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Probiotic supplementation during infancy may decrease infant gut microbial diversity and the abundance of bacteria associated with health risks

Samantha Krystal, Daniela Garcia, Hasti Haghdadi, Anna Gao

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

SUMMARY As more evidence points to the health benefits of probiotic supplementation in adults, there has been a growing interest in understanding the effects of probiotic supplementation in infants. The literature to date suggests that breast-feeding introduces some of the same beneficial bacteria to the infant gut as are present in probiotics supplements, and thus, supplementing formula-fed infants is especially of interest. However, thus far, there is no clear consensus on whether or not infant supplementation would be beneficial. The main purpose of this study was to provide further insight into the effects of probiotic intake on the microbial diversity and composition of the infant gut microbiome. Our analyses revealed that probiotic supplementation may significantly decrease infant gut microbial diversity. Although our study did not find an increase in the abundance of beneficial bacteria in the probiotic supplemented cohort, it did find an association between probiotic use and the reduction of *Clostridium sensu stricto* 1, *Collinsella, Acinetobacter,* and *Erysipelatochlostridium*, all of which have been linked to health risks. Additionally, the former three of these reduced genera are known to colonize breast milk, as well as the gut microbiome of breast-fed infants. Therefore, our findings suggest that probiotic supplementation may especially benefit breast-fed infants by reducing the abundance of potentially harmful bacteria genera.

INTRODUCTION

Probiotics refer to live microorganisms that, when ingested, can beneficially affect the consumer (1, 2). Over the past few decades, probiotics have gained popularity in the food industry, as more research points to their health benefits in humans. Thus far, studies have shown that probiotics can help prevent inflammatory bowel syndrome (IBS), improve the immune system, reduce symptoms of lactose intolerance, reduce traveler's diarrhea, and improve gut microbial balance (1, 2). More recent studies have investigated the use of probiotics to treat skin and oral diseases, as well as anxiety and depression via the gut-brain axis (1, 2). The most widely used probiotics include species of Lactobacillus and Bifidobacterium, with the acquired health benefits being strain-specific (1, 2).

Both Lactobacillus and Bifidobacterium have been identified within the lactating breast microflora (3). This microflora has been shown to pass into the breast milk, and subsequently the breastfeeding infant, where it colonizes the infant's gut (4). Breast milk has also been shown to promote optimal infant growth and development and may also help prevent metabolic diseases such as obesity and type 2 diabetes (5). The World Health Organization recommends that in the first six months of life, infants should be exclusively breastfed (5). However, breastfeeding is not always a viable option due to contraindications and infant formula is often used as a substitute (6).

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Address correspondence to: Anna Gao,

anna.gao78@gmail.com

Due to the recognized health benefits of breastfeeding and probiotics and the overlap in their bacterial strains, there is growing interest in supplementing formula-fed infants with probiotics (7). However, there is limited and conflicting evidence on the benefits of infant probiotic supplementation (8). In support of probiotics, Indrio *et al.* showed that supplementing preterm, formula-fed infants with *Lactobacillus reuteri* prevents feeding intolerance and improves gut motor and immune function development (9). In contrast, Topcuoglu *et al.* showed that probiotics could increase the risk of vancomycin-resistant Enterococcus colonization (10). Further, a retrospective clinical comparison by Quin et al., revealed a correlation between probiotic use in infants and an increased risk of mucosal infections (8).

Given the conflicting evidence, the main motivation for this study was to establish whether probiotic supplementation effects the diversity and composition of the infant gut microbiome. To do so, we used a dataset compiled by Dr. Kyung Rhee from the department of Pediatrics at the University of California (11). This dataset includes fecal samples collected from 82 infant-mother dyads along with associated dietary information, such as probiotic intake and mode of feeding (breast, formula, or combined). Based on previous studies, we expected to see no significant differences in the diversity of the infant gut microbiome between those taking versus not taking probiotics (8, 12). However, we did expect to see a higher relative abundance of probiotic genera in the supplementing cohort compared to the non-supplementing cohort (8, 12). Although the composition of probiotics used in Dr. Rhee's dataset is not provided, we expected to see an increase in the relative abundance of Lactobacillus and Bifidobacterium based on the most commonly used probiotics (1, 2). In addition, since recent studies have shown that dendritic cells may carry gut bacteria from the maternal gut microbiome to the breast microflora via the entero-mammary pathway (13-15), we looked at both direct probiotic use by the infant, and indirect probiotic use, whereby the mother was taking probiotic supplements and breastfeeding. If maternal gut bacteria are in fact being transferred into the breast milk, we would expect direct and indirect probiotic supplementation to have similar effects on the diversity and composition of the infant gut microbiome. Given the current contradictory findings on infant probiotic supplementation, our study may provide more clarity on the possible health benefits of probiotic use in infants.

METHODS AND MATERIALS

Dataset description. The dataset used in our study was obtained from Dr. Kyung Rhee, from the University of California. This unpublished dataset consists of Illumina sequences of the V4 region of 16S rRNA from the stool samples of 82 mother-infant dyads collected at 2 weeks, 2 months, 4 months, 6 months, 9 months, and 12 months of life (11). The Illumina sequences were obtained via the Earth Microbiome Protocol using the primers 515fbc and 806r (16). The metadata includes 171 accompanying medical and dietary categories, including information on infant and maternal probiotic use. The dataset files are publicly accessible on the European Nucleotide Archive (ENA) Browser via accession PRJEB39437 (11).

