40)	4.09 (5.10)	4.03 (5.64)	5.81 (6.31)	4.66 (5.56)
	Other bacteria	13.06 (13.50)	5.77 (7.12)	6.38 (8.83)	8.24 (6.81)	6.81 (7.39)
	Bifidobacteriaceae	17.24 (14.30)	54.45 (19.60)	50.97 (22.51)	43.07 (10.42)	49.44 (18.09)
	Bacteroidaceae	36.84 (25.57)	15.98 (21.77)	20.05 (26.53)	21.57 (21.74)	19.17 (22.61)
35	*Clostridiaceae*	6.53 (11.78)	1.41 (2.96)	0.95 (1.20)	1.91 (2.82)	1.44 (2.44)
	Enterobacteriaceae	17.23 (16.26)	13.46 (8.40)	14.56 (10.98)	12.30 (6.31)	13.40 (8.41)

	Enterococcaceae	0.53 (0.96)	1.25 (1.40)	1.51 (1.75)	2.54 (2.82)	1.78 (2.09)
	Lachnospiraceae	6.37 (9.59)	1.17 (1.82)	1.51 (2.15)	2.12 (2.65)	1.61 (2.19)
	Streptococcaceae	0.93 (2.52)	1.25 (1.79)	0.93 (1.16)	1.48 (2.08)	1.23 (1.69)
	Veillonellaceae	1.66 (1.68)	4.31 (5.64)	3.63 (5.20)	5.32 (4.60)	4.45 (5.03)
	Other bacteria	12.67 (14.34)	6.71 (9.98)	5.89 (7.79)	9.68 (6.66)	7.48 (8.15)
	Bifidobacteriaceae	23.74 (16.63)	61.67 (17.95)	55.46 (22.59)	46.26 (13.83)	54.43 (18.81)
	Bacteroidaceae	32.42 (23.81)	10.01 (15.42)	12.36 (20.67)	19.12 (22.46)	13.88 (19.41)
	Clostridiaceae	4.96 (12.72)	1.26 (1.79)	1.34 (2.02)	1.36 (3.11)	1.32 (2.30)
	Enterobacteriaceae	17.34 (15.91)	11.03 (5.38)	11.04 (3.55)	12.27 (7.68)	11.46 (5.68)
42	*Enterococcaceae*	0.35 (0.46)	0.90 (1.05)	1.30 (1.14)	2.39 (2.42)	1.53 (1.74)
	Lachnospiraceae	8.09 (11.95)	1.75 (3.20)	1.38 (2.82)	1.54 (1.93)	1.56 (2.61)
	Streptococcaceae	0.32 (0.68)	0.99 (1.07)	0.59 (0.68)	0.42 (0.63)	0.67 (0.83)
	Veillonellaceae	1.21 (1.87)	4.12 (6.77)	3.85 (4.65)	5.35 (4.59)	4.46 (5.30)
	Other bacteria	11.57 (13.43)	8.27 (10.78)	12.68 (18.24)	11.29 (7.30)	10.68 (12.37)
	Bifidobacteriaceae	25.54 (15.25)	50.40 (15.17)**	47.08 (19.45)*	51.35 (23.20)**	49.70 (18.93)**
	Bacteroidaceae	34.78 (24.47)	13.89 (19.44)	17.85 (24.06)	18.25 (21.28)	16.62 (20.90)
	Clostridiaceae	1.09 (2.03)	1.71 (2.23)	1.72 (2.35)	0.79 (2.09)	1.40 (2.18)
	Enterobacteriaceae	13.89 (8.82)	17.43 (10.10)	15.95 (10.32)	13.06 (14.04)	15.46 (11.40)
63	*Enterococcaceae*	0.53 (1.09)	1.64 (1.84)	1.24 (1.43)	0.98 (1.01)	1.29 (1.44)
	Lachnospiraceae	9.05 (12.88)	0.75 (1.22)	1.63 (2.36)	2.15 (2.73)	1.51 (2.20)
	Streptococcaceae	0.21 (0.40)	0.46 (0.72)	0.88 (1.09)	2.15 (2.68)	1.17 (1.84)
	Veillonellaceae	4.67 (9.94)	6.17 (6.38)	4.58 (4.21)	2.53 (2.56)	4.42 (4.76)
	Other bacteria	10.24 (11.07)	7.56 (8.96)	9.08 (13.62)	8.73 (8.89)	8.43 (10.23)

* $P < 0.05$, ** $P < 0.01$, ****$P < 0.0005$ for differences compared to placebo as determined by Wilcoxson rank sum test

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Discussion

The infant intestinal microbiome undergoes a profound change in diversity and stability in the first few months of life becoming more complex over time, reaching an adult-like configuration by age one year (1, 36). While we have previously demonstrated that *B. infantis* supplemented to breastfed infants within the first week of life are able to persistently change the intestinal microbiome one month and up to one year postnatal (33, 34), no studies have shown this effect in older infants who are thought to have a more stable microbiome. Therefore, this study was designed to determine the effect of a *B. infantis* probiotic supplementation in exclusively breastfed infants aged 2-4 months on fecal *B. infantis* levels and to determine the minimally effective dose required to significantly increase fecal *B. infantis.*

The current study showed that supplementation of *B. infantis* EVC001 for 28 day significantly increased fecal *B. infantis* levels in 2–4-month-old infants compared to Baseline and led to persistent colonization one-month post-supplementation. It has been reported, particularly in heathy adults, that persistent colonization following the intake of probiotics is limited and probiotics may have minimal effect on the overall intestinal microbial composition (54, 97-99). Evidence has emerged that the infant gut is likely more susceptible to colonization with probiotic bacteria because their intestinal microbiome is less stable and complex (1, 33, 36, 93). To date, only a paucity of studies in preterm and term infants show colonization of the gut with probiotic supplements containing different species of *Bifidobacterium* between weeks to up to five months postnatal (100-102). To our knowledge, this is the first study investigating the effect of probiotic supplementation in older infants who have a more mature and stable intestinal microbiome compared to preterm or term newborns (1, 36). In this study, all infants were exclusively breastfed

throughout the entire study period supporting the hypothesis that the persistent colonization we observed is largely due to the unique ability of *B. infantis* to utilize HMOs.

All supplement doses (10^9 to 10^{10} cfu/day) studied increased fecal *B. infantis* levels significantly when compared with placebo, however, there was no differences in fecal *B. infantis* levels between the low, medium and high dose groups. These results are consistent with Petschow et al (2009) who found that Lactobacillus GG (LGG) was able to transiently colonize the gut of infants who consumed formula containing 10^8 to 10^{10} cfu/day LGG for 2 weeks, regardless of the dose (103). By contrast, fecal recovery of probiotics appears to be dose-dependent in adults at doses ranging from 10^8 to 10^{11} cfu/day (104-108). Together, these results suggest that lower doses of probiotics can lead to colonization in infants compared to adults because the adult intestinal microbiome is more stable compared to infants. Thus, higher doses are likely needed in adults before any changes in microbial composition are observed (109).

The EVC (all doses) group had significantly higher *Bifidobacteriaceae* and *Enterococcaceae* and significantly lower *Ruminococcaceae* and *Erysipelotrichaceae* after *B. infantis* supplementation compared to placebo. These taxa differ in their preferences for substrates, metabolism of their preferred substrates into end-products, and their consequent biochemical effects in the gut and on infant health. For example, *Enterococcaceae* may play a role in lipid metabolism; previous studies have demonstrated that *Erysipelotrichaceae* are associated with inflammatory and metabolic disorders in animal models and human studies and are considered highly immunogenic (69, 110-114). Lin et al (2021) found that infants born to obese or overweight mothers had increased *Erysipelotrichaceae* compared to infants born to mothers with a normal BMI and hypothesized that these infants may have an increased risk of weight gain or metabolic disease (115). *Ruminococcaceae* may play a role in the development of food allergies and

sensitization, however results are inconsistent among studies. It has been reported that infants with cow milk allergy are significantly enriched for *Ruminococcaceae* and have a reduction in *Bifidobacteriaceae* (116) and, after a 6-month intervention with hypoallergenic formula, infants with cow milk allergy had significantly lower *Ruminococcaceae* compared with controls (117). Conversely, Azad et al (2015) found that at age one year, food sensitized infants had decreased abundance of *Ruminococcaceae* (118). Additionally, as infants age, the microbiome shifts from communities enriched in *Bifidobacteraceae, Enterococcaceae, Enterobacteraceae, Streptococcaceae, Lactobacillaceae,* and *Clostridiaceae* to communities enriched in *Ruminococcaceae, Bacteroidaceae, Prevotellaceae,* and *Lachnospiraceae,* indicating infants in the placebo group may have a more mature, adult-like microbiome compared to breastfed infants who were supplemented with *B. infantis* EVC001 (119).

Probiotic intake in pre-term and term infants is reported to be well tolerated (32, 120-122). We found no differences in the number of stools; stool size or consistency; number of sleep or crying hours; incidences of cold, runny nose, or cough; fever at or above 103°C; blood in stool; prolonged abdominal bloating or straining; vomiting; or diaper rash between treatment groups (all doses) compared with placebo, indicating that all *B. infantis* EVC001 doses were well tolerated in this study. These data are consistent Smilowitz et al. that showed that supplementation of the high dose of *B. infantis* EVC001 was well-tolerated and safe in term, breastfed newborns (32). For infants receiving any dose of *B. infantis* EVC001, the number of infant stools during the Intervention and Post-Intervention periods were significantly lower when compared to Baseline. These data are consistent with supplementation of the high dose *B. infantis* EVC001 in term, breastfed newborns whose fecal HMOs were reduced by 10-fold (33) and stool number decreased by 40% (32). *B. infantis* metabolizes HMOs into organic acids lowering intestinal pH, which is

correlated to decreased abundance of potentially harmful bacterial populations such as *Enterobacteriaceae, Clostridiaceae, Peptosteptoccocaceae*, and *Veillonellaceae* within the gut of infants (50, 123). Decreased fecal pH in infants has also been associated with markers of immune system development which may explain why infants colonized with *B. infantis* have reductions in the incidence of autoimmune diseases and better vaccine response (50, 60, 123-125). While we did not directly measure fecal pH within this study, the increased *B. infantis* levels we observed in the low medium and high *B. infantis* EVC001 dose groups suggest increased utilization of HMOs and increased production of organic acids in the guts of supplemented infants.

Early in life, the infant intestinal microbiome is highly variable between individuals and across time (126, 127). In this study, fecal *B. infantis* levels in the placebo group were highly variable throughout the duration of the study. Interestingly, there were several infants in the placebo group who, while having low levels of fecal *B. infantis* at Baseline, had increased *B. infanti*s levels during the Intervention period that were maintained throughout the Post-Intervention period. One mother-infant pair in this group who fecal *B. infantis* levels increased during the Intervention period spent a significant amount of time with a mother-infant pair in the medium dose group suggesting that horizontal transmission between infants is possible. Additionally, in the medium dose group, two infants had high levels of fecal *B. infantis* at Baseline that could not be readily explained. We hypothesize that these high levels of *B. infantis* at Baseline may be due to vertical transmission of *B. infantis* from mother to infant during delivery or horizontal transmission from other infants or siblings with high levels of *B. infantis*. Both *Bifidobacterium* breve and total bifidobacterial counts in maternal feces have been associated with increases in number of different bifidobacterial species and total bifidobacterial counts in infant feces (128, 129). One mother took a probiotic supplement during pregnancy and throughout the

course of the study and maternal intake of probiotic supplements has been shown to influence the infant intestinal microbiome. In particular, Lahtinen et al (2009) found that maternal intake of a probiotic supplement containing LGG during late pregnancy led more colonized with species of the *B. longum* group in infants (130). Additionally, sibling presence in the home is known to influence the composition of the infant microbiome and several studies have demonstrated that infants with older siblings are more likely to have a *Bifidobacterium* dominant fecal profile compared to infants without siblings in the home (131-135).

One infant in the EVC group consumed cephalosporin antibiotics between study Day 31-38 and *Bifidobacteria* species are susceptible to cephalosporins (136, 137). In this infant, fecal *B. infantis* levels decreased on Day 35 and rebounded on Day 45, one week after antibiotic treatment was complete. It is likely that the continued consumption of the EVC001 *B. infantis* supplement until Day 35 enabled *B. infantis* to persist in the gut and reestablish once treatment was completed. Although fecal *B. infantis* levels declined in this infant on Day 65 to Day 35 levels, levels remained high when compared to the placebo group. Taking probiotics and antibiotics simultaneously is likely beneficial as probiotics have shown to be effective at preventing antibiotic associated diarrhea in both children and adults (138-141). In very low birth weight infants who received post-natal antibiotics, probiotics have shown to be beneficial; infants who consumed *Lactobacillus acidophilus* or *B. infantis* probiotics following antibiotic treatment showed increased growth rates compared to infants who did not receive probiotics (142).

This study was designed to explore the effects of a daily probiotic supplement on the microbial composition in infants 2-4 months old and to determine the minimally effective dose to increase fecal *B. infantis* levels above Baseline. This was a randomized, double-blind, placebo-controlled trial that followed infants for one-month post-supplementation which allowed us to

determine persistence of *B. infantis* after supplementation ceased. One limitation is that this study included a small number of participants per group (n = 10) and was not powered to determine the effect of the probiotic supplement on other outcomes such as stooling patterns and health conditions. Additionally, the study followed participants for only one month after they stopped taking the supplement, thus, we do not know of the observed changes in microbial composition will persist long term. Lastly, this study only consisted of infants who were exclusively breastfed and future studies should focus on whether *B. infantis* is capable of colonizing the gut of infants fed formula that contains minimal amounts of HMOs when compared to breast milk.

In conclusion, the findings of this study demonstrated that supplementation with *B. infantis* EVC001 for 28 days in 2–4-month-old, exclusively breastfed infants across three dosing regimens was well tolerated and resulted in increased fecal *B. infantis* levels that persisted to at least one-month post-supplementation. All doses studied significantly increased fecal *B. infantis* levels above Baseline in older infants who have a more stable microbiome than newborns.

Chapter 3

Introduction of Solid Foods in Six-Month-Old Exclusively Breastfed Infants Leads to Changes in Microbial Metabolism

Introduction

Microbial composition and their functions in the gut are driven by the combination of bacterial inoculation and substrate availability. The first major intestinal colonization event occurs at birth and is heavily dependent on mode of delivery (5, 143) and diet (breastfed vs. formula-fed) (1, 10, 144). It is well documented that exclusively breastfed infants have lower diversity but higher levels of specific taxa from the protective bacterial class Actinobacteria compared with formula-fed infants who have higher microbial intestinal diversity but also higher levels of the pro-inflammatory bacterial class γ-Proteobacteria (1, 9-11). Additionally, breast milk delivers in large amounts (~10-20 g/L), human milk oligosaccharides (HMOs) that are non-digestible by the infant but selectively support the competitive growth of protective bifidobacterial species and subspecies within the intestine of the breastfed infant (12-16). The genus *Bifidobacterium* has historically dominated in the gut of breastfed infants until weaning (1, 21-23).

The second critical period in life when the intestinal microbiome dramatically shifts is during the introduction of complementary foods (1, 145). Weaning is a time of low diversity in the intestinal microbiota that is particularly responsive to the introduction of solid foods; thus, providing an opportunity to identify changes to gut microbial functions. In addition, the transition from breastfeeding to solid food represents an easy demarcation from which to compare a specific introduced food substrate—a result that is not easily witnessed in adult populations where

enterotypes (43) and other more nuanced assemblages can result in responders and non-responder populations (146). Finally, and most importantly, numerous studies have shown that the microbiota adopted during weaning can persist into adult life and; thus, these early microbiota interactions are tremendously important in setting the stage for health and/or disease in later life (41, 42).

During the weaning period, the introduction of solid foods drives a change in the intestinal microbial composition and their functions. While breast milk is still the primary source of nutrition in early weaning, species of *Bifidobacterium* that utilize HMOs remain dominant (1, 35). As complex plant-derived carbohydrates are introduced, increases in butyrate producing bacteria such as *Clostridium leptum*, *Eubacterium halli*, and the genus *Roseburia* s as well as the enrichment of genes associated with metabolism of plant-derived polysaccharides are observed, indicating an increased functional capacity for carbohydrate utilization within the microbiome (23, 35, 37, 147). Fermentation products of microbial metabolism, such as lactate, acetate, and butyrate, help to maintain a lower intestinal pH, support transport of these metabolites into the intestinal epithelium for use by the host, create an undesirable environment for potential pathogens, upregulate intestinal barrier function, and inhibit proinflammatory apoptotic responses (19, 20, 48, 85, 148-151). Recently, Henrick et al (2021) demonstrated that indole-3-lactic acid educates the immune system in infants by upregulating inhibitory galectin-1 in T cells in early life indicating a link between microbial metabolism and immune regulation (31).

Once breast milk intake ceases, the microbiota becomes more adult-like, shifting towards the bacterial phyla *Bacteriodetes* and *Firmicutes* (37). Specifically, several studies have observed an increase in *Clostridium*, *Akkermansia*, *Bacteroides*, *Lachnospiraceae* and *Ruminococcus* and a decrease in *Bifidobacterium*, *Lactobacillus*, and *Enterobacteriaceae* (23, 35, 152).

The U.S. Dietary Guidelines for 2020-2025 recommend introducing a variety of complementary foods to infants one at a time including infant cereals, meat or other proteins, fruits, vegetables, grains, yogurts and cheeses, and more beginning at approximately 6 months of age (153). Plant-derived complementary foods are particularly commonly used during weaning. These foods contain diverse and complex carbohydrates that cannot be broken down by humans and are uniquely suited to influence the development of the microbiome as microbial species differ in their glycan preferences (38-40). A deeper understanding of the carbohydrate-microbe interactions among the keystone colonizers in the weaning gut is critical to deduce the fundamental glycan-microbe relationships that persist into adulthood.

The introduction of different complementary foods during the infant's weaning period may either foster or disrupt the bacteria composition and functions in the intestine producing unknown outcomes. A gap in the scientific literature exists in identifying markers of a healthy microbiome trajectory uniquely linked to the specific weaning food. An accumulating knowledge base of specific food-microbiome responses will drive development of dietary strategies linked to healthy outcomes and simultaneously profile how specific weaning foods are differentially digested by different individuals. This information will help to refine and direct research on innovating effective weaning foods to promote and sustain a healthy microbiome trajectory from breastfeeding into early childhood.

In this randomized crossover study, we aim to determine how the infant intestinal microbiome and function shifts in response to ingesting two different complementary foods that differ in their complex carbohydrate content. Our hypothesis is that there will be interactions between the infant's gut microbiome and complementary foods, such that different foods will enrich different microbiota compositions and their functions.

Methods

Study Population

Between 2017 and 2019, healthy women who had vaginally delivered healthy full-term infants and lived within the Davis and Sacramento metropolitan region of Northern California (USA) were recruited to enroll in this study. Inclusion criteria for study participants were as follows: healthy women aged 21-45 years; healthy, term (>37 weeks gestation), singleton infants delivered vaginally, aged 5-7.5 months at time of enrollment, and developmentally ready for solid foods; women who exclusively (without solids or infant formula) breastfed (at the breast or feed breast milk by bottle) their infants for at least 5 months of age and continued to breastfeed with solids and/or infant formula until 12 months of age; women who were willing to either use their own breast pump, or hand-express, or use a manual pump provided by the study to collect milk samples; and women who were willing to refrain from feeding their infants infant formula, non-study solid foods; probiotic or iron supplements before the end of the feeding intervention period (first 18 days of the study). Exclusion criteria for study participants were as follows: infants with any gastrointestinal tract abnormalities or medical complications such as respiratory distress syndrome, birth defects, and infection or hypotonia; infants diagnosed with a medical or nutritional condition that required iron supplements; infants born by cesarean section; multiple infants born to one mother at the same time (i.e. twins or triplets); infants who were given antibiotics, iron supplements, or infant formula within 4 weeks of enrollment; infants who consumed probiotics containing *Bifidobacterium* within 4 weeks or other probiotics within 7 days of enrollment; infants who consumed infant formula for more than 10 days between birth and 4 weeks prior to screening; infants who consumed any solid foods; infants who, on average, passed less than one stool per

week; family history of immunodeficiency syndromes; women who smoked cigarettes within 1 month of becoming pregnant, during pregnancy, currently smoked, or planned to resume smoking during the study period; women who planned to feed their infant solid food before 5 months of age; women who planned to feed their infants probiotics during the feeding intervention period (first 18 days of the study); women who lived in more than one location (should only live in one house to ensure samples are correctly collected and stored); and women diagnosed with any of the following medical conditions: metabolic or endocrine, liver, kidney disease, any autoimmune disease, cirrhosis, hepatitis C, HIV, AIDs, cancer, obesity (pre-pregnancy BMI >34.9), PCOS (that requires management by oral steroid medication), celiac disease, Crohn's disease, heart disease; hyper- or hypothyroidism, hyper- or hypotension (including pre-eclampsia) that was not controlled (with medication or other intervention); type 1 or type 2 diabetes. Additional screening for eligibility to determine if the infant was developmentally ready for solid foods occurred at approximately 5-6 months of age to assess if infants could sit up well without support, were eager to participate in mealtime and tried to grab food and put it in their mouth, and exhibited good head control (154).

Study Design

This study is a combined randomized, crossover, interventional and observational trial. At the start of the study, infants entered a 7-day lead-in period to establish gut microbiome and gastrointestinal (GI) tolerability baselines. After the 7-day lead-in period, infants were randomized into one of two feeding arms (Arm 1: Days 8-14; Arm 2: Days 19-25) consisting of commercially available baby foods: sweet potato or pears consumed for 7 days followed by a 4-day washout period of exclusive breast milk before initiating the alternate 7-day feeding arm of sweet potato or pears. The second feeding arm was followed by an additional 4-day washout period of exclusive

breast milk before the introduction of any other food sources that parents choose to feed to their infants. Sweet potato (SP) (Plum Organics, *Just Sweet Potato*) and pears (P) (*Earth's Best, First Pears*) were selected as the interventional foods because they are one of the most commonly used first complementary baby foods and they vary dramatically in their carbohydrate complexity. Once the first month of the study was complete, infants entered an observational phase until they turned approximately 1 year old. A subset of study participants did not consume sweet potato as it was reported that various packaged baby food brands including Plum Organics sweet potatoes contained high levels of heavy metals (155). The second feeding arm was thus dropped from the study at this time and participants only consumed pears for 7 days. At the time of this consumer report, sixty infants had already completed both feeding arms of this study. Group A (n = 30) consumed pears first followed by sweet potatoes, Group B (n = 32) consumed sweet potatoes first followed by pears and Group C (n = 37) consumed only pears (**Figure 3.1, 3.2**).