Filtering and reformatting the metadata in R. To focus our study on probiotic use in infants, we filtered and reformatted the data using the tidyverse package in R version 4.0.2(17, 18). First, we generated two new columns in the metadata file based on the information provided on infant and maternal probiotic use, as well as on mode of feeding: 1) a 'probiotic_mode' column, in which the sample was labelled as "direct" if the infant was taking probiotics directly, and labelled as "indirect" if the mother was taking probiotics and breastfeeding, and 2) a 'probiotic' column in which the sample was labelled as "yes" to probiotics if taken directly or indirectly, and "no" to probiotics if not taken at all (Fig 1). Following this reformatting, we filtered out samples from mothers so that only samples from infants remained. Then, since samples from infants were collected at multiple time points, we filtered the data to only include the earliest collected sample from each infant. Therefore, each infant was only represented once in downstream analyses. Our reasoning for choosing the earliest time point was based on a study by Quin *et al.*, who found that probiotics have the most significant effect on the infant gut microbiome composition in the first week of life (8). After filtering, we were left with 52 samples from 0.5-month-old infants, 15 samples from 2-

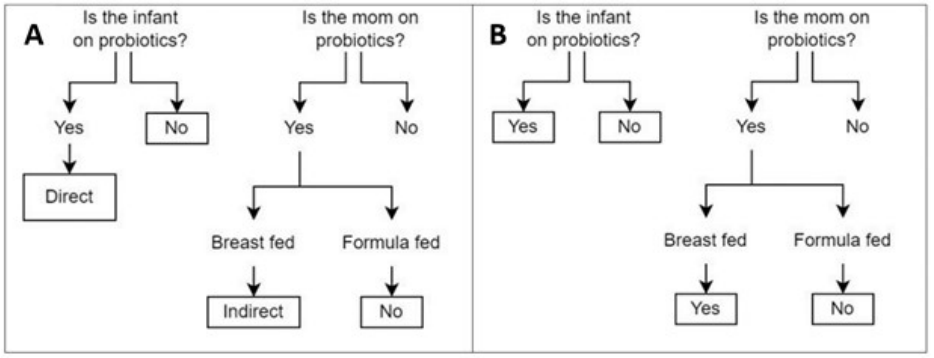

FIG. 1 New columns were generated in the metadata file. In the metadata file, we generated two new columns in R: **(A) '**probiotic_mode' and **(B)** 'probiotic'. The words highlighted in boxes were used to label the samples in their respective columns.

month-old infants, and 5 samples from 4-month-old infants, all of which were breastfed or combined-fed. More detailed steps on how we filtered and reformatted the metadata are outlined in Script 1.

Filtering the manifest file in R. The Illumina sequences from ENA were previously demultiplexed, courtesy of Mihai Cirstea from the University of British Columbia's department of Microbiology and Immunology. These demultiplexed sequences were available as a manifest file in the MICB 447 infant directory in QIIME2 version 2021.4 (19). We imported the manifest file into R and filtered out the same samples that were filtered out in the metadata file to make the artifacts compatible in QIIME2. This step is outlined in Script 1.

Preliminary processing in QIIME2. To work within the QIIME2 pipeline, we imported the filtered and reformatted metadata file and the filtered manifest file using the semantic type, 'SampleData[SequencesWithQuality]'. We then used the demultiplexed sequences within the manifest file to visualize the quality of the sample reads. Since all quality scores were above 30, we did not truncate the reads during the sequence quality control step, which was performed with Divisive Amplicon Denoising Algorithm 2 (DADA2) to output representative sequences and a feature table of amplicon sequence variants (ASVs) (20). We then taxonomically classified the data using the SILVA database and filtered out mitochondria and chloroplast ASVs from the feature table (21). To analyze differences in the infant gut microbiome based on probiotic use, we filtered out samples that were missing information in the 'probiotic_mode' and 'probiotic' columns. Preliminary processing steps are outlined in detail in Script 2.

Alpha and beta diversity analyses in QIIME2. To run diversity metrics that consider phylogenetic distance, we made a phylogenetic tree using the representative sequences generated during the quality control step. We then chose a rarefaction depth of 21,146 to maximize the number of features (52.65%) and the number of samples (83.10%) retained, as well as to keep at least 3 samples within each category of the 'probiotic_mode' column. We subsequently used the qiime diversity core-metrics-phylogenetic method to calculate alpha and beta diversity metrics and to produce beta diversity PCoA plots. The PCoA plots were regenerated in R as outlined in Script 3. We used the alpha diversity metrics to generate boxplots for Pielou's evenness, Shannon's index, observed features, and Faith's phylogenetic diversity and Kruskal-Wallis pairwise comparisons to evaluate q-values and significance ($q < 0.05$). We then used the beta diversity metrics to generate boxplots for unweighted UniFrac, weighted UniFrac, Bray-Curtis, and Jaccard, and used PERMANOVA to evaluate q-values and significance ($q < 0.05$). Subsequently, we used adonis to account for the confounding variables, mode of feeding (breast, formula, or combined), age (0.5, 2, or 4 months), and mode of delivery (Caesarian section versus vaginal), for unweighted UniFrac. These steps are outlined in Script 2.

Generating differential and relative abundance plots in R. To make a differential abundance plot of those taking probiotics relative to those not taking probiotics, we generated and exported a biom and a tree file in QIIME2 and imported these files into R along with the metadata. These files were then combined into a phyloseq object using the phyloseq package

(22). Based on the location of an abrupt drop in the number of reads per sample, we decided on a pruning depth of 1533 to keep samples with at least 1533 reads. Additionally, features with a relative abundance below the set threshold (0.0005) were removed. Using the DESeq2 package (23), we set the taxonomic level for analysis to genus and the alpha level to 0.05. These steps outputted genera significantly different in abundance ($p < 0.05$) between those taking versus not taking probiotics. These genera were then used to generate a differential abundance plot with ggplot2 (24). Next, we calculated the relative abundance of the genera outputted by the differential abundance analysis and again, excluded features with a relative abundance below the set threshold (0.0005). The relative abundance plots were subsequently made with ggplot2 (24) by using the calculated relative abundance of the genera. Notably, the tidyverse, vegan, and ape packages were also used for these analyses (17, 25-26). Details of these steps are outlined in Script 2 and Script 3.

RESULTS

Probiotic use may decrease the average abundance and richness of microbes in the infant gut microbiome. To compare the diversity of the gut microbiome of infants taking versus not taking probiotics, we ran alpha diversity analyses using both the 'probiotic_mode' and 'probiotic' columns in the metadata. The results showed no significant differences between those taking probiotics directly versus indirectly. Therefore, we decided not to differentiate between direct and indirect probiotic use in downstream analysis. There were, however, significant differences in Pielou's evenness and Shannon's index for "yes" versus "no" probiotic use and "indirect" versus "no" probiotic use, as well as in observed features for "yes" versus "no" probiotic use and "direct" versus "no" probiotic use. There were no significant differences in Faith's phylogenetic diversity. q-values for each comparison are summarized in Table 1. The alpha diversity boxplots (Fig. 2, S1) revealed that the median of those taking probiotics was lower than the median of those not taking probiotics for all significantly different alpha metrics. Therefore, these results suggest that probiotic use in infants may decrease the evenness, abundance, and richness of bacterial species in the gut, regardless of whether they are taken directly or indirectly.

TABLE. 1 Alpha diversity metrics yield significant differences between those taking versus not taking probiotics regardless of probiotic mode. q-values for alpha diversity metrics were determined using Kruskal-Wallis pairwise comparisons. Columns indicate q-values for comparisons between specified groups with significant values bolded and referenced as * < 0.05 and ** < 0.01. Alpha diversity boxplots of those taking versus not taking probiotics are shown in Figure 2. Refer to Supplementary Figure 1 for all other alpha diversity box plots.

Metric	**Direct versus Indirect probiotic intake (q-value)**	**Taking versus not taking probiotics (q-value)**	**Direct intake versus not taking probiotics (q-value)**	**Indirect intake versus not taking probiotics (q-value)**
Pielou's evenness	0.236	**0.035***	0.721	**0.033***
Shannon's index	0.236	**0.011***	0.268	**0.036***
Observed features	0.289	**0.004****	**0.028***	0.137
Faith's phylogenetic distance	0.433	0.469	0.433	0.878

Probiotic use may affect the phylogenetic relatedness between bacterial species in the infant gut microbiome. To further analyze the diversity of the infant gut microbiome, we generated beta diversity PCoA plots and boxplots using the 'probiotic_mode' and 'probiotic' columns in the metadata (Fig 3, S2). The PCoA plots revealed some clustering of data points from the "yes" probiotic cohort in both the unweighted and weighted UniFrac plots. However, there were only 7 "yes" data points, and they overlapped with the data points from the "no" probiotic cohort. The statistical tests showed no significant differences between those taking probiotics directly versus indirectly but did reveal a significant difference in unweighted UniFrac diversity for "yes" versus "no" probiotic use as summarized in Table 2. After using adonis to adjust for the confounding variables of mode of feeding (breast, formula, or combined), age (0.5, 2, and 4 months), and mode of delivery (Caesarean section or vaginal), there was still a significant difference in unweighted UniFrac diversity.

Therefore, these results suggest that probiotic use may increase or decrease the presence of certain taxa, thereby affecting the phylogenetic relatedness of microbes in the infant gut microbiome regardless of feed type.