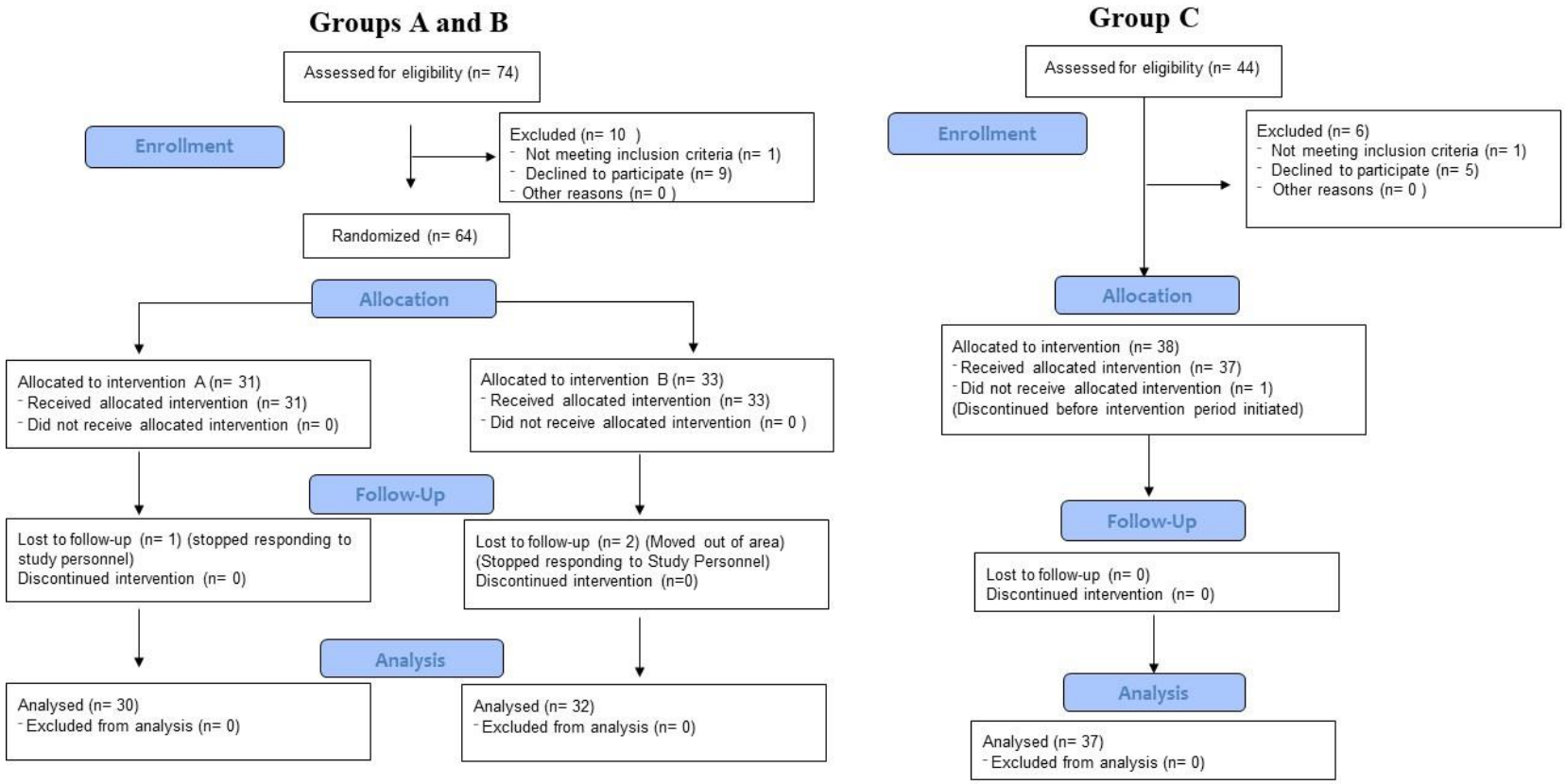

Figure 3.1: Consort diagram describing Group A, B, and C.

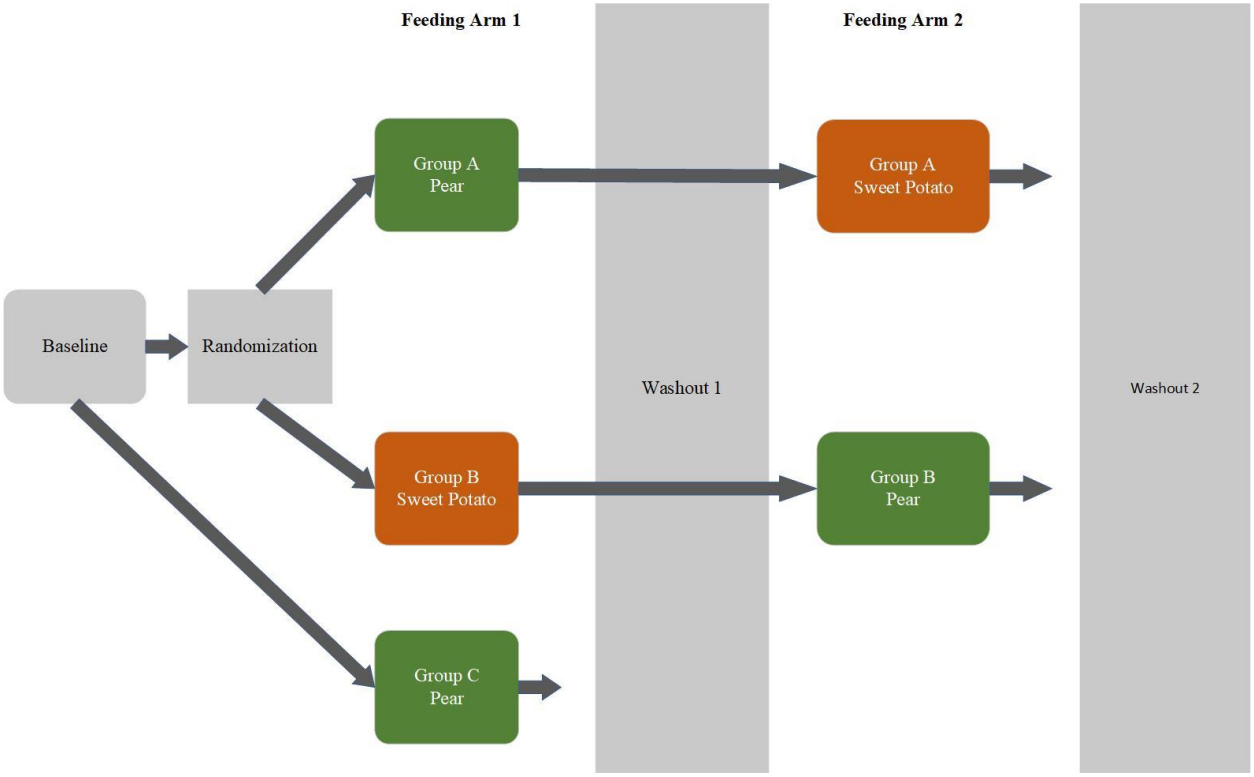

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Figure 3.2: Crossover study design.

Enrollment (Day 0)

On the day of enrollment, mothers provided written informed consent and infants were assessed for developmental readiness to begin consuming solid foods using a portion of the Alberta Infant Motor Scale (AIMS) assessment. This assessment is a standard assessment of infant motor development used by physical therapists. As part of this assessment, infants who are not yet able to sit independent are tested in a 'pull-to-sit', and their head control was observed. Infants who did not pass portions of the AIMS assessment during the enrollment visit were scheduled for a re-test

the following week and weekly if necessary. Only once infants could sit with good head control or could demonstrate good head control during the pull-to-sit assessment were they able to begin the Lead-in period of the study.

Lead-In Period

After enrollment (Day 0), participants entered a 7-day Lead-In Period to acquire baseline dietary, GI, and health data. On Day 7, participants were provided with the study food and a food scale to weigh each the food before and after intake to determine how much food was consumed by their infant at each feeding. Groups A and B were randomized by a random number generator to feeding intervention in blocks of 10 and groups were matched for number of siblings as 0 siblings versus 1 or more siblings. Group C was not randomized to feeding intervention and only consumed pears during the study. The feeding intervention started on Day 8.

Feeding Intervention (Arm 1 and Arm 2)

Participants were asked to feed the study food to their infants for 7 consecutive days by offering at least 1 tablespoon of the study food to their infant up to three times per day. Participants were instructed to offer their infants the study foods in the morning and early afternoon on the first day of each feeding arm (Days 8 and 19), to monitor any potential adverse reactions. Participants were instructed to weigh the food in its original packaging before and after each feeding and to record these weights on a food log. If the infant developed a minor adverse reaction to the study food (i.e. diaper rash), participants were allowed to stop feeding the study food to their infants until if the symptoms disappeared during a one-week washout period before restarting the study intervention. All participants participated in Feeding Arm 1 but only a subset of participants

participated in Feeding Arm 2 (n = 60) because sweet potato was dropped from the intervention during the course of the study.

Washout Periods 1 and 2

Upon completing each Feeding Intervention, participants entered two 4-day Washout Periods (Days 15-18 and 26-29) of exclusively breast milk feeding in order to return the gut microbiota back to baseline. All participants participated in Washout period 1 but only a subset of participants participated in Washout Period 2 (Groups A and B, n = 60) because sweet potato was dropped from the intervention during the course of the study.

Observation Period

After completion of the Intervention and Washout Periods, participants entered an Observational Period until their infant turns approximately one year old (Day 182). During this period, participants were asked to feed their infants as they normally would.

Infant weight was measure on Days 1-2, 7, 14, 30, 60, 61 and 182 using a digital Pediatric scale (Tanita); parents were asked to remove their infant's clothes and diaper prior to study personnel taking this measurement.

Questionnaires

At the Enrollment Visit on Day 0, mothers completed questionnaires regarding their pregnancy, labor, and delivery experience, reproductive health, and their infant's health and diet since birth. Throughout the course of the study, mothers completed a monthly questionnaire about their intake of antibiotics, supplements and medications and their infant's health and diet. On each day through Day 29, participants were asked to keep prospective, daily logs regarding their infant's general health, stool patterns, diet, and medication usage. Mothers were asked for their infant's

height and weight that was measured during their infant's 6-month well-baby visit on the last questionnaire they completed for the study.

Samples

Study Foods

Foods used in this study were Earth's Best Pears (Hain Celestial Group, Inc., Boulder, CO) and Plum Organics Sweet Potatoes (Sun-Maid, Fresno, CA).

Infant Stool

Participants collected their infant stool from their diapers using PurFlock Ultra Flocked Swabs (Puritan, Guilford, ME) on 13 occasions: two samples were collected during Lead-In (Day 1 and Day 5), two during Feeding Arm 1 (Group A and B – Day 9, 11, and 13; Group C - Day 11 and 14), two during Washout Period 1 (Day 16 and 18), two during Feeding Arm 2 (Group A and B – Day 20, 22, and 24; Group C - Day 11 and 14), two during the Washout Period 2 (Group A and B - Day 27 and 29) and on Day 29, 60, and 180 during the Observation Phase. For infants who did not complete the sweet potato feeding arm (Group C), 9 stool samples were collected during the study period: Lead-In (Day 1 and 5), Feeding Arm 1 (Day 11 and 14) Washout Period 1 (Day 16 and 18) and during the Observation Phase (Days 29, 60, and 180). All stool samples were stored in participants' home freezers until transported to UC Davis on dry ice and stored at -80C until processed for analysis.