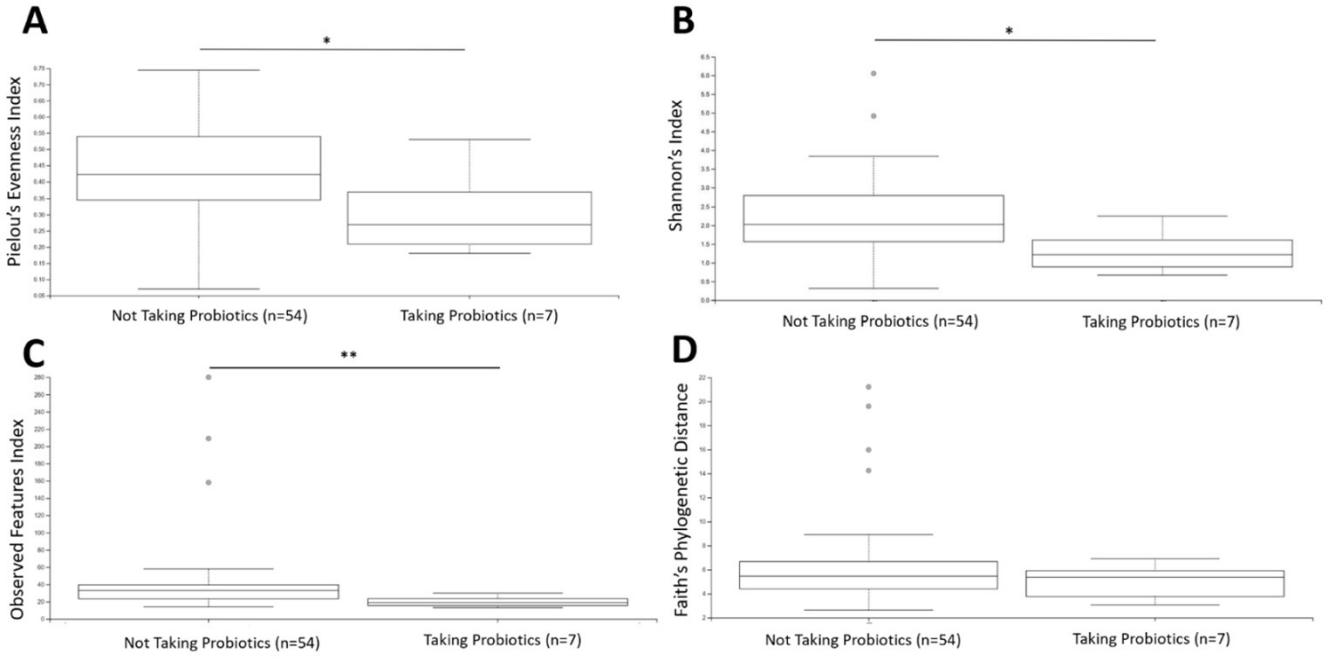

FIG. 2 Probiotic use may decrease the evenness, abundance, and richness of bacterial species in the infant gut microbiome. Alpha diversity metrics between those taking versus not taking probiotics were visualized as boxplots. Kruskal-Wallis pairwise comparisons were used to calculate q-values and significance (* = $q < 0.05$, ** = $q < 0.01$). All q-values for alpha diversity analyses are outlined in Table 1. The overall alpha diversity of the infant gut microbiome is significantly different between those taking (n = 7) versus not taking probiotics (n = 54) for **(A)** Pielou's evenness (q-value = 0.035)**, (B)** Shannon's index (q-value = 0.011), and **(C)** observed features (q-value = 0.004)**.** The median for those taking probiotics is lower than for those not taking probiotics for all three metrics. **(D)** There is no significant difference for Faith's phylogenetic distance (q-value = 0.469).

TABLE. 2 Unweighted UniFrac beta diversity metric yields significant difference between those taking versus not taking probiotics. q-values for beta diversity metrics were determined using PERMANOVA. Columns indicate q-values for comparisons between specified groups with significant values bolded and referenced as * < 0.05. The unweighted UniFrac beta diversity box plot for those taking versus not taking probiotics is shown in Figure 3. Refer to Supplementary Figure 2 for all other beta diversity boxplots.

Metric	**Direct versus Indirect probiotic intake (q-value)**	**Taking versus not taking probiotics (q-value)**	**Direct intake versus not taking probiotics (q-value)**	**Indirect intake versus not taking probiotics (q-value)**
Unweighted UniFrac	0.467	**0.042***	0.390	0.387
Weighted UniFrac	0.315	0.096	0.466	0.466
Bray-Curtis	0.875	0.541	0.875	0.875
Jaccard	0.281	0.115	0.060	0.606

Probiotic supplementation may decrease the abundance of *Clostridium sensu stricto 1, Collinsella, Acinetobacter,* and *Erysipelatochlostridium* in the infant gut microbiome. To identify the taxa responsible for the significant differences revealed in the diversity analyses, we generated a differential abundance plot of those taking probiotics relative to those not taking probiotics (Fig 4). The plot revealed a significantly lower ($p < 0.05$) abundance of the genera, *Clostridium sensu stricto 1, Collinsella, Acinetobacter,* and *Erysipelatochlostridium* in the gut microbiome of those taking probiotics. To further investigate the genera identified in the differential abundance plot, we then ran relative

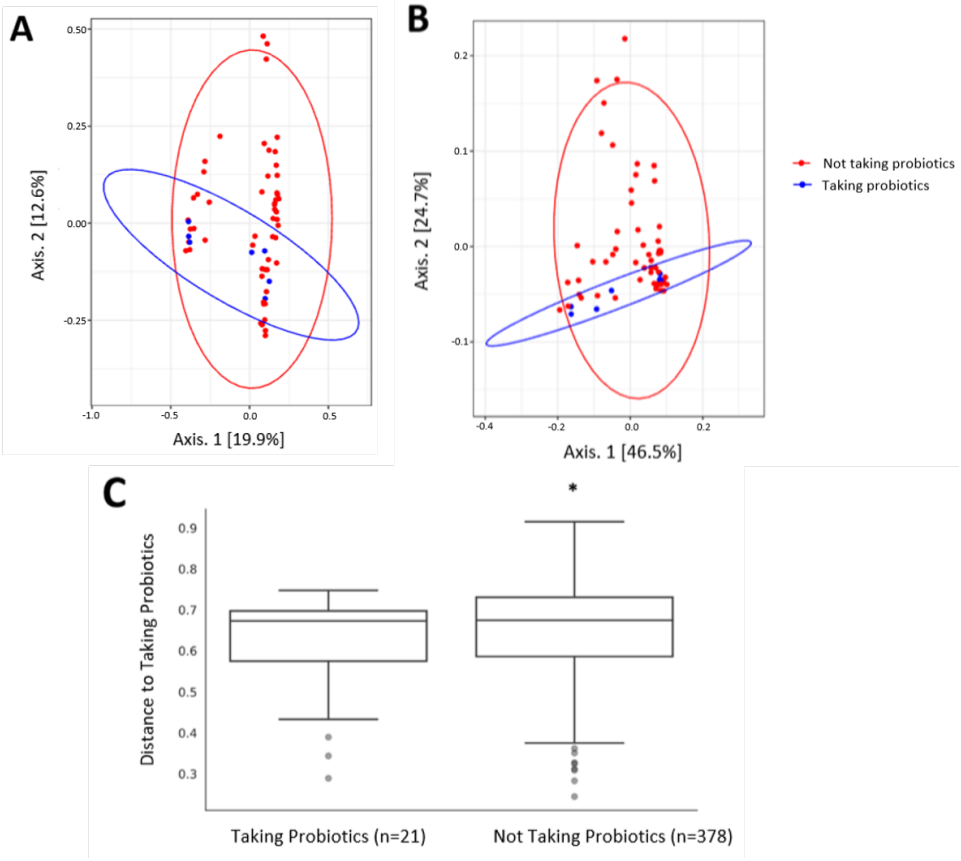

FIG. 3 Probiotic use may change the phylogenetic relatedness of bacterial species in the infant gut microbiome. The clustering patterns of infant gut microbiome samples were visualized on PCoA plots using **(A)** unweighted UniFrac and (**B)** weighted UniFrac analyses in R. Samples for those taking probiotics (n = 7) are represented in blue and samples from those not taking probiotics are represented in red (n = 54). Ellipses represent 95% confidence intervals. **(C)** The unweighted UniFrac beta diversity between those taking versus not taking probiotics, relative to the distance of those taking probiotics, was visualized as a boxplot. Using PERMANOVA, the q-value was determined to be 0.042 (* = q < 0.05). All q-values for beta diversity analyses are outlined in Table 2.

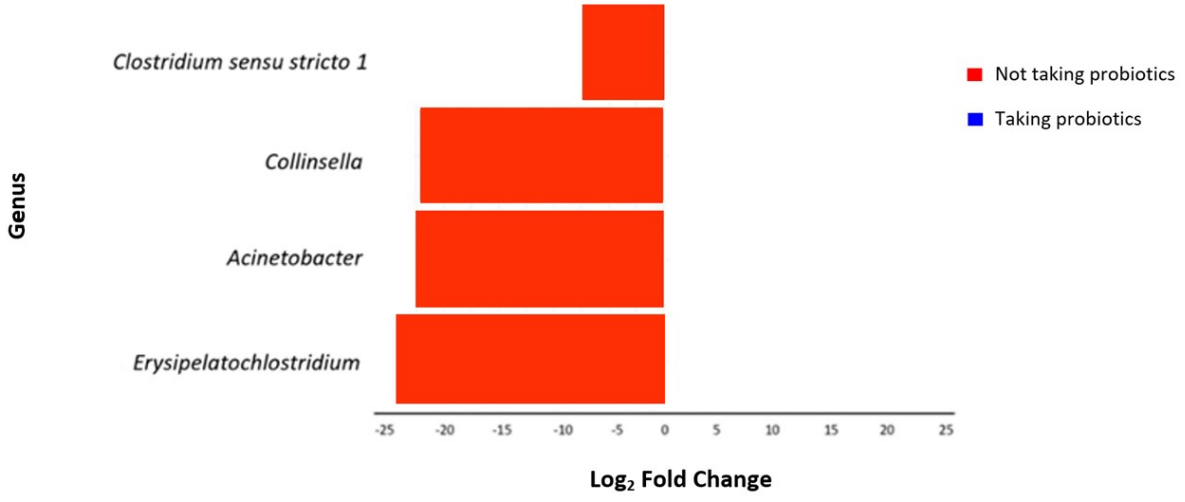

FIG. 4 Probiotic use may decrease the differential abundance of *Clostridium sensu stricto 1*, *Collinsella*, *Acinetobacter*, and *Erysipelatochlostridium* in the infant gut microbiome. Taxonomic differences at the genus level were visualized on a differential abundance plot to look at those taking probiotics (n = 7; blue) relative to those not taking probiotics (n = 54; red). *Clostridium* sensu *stricto 1*, *Collinsella*, *Acinetobacter*, and *Erysipelatochlostridium* are less abundant in those taking probiotics compared to those not taking probiotics by 7.3, 22.1 22.5, and 24.2 Log_2 fold change, respectively ($\alpha = 0.05$).