Participants were required to collect at least one stool during each study period (Lead-In, Feeding Arm 1, Washout 1, Feeding Arm 2, and Washout 2) before their infants moved on to the subsequent period.

Molecular Methods and Analysis

DNA from 200 mg feces were extracted using the ZymoBIOMICS-96 MagBead DNA Kit (Zymo Research). Briefly, the feces were placed in lysis tubes with 750 µL of ZymoBIOMICS Lysis Solution and bead beating was performed using the Disrupter Genie FastPrep 24 for 3 cycles of 1 minute bead beating (6.5 m/s) and 5 minutes on ice. Samples were then centrifuged at 10000xg for 1 minute, and 200 µL of the supernatant was transferred to a deep-well sample plate containing 600 µL of ZymoBIOMICS MagBinding buffer and 75 µL of MagBeads per well. In addition, a deep-well plate containing 900 µL per well Wash 1, two deep-well plates containing 900 µL Wash 2 per well, and a standard plate containing 150 µL DNAse/RNAse free water per well (the elution plate) were prepared for use with the KingFisher. The KingFisher robot was run with the following conditions: mix sample in sample plate on fast setting for 10 seconds and collect beads on count 2 for 5 seconds. Move to Wash 1 plate and release beads on fast setting for 10 seconds, then mix for 5 seconds on the fast setting, and collect beads on count 2 for 5 seconds. Move to the first Wash 2 plate and repeat conditions for Wash 1. Repeat with second Wash 2 plate. Dry beads outside the well for 10 seconds, then release beads into the elution plate for 10 seconds using the bottom mix speed. Then mix on fast speed for minutes, pause for 2 minutes, and mix on fast speed for 5 minutes using the tip edge in well setting. Finally, the beads were collected using count 2 for 5 seconds. The V4 region of the 16S rRNA gene was then amplified and sequenced using published methods (doi: 10.1073/pnas.1317377110; doi: 10.1016/j.jhep.2015.09.022). Raw sequence data was demultiplexed using sabre (https://github.com/najoshi/sabre), and imported into QIIME2 version 2017.12.0 (https://doi.org/10.1038/s41587-019-0209-9). Reads were quality trimmed to 190 bp on the forward read and 205 bp on the reverse read, and the primer sequence was removed from each read before processing the reads using DADA2 (https://doi.org/10.1038/nmeth.3869).

Samples with fewer than 4600 reads were excluded from further analysis. Subjects without a successfully sequenced baseline sample were excluded from further analysis. Samples were rarefied to a depth of 4658 reads per sample, and alpha diversity was calculated using the Shannon index as available in the vegan package in R. As alpha diversity was not normally distributed a Wilcoxon paired test was used to test for differences in alpha diversity between baseline samples and after the consumption of the first food. To assess beta-diversity, both weighted and unweighted UniFrac distances were used in combination with PERMANOVA as implemented in the adonis command in the vegan package in R. Strata was set to Subject ID, and beta-diversity was compared between the baseline samples and the samples collected after the first food was consumed. Beta-diversity was visualized using non-metric multidimensional scaling. Differential abundance testing at the genus level was completed using ANCOM-II (https://doi.org/10.3389/fmicb.2017.02114), using Subject ID as a random factor and comparing between baseline samples and samples after the consumption of the first food using an alpha of 0.05 and a Benjamini-Hochberg correction with the default cut-off for W.

Monosaccharide Analysis of Infant Foods

Monosaccharides in the study foods were analyzed in the manner of Amicucci et al (156). Briefly, food samples were freeze dried and massed to 10 mg. Samples were then brought up in a 4 M trifluoroacetic acid (TFA) solution and hydrolyzed at 100 °C for two hours and dried under vacuum centrifugation. Hydrolyzed monosaccharides were then derivatized with 3-methyl-1-phenyl-2-pyrazoline-5-one (PMP) and extracted in chloroform. Samples were analyzed in MRM mode on an Agilent UHPLC-QqQ mass spectrometer and quantification was performed by comparison to a standard curve.

Monosaccharide Analysis of Infant Feces

On dry ice, fecal material was cut and weighed to 10 mg and placed into 1 ml of water. Analysis of fecal products was done in the manner of Amicucci et al (156) and Xu et al (157). Briefly, Samples were then brought up in a 4 M trifluoroacetic acid (TFA) solution and hydrolyzed at 100 °C for two hours and dried under vacuum centrifugation. Hydrolyzed monosaccharides were then derivatized with 3-methyl-1-phenyl-2-pyrazoline-5-one (PMP) and extracted in chloroform. Samples were analyzed in MRM mode on an Agilent UHPLC-QqQ mass spectrometer and quantification was performed by comparison to a standard curve.

Organic Acid Analysis

Organic acid analysis was performed in the manner of Rivera-Chávez et al (158). Briefly, fecal material was cut and weighed to 10 mg and diluted with 80% ethanol and rocked in a 96-well plate overnight at 4 °C. The samples were centrifuged, and the supernatant transferred for derivatization with N-(3-methylaminopropyl-N'-ethylcarbodiimide hydrochloride (1-EDC HCL) and 2-Nitrophenylhydrazine (2-NPH) in the presence of pyridine, Acetonitrile (ACN), and HCl. The derivatization proceeded at 40 °C for 30 minutes. Upon completion, 400 µl of 10% ACN was added to the solution to precipitate the remaining derivatization reagents. The solution was centrifuged, and the supernatant transferred for analysis. Analysis was performed on a UHPLC-QqQ mass spectrometer and quantitation was performed by comparison to a standard curve.

Statistics

All hypothesis testing was two-sided, and the family-wise type I error rate (α) was maintained at 0.05.

Baseline characteristics

Differences in maternal and infant baseline characteristics between Groups A, B and were determined using Kruskal Wallis and pairwise comparisons between the three groups were conducted using Wilcoxson rank sum test when significant.

Food consumption

Differences in the total amount of pears consumed between Groups A, B, and C; and sweet potato between Groups A and B were determined using Kruskal Wallis, and when significant pairwise comparisons were conducted using Wilcoxson rank sum test .

Wilcoxson rank sum test was also used to determine differences between the total amount of pear and sweet potato consumed in Groups A and B; the total amount of sweet potato consumed when sweet potato was the first food compared to the second food; and the total amount of pear consumed when pear was the first food compared to the second food. Wilcoxson signed rank test was used to determine differences in the total amount of food consumed in Feeding Arm 1 compared to Feeding Arm 2 for Groups A and B.

GI Tolerability

These data were binned across the three study time periods Baseline, Feeding Arm 1, Washout 1, and Feeding Arm 2, means and proportions were calculated for continuous and categorical variables across each study period. Proportions for binary categorical variables were calculated as the number of days reported/total number of days in each study period. The calculated values were multiplied by 100 to generate percentages.

Differences in the number of infant stools, stool size and stool consistency; mean irritability, discomfort, and flatulence scores; hours spent with a care giver; number of crying hours; the proportion of days infants experienced symptoms of teething, illness, or fever at or

above 103°C; and the proportion of days infants consumed breast milk, infant formula, vitamins, probiotics, medications, or other liquids before and after the introduction of any solid foods (Baseline versus Feeding Arm 1) was determined using Wilcoxson rank sum test. When participants were missing data for crying hours and number of hours spent with a caretaker, they were excluded from those analyses.

When an outcome variable was significantly different before and after the introduction of solid foods, a linear mixed-effects model (LME) was conducted for Groups A and B. Group, time, and the interaction between group and time were used as predictors with subject as a random variable. Group C was excluded from this analysis because they did not participate in Feeding Arm 2. Prior to conducting this LME, response variables were log transformed to approximate a normal distribution and normality was assessed by histograms, QQ-plots, and Shapiro-Wilk's test. When a predictor had a significant effect on the outcome variable, a post-hoc analysis using Tukey HSD was performed.

Fecal Organic Acids and Glycans

Fecal organic acids and fecal glycans detected in greater than 50% of participants across all time points were included in this analysis. Imputed values were calculated by limit of detection (LOD)/√2. Differences in fecal organic acids (absolute and relative abundances) between Baseline and at the end of Feeding Arm 1 (after the introduction of any solid food) were determined using Wilcoxson rank sum test using an alpha of 0.05 and a Benjamini-Hochberg correction.

Fecal bound glycans (absolute and relative abundances) and fecal free glycans (absolute and relative abundances) between Baseline and after consumption of pears (Feeding Arm 1 and 2) and between Baseline and after consumption of sweet potato (Feeding Arm 1 and 2) were

determined using Wilcoxson rank sum test using an alpha of 0.05 and a Benjamini-Hochberg correction.

Results

Study food:

Pear and sweet potato were chosen for this study as they differed in their carbohydrate composition. Pear contains more fructose and arabinose than sweet potato which contains higher levels of glucose (**Figure 3.3**).

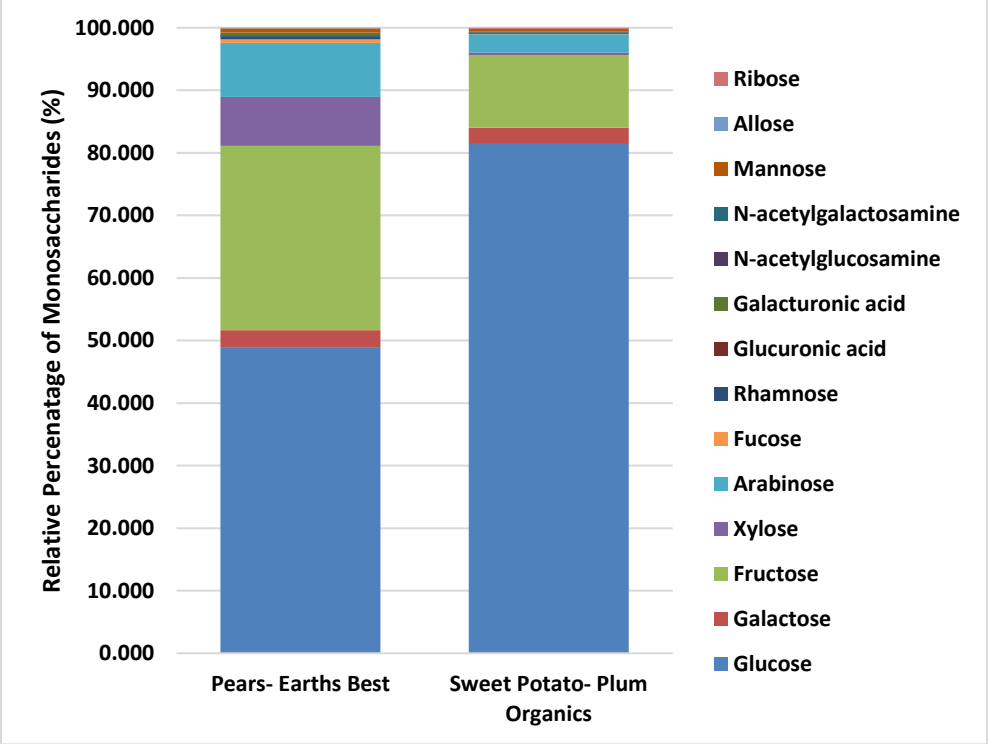

Figure 3.3 – Relative percentage of monosaccharides in pear and sweet potato first infant foods.