abundance analyses for each (Fig 5). For all four genera, we were unable to resolve the medians because they were close to zero on the y-axis, with significant differences being almost entirely accounted for by outliers in the no probiotic cohort. Therefore, although the

differential abundance plot suggests that probiotic use may decrease the abundance of *Clostridium sensu stricto 1*, *Collinsella, Acinetobacter,* and *Erysipelatochlostiridum,* the relative abundance plots suggest that these genera are present in a relatively low abundance for both cohorts, and the significant differences between cohorts may simply be the result of outliers.

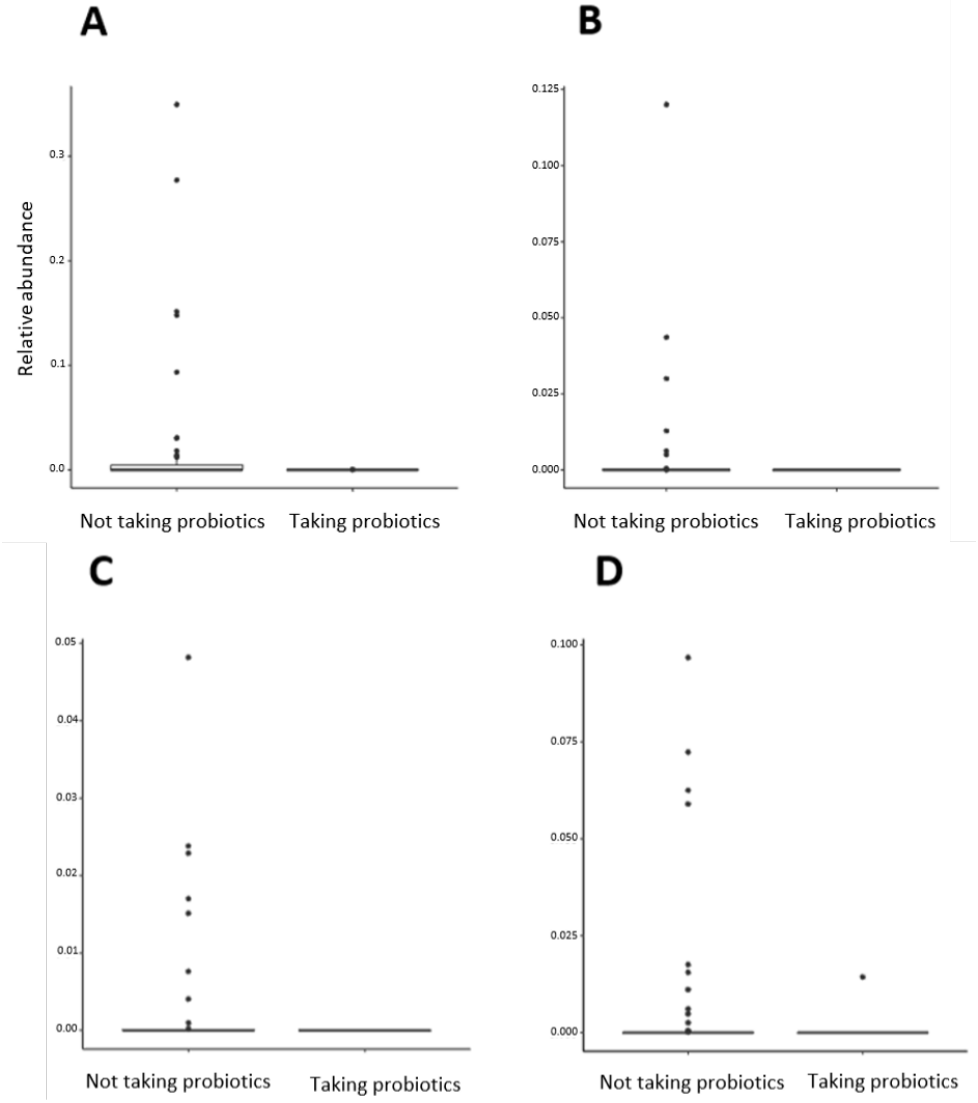

FIG. 5 Probiotic use may decrease the relative abundance of *Clostridium sensu stricto 1*, *Collinsella*, *Acinetobacter*, and *Erysipelatochlostridium* in the infant gut microbiome. Relative abundance plots for those taking versus not taking probiotics were visualized for **(A)** *Clostridium sensu stricto 1* (2 reads for taking probiotics; 23 reads for not taking probiotics) **(B)** *Collinsella* (0 reads for taking probiotics; 9 reads for not taking probiotics) **(C)** *Acinetobacter* (0 reads for taking probiotics; 9 reads for not taking probiotics) and **(D)** *Erysipelatochlostridium* (1 read for taking probiotics; 14 reads for not taking probiotics).