Maternal baseline characteristics

Maternal age at enrollment, pre-pregnancy BMI, number of previous pregnancies, number of live births, and number of other children (not including the infant enrolled in this study) were not statistically significant between groups. Maternal weight gain during pregnancy was significantly different between Groups A, B, and C (P <0.05), however, there were no significant differences between groups after pairwise comparisons, likely because of low statistical power (**Table 3.1**).

Table 3.1 – Maternal Baseline Characteristics

Maternal Baseline Characteristics		Group A (*n* = 31)		Group B (*n* = 34)		Group C (*n* = 38)	
		Mean	SD	Mean	SD	Mean	SD
Age at Enrollment (yr)		31.4	3.5	32.4	3.6	32.9	4
Pre-Pregnancy BMI		38.3	5.7	41.1	6.5	41.8	6.2
Pregnancy Weight Gain (lb) *		34.3	10.3	28..3	10.3	36.2	26.6
Number of Pregnancies (other than infant enrolled in this study)		1.3	1.1	1.4	1.4	2	1.5
Number of Live Births (other than infant enrolled in this study)		1.2	0.7	1.2	0.9	1.3	1
Number of Children (not including infant enrolled in this study)		1.2	1.2	0.9	0.9	1.1	1.1
Blood Type							
	A Positive	10 (32.3)		9 (26.5)		12 (31.6)	
	A Negative	0 (0.0)		3 (8.8)		2 (5.3)	
	B Positive	4 (12.9)		3 (8.8)		3 (7.9)	
	B Negative	0 (0.0)		1 (2.9)		1 (2.6)	
	AB Positive	5 (16.1)		6 (17.6)		10 (26.3)	
	AB Negative	1 (3.2)		1 (2.9)		4 (10.5)	
	O Positive	0 (0.0)		0 (0.0)		1 (2.6)	
	O Negative	3 (9.7)		3 (8.8)		1 (2.6)	
	Unsure	8 (25.8)		8 (23.5)		4 (10.5)	
Parity, *n* (%)							
	Primiparous	10 (32.3)		13 (38.2)		13 (34.2)	
	Multiparous	21 (67.7)		21 (61.8)		25 (65.8)	
Ethnicity, *n* (%)							
	Not Hispanic	25 (80.6)		27 (79.4)		32 (84.2)	
	Hispanic	6 (19.4)		6 (17.6)		4 (10.5)	
	Unsure	0 (0.0)		1 (2.9)		0 (0.0)	
	Refuse	0 (0.0)		0 (0.0)		2 (5.3)	
Race, *n* (%)							

Asian	2 (6.5)	3 (9.4)	1 (2.6)
Black or African American	1 (3.2)	0 (0.0)	0 (0.0)
White (including Middle Eastern)	26 (83.9)	27 (84.4)	31 (81.6)
2 or More Races	0 (0.0)	1 (3.1)	4 (10.5)
Other	1 (3.2)	1 (3.1)	1 (0.0)
Refuse	1 (3.2)	0 (0.0)	1 (0.0)
Education, *n* (%)			
High school graduate (or equivalent)	1 (3.2)	0 (0.0)	0 (0.0)
Associate's degree (including occupational or academic degrees)	2 (6.5)	1 (2.9)	6 (15.8)
Some college (1-4 years, no degree)	5 (16.1)	5 (14.7)	6 (15.8)
Bachelor's degree (BA, BS, etc.)	14 (45.2)	18 (52.9)	14 (36.8)
Master's degree (MA, MS, MEng, MSW, etc.)	7 (22.6)	6 (17.6)	8 (21.1)
Professional or doctorate (MD, DDS, JD, PhD, EdD, etc)	2 (6.5)	4 (11.8)	3 (7.9)
Refuse	0 (0.0)	0 (0.0)	1 (2.6)
Marital Status, *n* (%)			
Married/Couple	29 (93.5)	31 (91.2)	38 (100.0)
Divorced/Separated	1 (3.2)	1 (2.9)	0 (0.0)
Never Married	1 (3.2)	2 (5.9)	0 (0.0)

* $P < 0.05$ for differences between Groups A, B, and C (Kruskal-Wallis)

Infant characteristics

Infant gestational age at birth, birth weight, birth length and gender were not significantly different between treatment groups. Infant age at enrollment was significantly different between Groups A, B, and C ($P < 0.05$). Pairwise comparisons showed that there was a significant difference between Group B and Group C ($P < 0.05$) (**Table 3.2**). Differences in age at enrollment are not considered biologically significant as all infants in this study were developmentally ready for solid foods.

Table 3.2 – Infant Baseline Characteristics

Infant Baseline Characteristics	Group A (n = 31)		Group B (n = 34)		Group C (n = 38)	
	Mean	SD	Mean	SD	Mean	SD
Age at Enrollment (mo) *	5.9	0.6	6.1	0.6	5.8	0.4
Gestational Age (wk)	39.7	1.3	40.2	0.9	40.1	0.9
Birth Weight (g)	3536	447	3469	479	3647	405
Birth Length (cm)	51.7	2.8	51.5	3.2	52.4	2.3
Infant Gender, n (%)						
Male	15 (48.4)		16 (47.1)		19 (50.0)	
Female	16 (51.6)		18 (52.9)		19 (50.0)	
Ethnicity, n (%)						
Not Hispanic	25 (80.6)		25 (73.5)		28 (73.7)	
Hispanic	5 (16.1)		8 (23.5)		7 (18.4)	
Unsure	1 (3.2)		1 (2.9)		1 (2.6)	
Refuse	0 (0.0)		0 (0.0)		2 (5.3)	
Race, n (%)						
Asian	0 (0.0)		2 (5.9)		0 (0.0)	
Black or African American	1 (3.2)		0 (0.0)		0 (0.0)	
White (including Middle Eastern)	21 (67.7)		26 (76.5)		31 (81.6)	
2 or More Races	7 (22.6)		5 (14.7)		6 (15.8)	
Other	1 (3.2)		1 (2.9)		0 (0.0)	
Refuse	1 (3.2)		0 (0.0)		1 (2.6)	

* P <0.05 for differences between Groups A, B, and C (Kruskal-Wallis). Pairwise comparisons showed that there was a significant difference between Group B and Group C (Wilcoxson rank sum, P <0.05).

Food Consumption

When looking at total food consumption (pear plus sweet potato), Group A consumed significantly more food across the study period compared to Group B (P <0.01) (**Table 3.3**).

There was a significant difference in the total amount of pears consumed between Groups A, B, and C (P <0.01). Post-hoc analysis showed significant differences in the amount of pears consumed; Group A consumed significantly more pear than Group B (P <0.0005) and Group C (P <0.01). There was no significant difference in the amount of pear consumed between Groups B and C. When looking at the total sweet potato consumption between Groups A and B, Group A consumed significantly more sweet potato across the study period (P <0.05) (**Table 3.3**).

Across Groups A and B, there was no difference between the total amount of pear and sweet potato consumed and no difference in the total amount of pear consumed when pear was the first food or second food. Significantly more sweet potato was consumed when sweet potato was the second food (P <0.01). Across Groups A and B, significantly more food was consumed in Feeding Arm 2 compared to Feeding Arm 1 (P <0.0005).

Table 3.3 – Total amount of food consumed, and food containers used in Group A, B, and C in Feeding Arm 1 and Feeding Arm 2.

	Group A							
	Pear (n = 29)			Sweet Potato (n = 29)			Total (n = 29)	
	Mean	SD	Range	Mean	SD	Range	Mean	SD
Containers Used	14.0	4.1	4.0, 22.0	15.1	4.3	6.0, 24.0	29.2	7.5
Solid Food Consumed (g)	354.7[b]	247.4	7.5, 1034.1	523.5[c]	446.3	13.1, 1653.1	878.1[a]	673.6

	Group B							
	Pear (n = 31)			Sweet Potato (n = 31)			Total (n = 31)	
	Mean	SD	Range	Mean	SD	Range	Mean	SD
Containers Used	16.5	6.1	5.0, 41.0	15.1	4.7	5.0, 23.0	31.1	9.7
Solid Food Consumed (g)	283.7[b]	561.2	13.7, 2993.4	487.4[c]	233.5	34.4, 894.2	765.2[a]	745.6

	Group C		
	Pear (*n* = 36)		
	Mean	SD	Range
Containers Used	15.2	3.0	7.0, 19.0
Solid Food Consumed (g)	361.4[b]	221.5	69.3, 913.0

[a] $P<0.01$for differences in total amount of food consumed across all study periods between Groups A and B as determined by Wilcoxson signed rank test.

[b] $P<0.01$for differences in total amount of pears consumed between Groups A, B, and C as determined by Kruskal-Wallis. Pairwise comparisons showed that Group A consumed significantly more pear than Group B ($P<0.0005$) and C ($P<0.01$).

[c] $P<0.05$ for differences in total amount of sweet potato consumed between Groups A and B as determined by Wilcoxson signed rank test.

GI Tolerability

No infants consumed infant formula, probiotics, or antibiotics during the intervention period.

There were significant differences in mean flatulence score (P <0.05) (**Figure 3.4)**, mean discomfort score (P <0.0005) (**Figure 3.5a)**, and mean stool consistency (P <0.0005) (**Figure 3.6a)** before and after the introduction of any solid foods. No significant differences in number of crying hours; number of hours spent with a caregiver; proportion of days infants consumed breast milk, vitamins, or medications; the proportion of days infants experienced symptoms of teething, illness, or fever; mean irritability score or stool number and stool size before and after the introduction of any solid food (**Supplemental Table 3.1**).

Linear mixed effect models were conducted for Groups A and B for response variables mean flatulence score, mean discomfort score, and stool consistency with predictors: Group, time, Group*time with subject as a random variable. Time had a significant effect on mean discomfort score (DF = 182, F = 2.95, P < 0.05*).* Post-hoc analysis showed a significant difference in mean discomfort score between Baseline and Feeding Arm 2 (P <0.05) (**Figure 3.5b**). Time had a significant effect on mean stool consistency (DF = 182, F = 34.384, P < 0.0005*).* Post-hoc analysis showed a significant difference in mean stool consistency between Baseline and Feeding Arm 1 (P <0.0005), Baseline and Washout 1 (P <0.0005), Baseline and Feeding Arm 2 (P <0.0005), Feeding Arm 1 and Feeding Arm 2 (P <0.05), and Washout 1 and Feeding Arm 2 (P <0.05) (**Figure 3.6b**). Group and time had no significant effect on mean flatulence score.

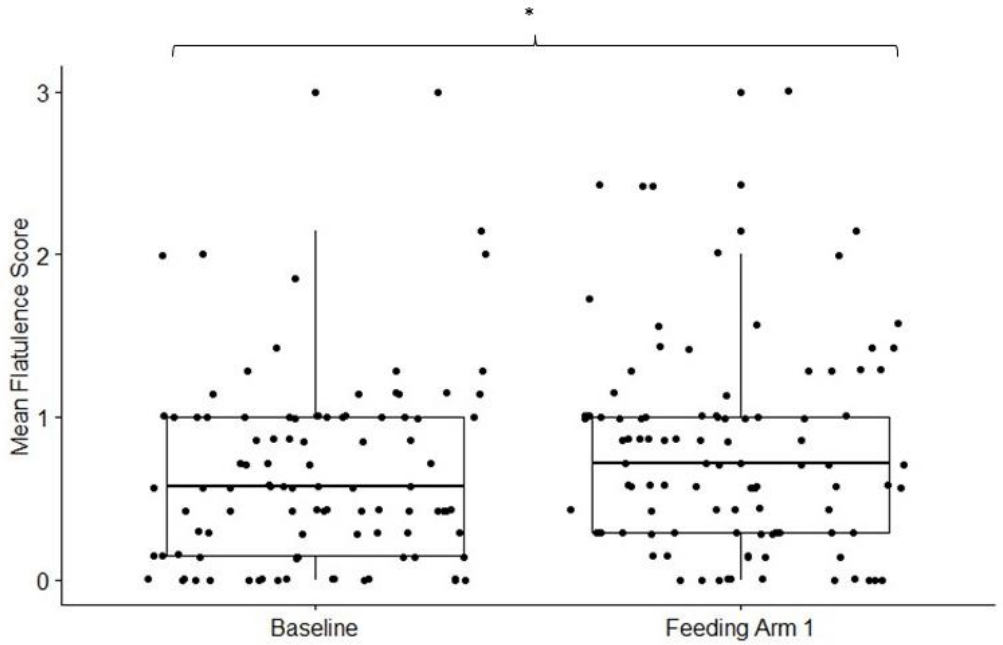

Figure 3.4. Mean flatulence score. Compared to Baseline, there was a significant increase in mean flatulence score after the consumption of the first food (Feeding Arm 1) for Groups A, B, and C combined. * P <0.05, Wilcoxson signed rank test.

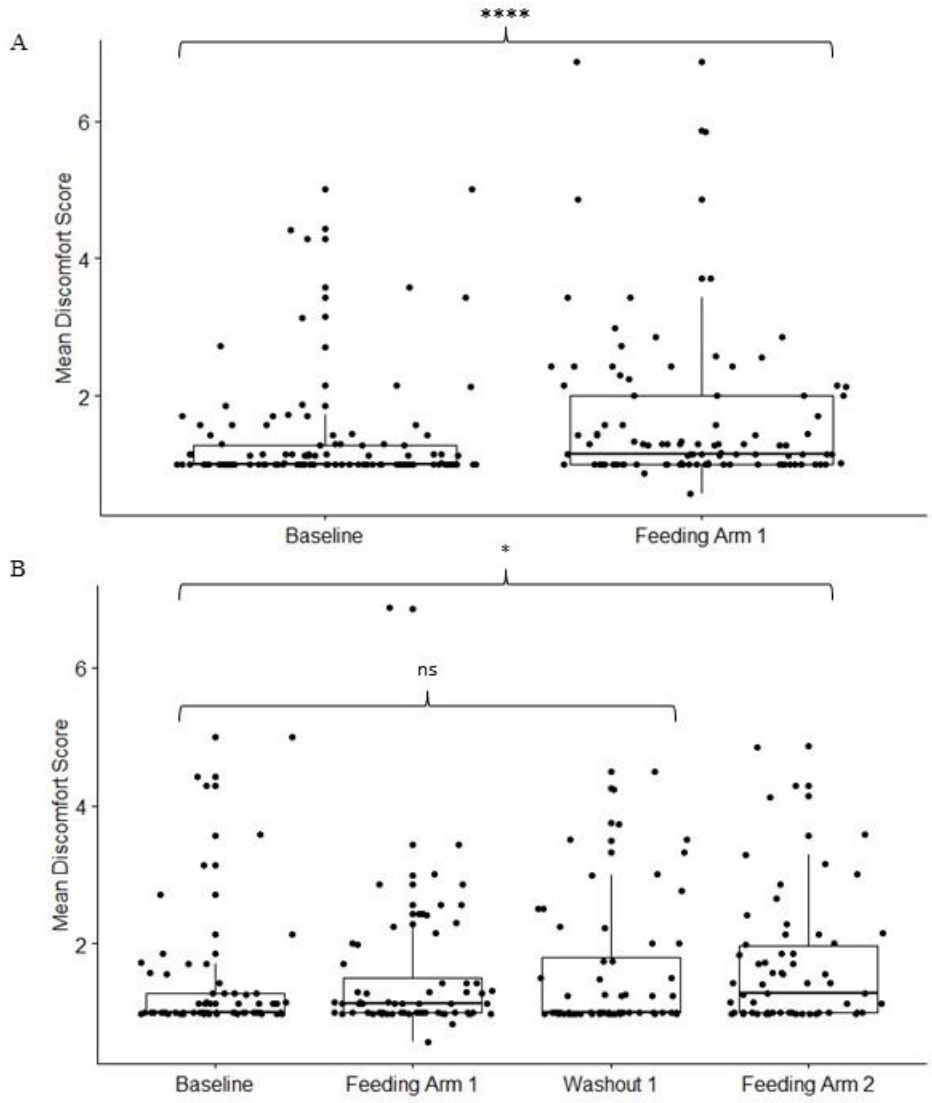

Figure 3.5. Mean discomfort score. A – Compared to Baseline, there was a significant increase in mean discomfort score after the consumption of the first food (Feeding Arm 1) for Groups A, B, and C combined, **** P <0.0005, Wilcoxson signed rank test. B – There was a significant time effect (P <0.05) for Groups A and B as determined by a linear mixed effects model. Based on multiple comparison post hoc analysis, compared to Baseline there was a significant increase in mean discomfort score during Feeding Arm 2, * P <0.05, ns = not significant.

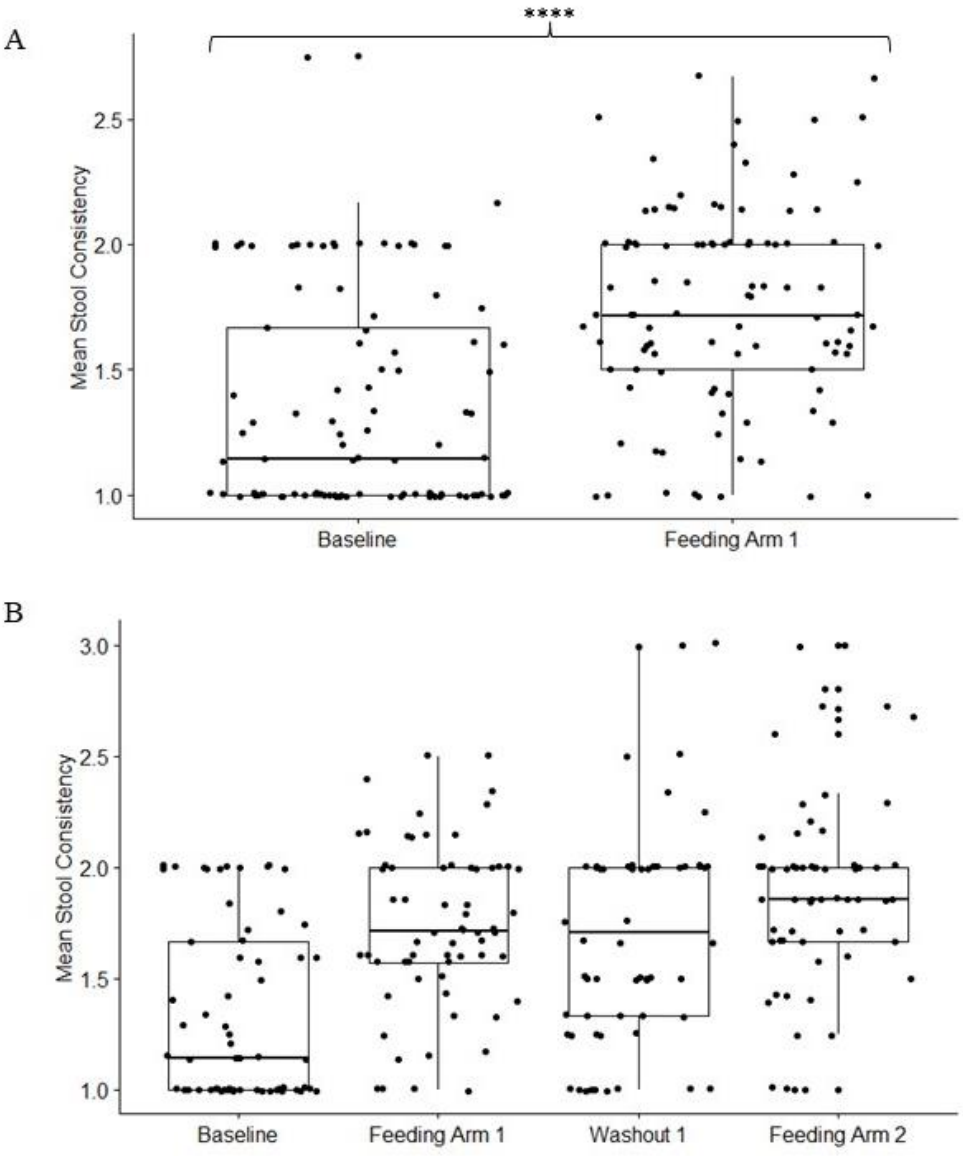

Figure 3.6. Mean stool consistency, on a scale from 1 to 4 where 1 is watery and 4 is hard. A – Compared to Baseline, there was a significant increase in mean stool consistency resulting in firmer stools after the consumption of the first food (Feeding Arm 1) for Groups A, B, and C combined, **** P <0.0005, Wilcoxson signed rank test. B –There was a significant time effect (P <0.0005) for Groups A and B as determined by a linear mixed effects model. Based on multiple

comparison post hoc analysis, compared with Baseline, mean stool consistency increased during Feeding Arm 1, Washout 1 and Feeding Arm 2 (P <0.0005). Mean stool consistency was significantly different between Feeding Arm 1 and Feeding Arm 2 (P <0.05) and Washout 1 and Feeding Arm 2 (P <0.05). There were no differences between Feeding Arm 1 and Washout 1.

Fecal Organic Acids and Glycans

There was a significant decrease in the sum of fecal absolute organic acids (P <0.05), absolute lactic acid (P <0.01), and relative lactic acid (P <0.01) between Baseline and Feeding Arm 1, after the consumption of the first solid food (**Figure 3.7a)**. There was a significant increase in fecal relative isobutyric acid (P <0.05), relative valeric acid (P <0.05), and relative pyruvic acid (P <0.05) between Baseline and Feeding Arm 1, after the consumption of the first solid food (**Figure 3.7b**).

There was a significant increase in fecal bound absolute arabinose (P <0.01), bound absolute fructose (P <0.05), and bound relative arabinose (P <0.05) between Baseline and after the consumption of pears. There was a significant decrease in bound relative sialic acid (P <0.05) and bound relative glucose (P <0.05) between Baseline and after the consumption of pears during Feeding Arm 1 and Feeding Arm 2 (**Figure 3.8a and 3.8b**). There were no significant differences in fecal free glycans (absolute or relative) between Baseline and after the consumption of pear or fecal free and bound glycans (absolute or relative) between Baseline and after the consumption of sweet potato.