DISCUSSION

Our study aimed to investigate the effects of probiotic supplementation on the infant gut microbiome. Using the dataset compiled by Dr. Rhee, we found significant differences in the diversity and composition of the infant gut microbiome between those taking versus not taking probiotics.

Probiotic use may decrease the presence of low abundant taxa and change the phylogenetic relatedness of microbes in the infant gut microbiome. Alpha diversity analyses revealed significant differences in Pielou's evenness, Shannon's index, and observed features for those taking versus not taking probiotics, with a lower median diversity across

all three metrics for those taking probiotics. However, there was no significant difference in Faith's phylogenetic distance. These results suggest that probiotics may decrease the average abundance of certain taxa. Furthermore, these findings are consistent with our differential abundance analysis, which found a decrease in the abundance of four specific genera in the supplementing cohort.

Beta diversity analyses showed a significant difference in unweighted UniFrac diversity between those taking versus not taking probiotics, even after accounting for mode of feeding (breast, formula, or combined), age (0.5, 2, and 4 months), and mode of delivery (Caesarean section or vaginal). This result suggests that probiotics may increase or decrease the presence of certain taxa, resulting in changes to the phylogenetic relatedness of microbial communities in the gut. This is inconsistent with the alpha diversity analysis, which found no significant differences for Faith's phylogenetic diversity: a metric that considers phylogenetic relatedness. However, alpha diversity analyses compare the average diversity between groups, whereas beta diversity analyses are more complex as they compare multiple samples in one group to multiple samples in another group.

Weighted UniFrac diversity considers the phylogenetic relatedness between species as well as the abundance of those species. Therefore, low abundant taxa carry little weight when calculating this metric. In contrast, unweighted UniFrac diversity only considers phylogenetic relatedness; therefore, low abundant taxa carry just as much weight as highly abundant taxa. Since there was no significant difference in weighted UniFrac diversity, but there was a significant difference in unweighted UniFrac diversity, these results suggest that probiotics may be affecting rare or low abundant microbes. This finding is consistent with our abundance analyses; specifically, our differential abundance plot showed a decrease in certain taxa, explaining the changes to phylogenetic relatedness, and our relative abundance plots showed medians close to zero, supporting that the affected taxa are present at a relatively low abundance for both cohorts.

Based on the studies by Quin *et al.* (8) and Yousuf *et al*. (12), we did not expect to see significant differences in diversity between those taking versus not taking probiotics. However, upon further review of the literature, we found two additional sources on infant probiotic supplementation that are congruent with our findings. Specifically, Hui *et al.* found no significant differences in Inverse Simpson, but found a slight decrease in Shannon's index in preterm infants taking versus not taking probiotics (27). Since Shannon's index is more sensitive to rare taxa than Inverse Simpson, the authors concluded that the decline in alpha diversity is due to the reduction of rare species (27). Additionally, they found that probiotics had a greater effect on the unweighted UniFrac diversity of the infant gut microbiome compared to the weighted UniFrac diversity, which they also attributed to changes in rare taxa (27). As well, Gong *et al.* found a decrease in the alpha diversity of the gut microbiome of preterm infants taking probiotics compared to those not taking probiotics and concluded that this finding was due to a decrease in the presence of harmful bacteria (28).

Furthermore, our study found no significant differences in the alpha or beta diversity of the gut microbiome between infants taking probiotics directly versus indirectly. Based on studies regarding the entero-mammary pathway (13-15), as well as the study by Quin *et al.* (8), this lack of significance was as expected. Since there were no significant differences in diversity between these two groups, we decided not to differentiate between direct and indirect probiotics in downstream analyses.

Probiotic supplementation might not increase the abundance of probiotic genera in the infant gut microbiome. Based on previous studies (8, 12) and the most commonly used probiotics (1, 2), we expected to see a higher abundance of *Bifidobacterium* and *Lactobacillus* in the supplementing cohort compared to the non-supplementing cohort. However, a differential abundance analysis did not show a significant increase in either of these genera. Since the composition of probiotics in Dr. Rhee's dataset was not provided, it is possible that each infant in the probiotic supplemented cohort was administered a different type of probiotic and, as a result, no specific genus was present at a high enough level to cause a measurable increase.

Another possible explanation is the natural acquisition of probiotic bacteria that occurs with age. Previous studies have shown that *Bifidobacterium* start colonizing the gut post gestation, with measurable amounts being detected in fecal samples after one week of life

(29) . In the study by Quin *et al.,* significant differences in the abundance of *Bifidobacterium* in those taking versus not taking probiotics were only observed within the first week of life (8). Furthermore, although Yousef *et al.* found significant increases in *Bifidobacterium*, they were looking at pre-term infants, who often have delayed colonization of probiotic species (12). Since the infants in our study were 2+ weeks of age, the natural acquisition of probiotic bacteria over time may have narrowed the gap in probiotic abundance between those taking versus not taking probiotic supplements.

Lastly, the lack of increase in probiotic genera may suggest that the supplemented bacteria did not colonize the infant gut microbiome. However, the likelihood of this interpretation cannot be determined, as information on factors that impact the persistence or transience of supplemented bacteria (e.g., probiotic composition, dosage, and length of administration) (12) were not provided in Dr. Rhee's dataset.