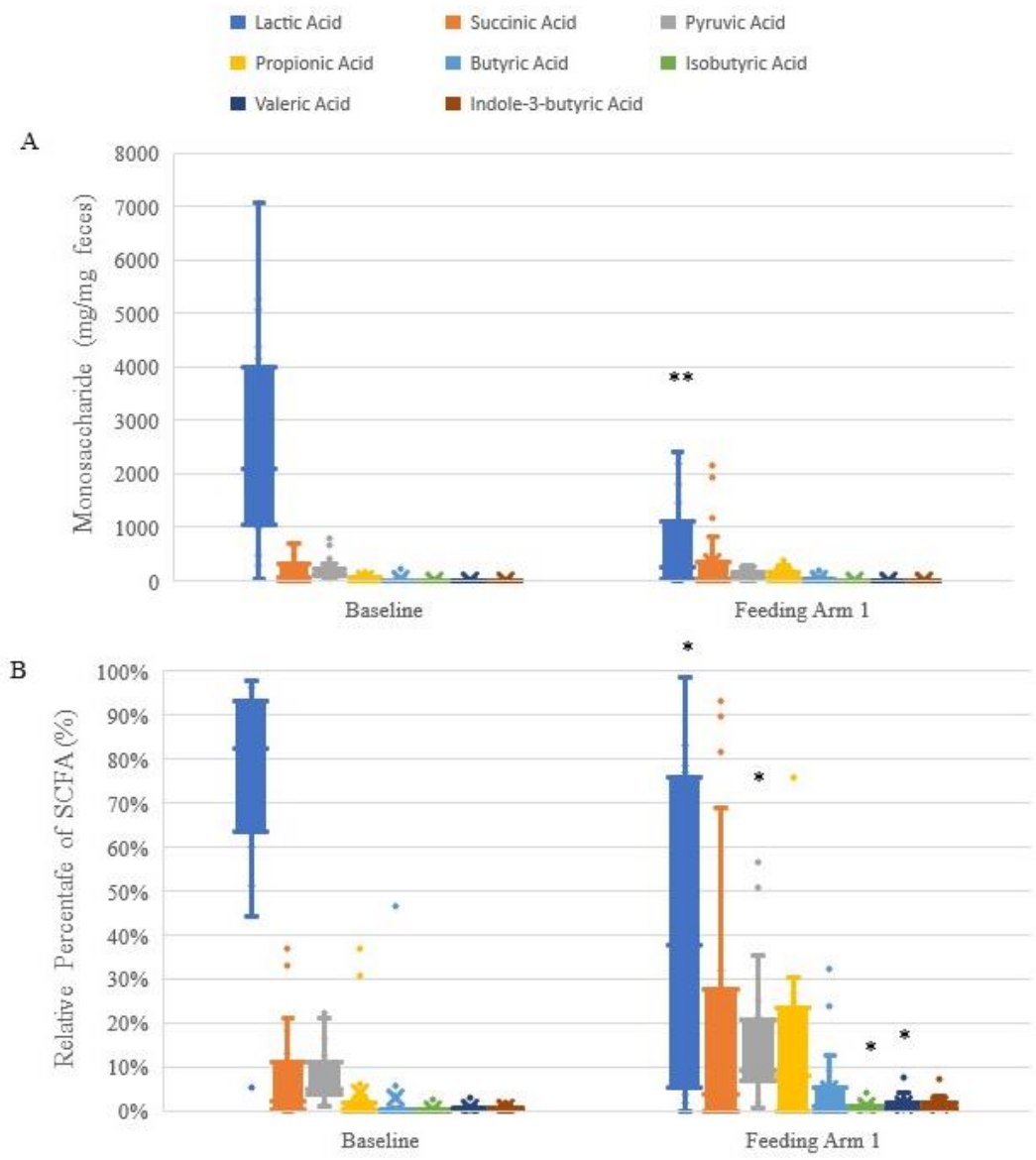

Figure 3.7 Fecal organic acids at Baseline and Feeding Arm 1 (after the consumption of any solid foods). **A**: Fecal organic acid levels (mg/mg feces) at Baseline and Feeding Arm 1. After consuming the first food, there was a significant decrease the sum of organic acids and lactic acid. ** P <0.01 significantly different from baseline using Wilcoxson rank sum test and Benjamini-Hochberg correction. **B**: Relative percentage organic acid (%) at Baseline and Feeding Arm 1. After the consumption of the first food, there was an increase in relative

isobutyric, valeric and pyruvic acid and a decrease in relative lactic acid. * indicates different from Baseline.

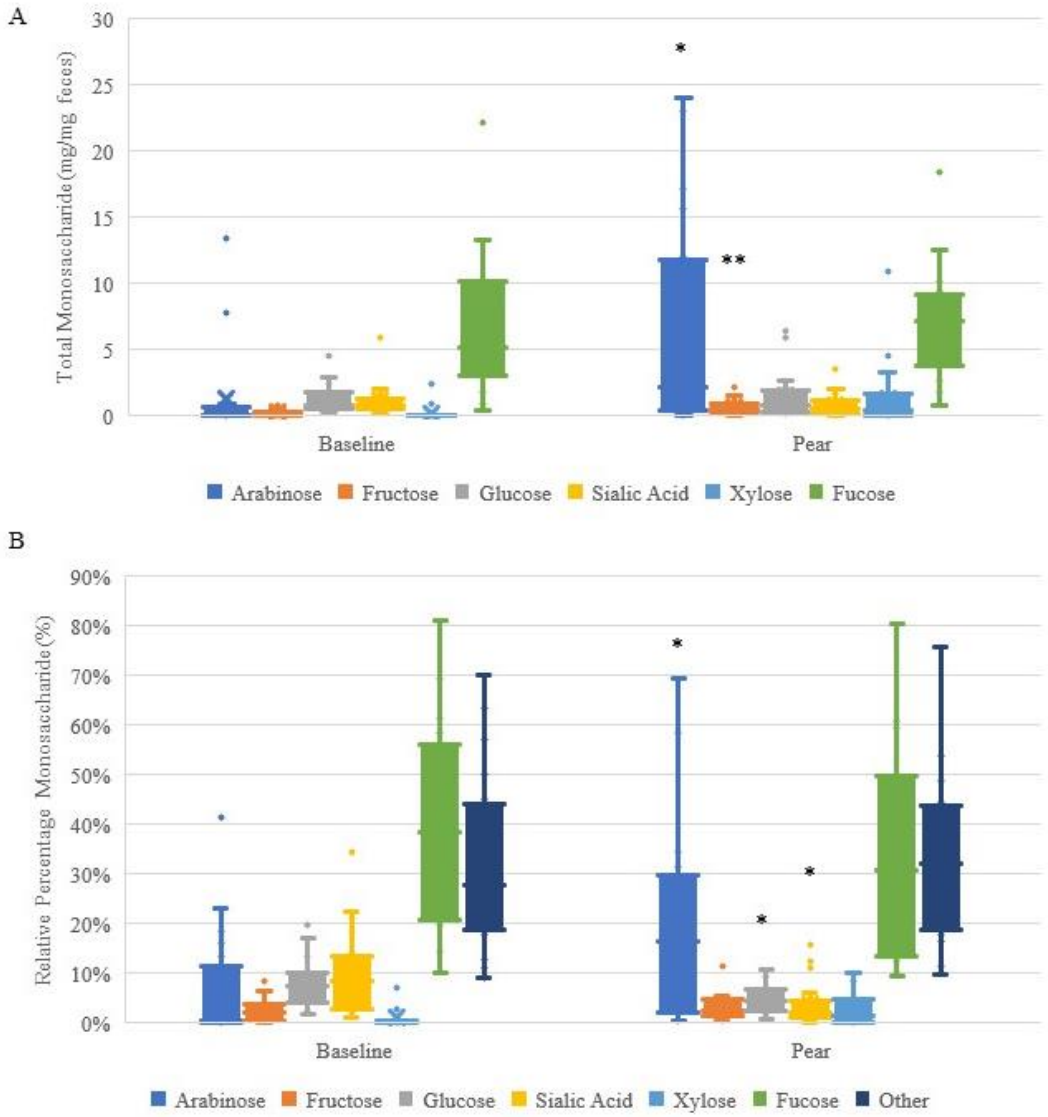

Figure 3.8. Fecal monosaccharides at Baseline and after the consumption of pears. **A**: Fecal monosaccharide levels (mg/mg feces) at Baseline and after consumption of Pears. After consuming pears, there were significant increases in bound absolute arabinose and bound

absolute fructose. ** P <0.01, * P <0.05, significantly different from Baseline using Wilcoxson rank sum test and Benjamini-Hochberg correction. **B**: Relative percentage monosaccharides (%) at Baseline and after consumption of Pears. After consuming pears, there was an increase in relative bound arabinose and decrease in relative bound glucose and sialic acid. "Other" was calculated by adding all additional measured monosaccharides together. * indicates different from Baseline.

Microbiome

Alpha diversity, as measured by the Shannon index, did not differ significantly between the baseline sample (pre-first food) and samples collected after the consumption of the first food (post-first food, Wilcoxon paired test, $P < 0.05$, **Figure 3.9**). There was no significant difference in beta-diversity by weighted UniFrac ($P > 0.05$), but there was a significant difference between baseline and first food using unweighted UniFrac ($P < 0.05$, **Figure 3.10**). There were no genera that exceeded the W threshold for significance using ANCOM-II.

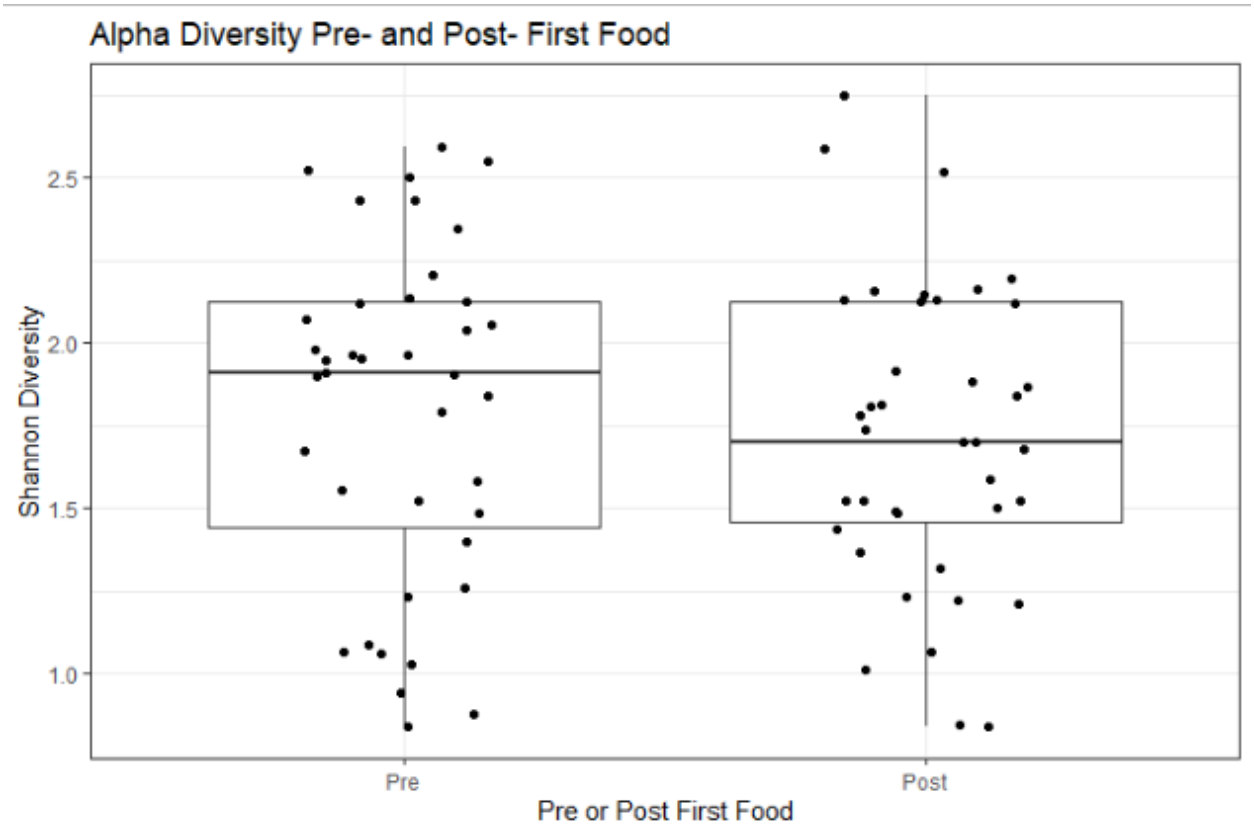

Figure 3.9: Alpha diversity at baseline and after feeding the first of the two weaning foods.

After feeding the first food, alpha diversity was not significantly different by the Shannon Index.

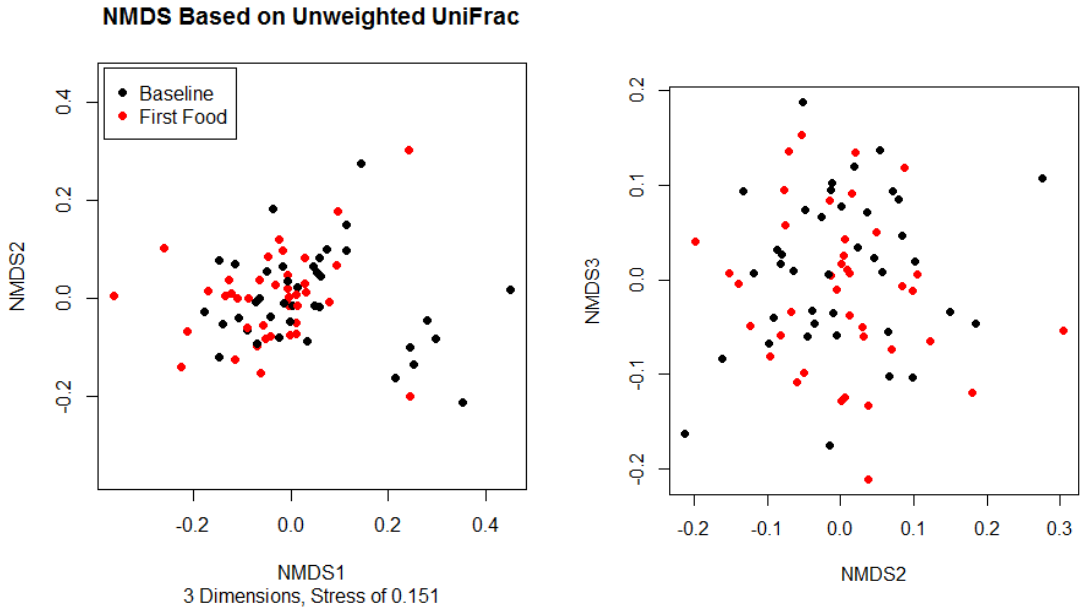

Figure 3.10: Unweighted UniFrac results. There is no visible separation between baseline and first food samples, despite the detected significant difference.

Discussion

The weaning period, when infants begin eating solid foods, is a critical period of development for the intestinal microbiome. The microbiome of breastfed infants is characterized by low diversity which makes it particularly susceptible to changes in microbial composition and function as new food substrates are introduced. Additionally, since it is recommended that infants be introduced to solid foods one at a time, studies on infants during the weaning period can offer unique insights into food-microbe relationships that are more difficult to observe in adult populations (43, 44).

In this randomized, cross-over study, infants were fed pears or sweet potatoes for 7 consecutive days, followed by a 4-day washout where they consumed breast milk only and then

they were fed the subsequent food for 7 consecutive days. Infants were followed until they were approximately one year old. Pears and sweet potatoes were chosen as the study foods because they differed greatly in their carbohydrate complexity. Analysis of glycans in pear and sweet potato showed that pears had significantly higher levels of fructose and arabinose than sweet potatoes which had higher levels of glucose and galactose. Microorganisms differ in their ability to utilize these glycans which are indigestible by the infant and, as such, glycans in foods may have the ability to modulate intestinal microbial community composition and function (38-40).

We found no differences in α-diversity or β-diversity before and after the consumption of the first food. However, we did observe changes in fecal organic acids (lactic, isobutyric, pyruvic, and valeric acids) and total glycans (arabinose, fructose, glucose, and sialic acid) before and after the consumption of the first food. A study conducted by Parkar et al (2021) showed that incubation of pre-digested foods with infant fecal bacteria for 10 hours resulted in changes in short chain fatty acid concentrations and glycosidase activity indicating altered microbial metabolism. Additionally, α-diversity significantly decreased and changes in β-diversity before and after fermentation were driven by *Veillonella*, *Bifidobacterium,* and *Enterobacteriaceae* (159). Our lack of observed changes in microbial community structure may be due to the relatively short feeding intervention time (7 days) or low consumption of the solid food compared to breast milk as breast milk was still the sole source of nutrition for these infants. While Group A consumed significantly more pear, sweet potato, and total food across the study period compared to Group B, this increase in solid food was likely not significant when compared to the amount of breast milk infants were consuming. As has been previously reported, *Bifidobacterium* strains were likely in the gut of all infants in this study as they continued to breastfeed throughout the course of the study (1, 21-23).

While we did not see any changes in microbial composition in this study, the changes in fecal organic acids and glycans we observed indicate a change in microbial functional metabolism as microbes already present in the gut are able to utilize new substrates for energy. Studies in animal models and humans have shown changes in microbial metabolism in response to the substrates available from the host diet. In a study conducted by Turnbaugh et al. (2014), participants who consumed a plant-based diet compared with an animal based-diet had significant increases in fecal acetate and butyrate. Microbial gene expression was strongly linked to diet composition indicating that changes in host diet altered microbial metabolic activity (160). Additionally, Gordon et al (2021) demonstrated that different fiber snacks altered microbial carbohydrate-active enzymes and metabolism both in gnotobiotic mice and humans (161). Snacks containing different fiber types differentially altered carbohydrate-active enzymes in both study groups. Notably, increases genes encoding enzymes involved in arabinose and xylose metabolism were observed after pea fiber consumption; and increases in glucanases and decreases in arabinan metabolizing enzymes were observed following barley bran consumption (161). The changes in microbial metabolites, specifically fecal organic acids, observed in our study following consumption of pear and sweet potato suggest changes in microbial metabolic activity associated with each dietary intervention. These organic acids, produced by microbial fermentation within the gut, have functional effects on the host including decreasing intestinal pH, creating an unfavorable environment for pathogens, modulating the immune system, upregulating intestinal barrier function, inhibiting pro-apoptotic responses, and transportation of these metabolites into the intestinal epithelium where they can be utilized by the host (19, 20, 31, 48, 85, 148-151).

In this study, parents reported increased in discomfort, flatulence and stool consistency (parents reported firmer stools more frequently) after consumption of solid foods. These effects may be explained by the appearance of undigested, bound glycans in the colon following consumption of pear and sweet potato. Studies on the effect of weaning cereals on GI tolerability have found no differences in GI symptoms or stooling patterns (162-164). These studies, however, did not investigate changes in microbial composition or metabolism. It is likely the changes we see in GI tolerability are due to the more complex carbohydrate composition of our study food compared to weaning cereal.

Together, these data suggest a novel approach in using foods with differing carbohydrate complexity to modulate the functional capacity and metabolic capabilities of the microbiome and is one of the first studies to investigate the effect of specific foods on microbial composition and metabolism in humans. One limitation of this study is the small sample size; the microbiome was only measured in 40 infants and fecal SCFA and glycans in 20 infants. Additionally, a longer intervention period may be necessary in order to see changes in microbial composition as solid food consumption is small compared to breast milk. Future studies should include more diverse classes of weaning foods to better understand the impact solids have on the infant microbiome.

Conclusion

A healthy intestinal microbiome in infancy is critical for lifelong health. The microbiome plays an important role in the development and maturation of the immune system which is not fully functional at birth (165). Beneficial bacterial such as *Bifidobacterium* play a key role in modulating the immune system and protecting the infant from potential pathogens by creating unfavorable environments for potential pathogens and upregulating intestinal barrier function and proinflammatory and apoptotic responses (19, 20). Intestinal dysbiosis in early life is of particular concern because perturbations in the infant microbiome have been associated with increased risk for metabolic, allergic, and auto-immune diseases later in life (28-30). Additionally, evidence suggests that abundances of beneficial *Bifidobacterium* species have decreased in the guts of breastfed babies from resource-rich nations over the past 100 years (24, 25). Intervention in early infancy when the microbiome undergoes profound shifts in composition, such as in early infancy and during weaning, may help to combat this dysbiosis. Additionally, studies in infants consuming solid foods for the first time are uniquely able to investigate individual food-microbe interactions. As microbiota adopted during the weaning period can persist into adulthood, a better understanding of food-microbe interactions may be important for understanding health and disease risk.

The infant microbiome may be uniquely susceptible to colonization with probiotic supplements. While studies in healthy adults have shown that probiotics likely have minimal effect on the overall intestinal microbial composition, several studies have shown that in preterm and term infants, colonization of the gut with supplemented bacteria can last up to one year postnatal (33, 34, 54, 97-102). This work further demonstrates that probiotic supplementation with *B. infantis* during infancy in exclusively breastfed infants can lead to persistent colonization up to

one-year postnatal and at one-year, differences in *B. infantis* colonization between supplemented and supplemented infants were seen even after cessation of breastfeeding.

Additionally, we have demonstrated that solid foods containing different plant-derived polysaccharides are able to modulate the functional capacity of the microbiome by altering microbial metabolism. Organic acids, which are produced by microbial fermentation of partially or non-digested polysaccharides, influence intestinal pH, provide energy for host cells, educate the immune system, and have anti-inflammatory and anti-microbial effects in the host. While changes in microbial composition and functional metabolism have previously been demonstrated in-vitro and in adults, to our knowledge, this is the first study to investigate the effects of specific foods with different plant-derived complex carbohydrates on the composition of the intestinal microbiome and microbial metabolism in infants (159-161). Although we did not see changes in microbial community structure, we observed changes in fecal organic acids and fecal glycans before and after the introduction of solid foods indicating changes in microbial utilization of new carbohydrate substrates and subsequent fermentation within the gut.

Future studies should focus on the effectiveness of probiotics to colonize the gut in formula-fed infants and should investigate the effects of probiotic supplements and changes in the microbiome on long-term health outcomes. Additionally, future work should further investigate food-microbe interactions in weaning infants to better understand different feeding practices on older infants. Longer feeding intervention periods and larger sample sizes may be required to see changes in microbial composition during weaning.

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