Probiotics may decrease the abundance of genera associated with health risks. Despite not observing an increase in probiotic genera, differential abundance analysis revealed a decrease in the relative abundance of *Clostridium sensu stricto 1, Collinsella, Acinetobacter,* and *Erysipelatochlostridium* in the gut microbiome of those taking probiotics compared to those not taking probiotics. However, it is important to note that based on the relative abundance plots, these differences may simply be the result of outliers in the non-supplementing cohort.

According to the literature, all of these reduced genera have primarily been associated with health risks when overly represented in the gut. For example, an overabundance of *Clostridium sensu stricto 1* has been linked to antigen-specific IgE in infants with food allergies (30). As well, this genus has been shown to be more abundant in rats with visceral hypersensitivity (VH), which is a characteristic of IBS (31). Interestingly, specific probiotic administration in VH rats has been shown to reduce the abundance of *Clostridium sensu stricto 1* along with a reduction in *visceral sensitivity* (27). This notable reduction in the abundance of *Clostridium sensu stricto 1* is consistent with our findings, which suggest that probiotics may decrease this genus.

Furthermore, an over representation of *Collinsella* has been linked to obesity, type 2 diabetes mellitus, and atherosclerosis, as well as increased levels of cholesterol and low-density lipoprotein (LDL) (32). Additionally, both *Collinsella* and *Acinetobacter* have been found in a higher abundance in patients with non-alcoholic hepatic steatosis (33). As well, an accumulation of *Collinsella* and/or *Erysipelatochlostridium* in the gut has been linked to infant bronchiolitis (34). High levels of *Erysipelatochlostridium* have also been associated with gout (35) and Crohn's disease (36). Further, an overabundance of this genus may be a marker for the onset of pro-inflammatory diseases, obesity, and metabolic disorders (37-38), and has been shown to correlate with elevated host cholesterol metabolites (39).

Although *Acinetobacter* may have an allergy protecting effect in infants living in rural environments (40), high levels of *Acinetobacter* have been linked to obesity (41) and multiple sclerosis (42). Further, in preterm infants, *Acinetobacter* has been shown to be the most common cause of nosocomial infections (43) and the gut microbiome of infants with acute Kawasaki disease has been characterized by a significantly higher abundance of *Acinetobacter* compared to healthy controls (44). Therefore, taken together, these results suggest that probiotic use in infants may reduce the abundance of harmful bacteria, which may be beneficial to their health.

Probiotics may be beneficial to breastfed infants. According to the literature, *Clostridium sensu stricto 1, Collinsella,* and *Acinetobacter,* are typically more abundant in the gut microbiome of breastfed infants compared to formula-fed infants (30, 45-52). Therefore, although there is a growing interest in probiotic supplementation for formula-fed infants, our study suggests that probiotics may be beneficial to breastfed infants; specifically, because these potentially harmful genera are typically more abundant in breastfed infants and our findings illustrate an association between probiotic use and a lower incidence of these genera. Notably, since all of the probiotic supplemented infants in Dr. Rhee's dataset were breastfed or combined-fed, we cannot make any conclusions on the benefits of supplementing formula-fed infants.

Interestingly, by using fecal pH as a proxy for the abundance of infant-associated *Bifidobacterium,* Henrick *et al.* reported a generational loss of *Bifidobacterium* in breastfed

infants, in resource-rich nations, within the last 100 years (53). This notable decrease in highly specialized *Bifidobacterium* has been linked to infant intestinal dysbiosis, accompanied by higher levels of *Enterobacteriaceae*, *Clostridiaceae*, *Peptostreptococcaceae*, and *Veillonellaceae* (53). Based on this study, O'Brien *et al.* looked at the use of probiotics for the restoration of the gut microbiome of breastfed infants (54). They found that supplementing breastfed infants with the probiotic *Bifidobacterium longum* subsp. *Infantis* within the first month of life resulted in the stable colonization of *B. infantis* even one year later (54). These studies support our findings, which suggest that probiotics may be useful in optimizing the gut microbial composition and consequently, the health of breastfed infants.

Limitations Although our study provides a preliminary analysis on the potential benefits probiotic supplementation may have on the infant gut microbiome, there are several limitations to be considered. Firstly, as the participant samples were collected from fecal material, they may not fully represent the microbiome that remains within the infant's gut. Rather, the microbiome reflected by the fecal matter may be the result of more transient microbes or feature a skewed representation of the composition of the colonic microbiome. Additionally, as this dataset featured a limited number of infants taking probiotics, the significant findings featured in this study may not be representative of a larger population and may instead be influenced by the small sample size. This limitation is illustrated in the relative abundance plots, where we could not confidently conclude whether the outliers in the non-supplemented group were true outliers or if they were representative of the differences between the cohorts. A larger sample size of those on probiotics would make this uncertainty clearer. Furthermore, since Dr. Rhee's study was not focused on probiotics, there is no information provided on the composition, dose, or timing of probiotic supplementation. Any inconsistencies within these metrics may influence potential correlations that exist with probiotic use. Although we were able to account for age differences, mode of delivery, and mode of feeding by using adonis, there are many other confounding variables that we did not account for, such as infant-to-infant biological variation, infant weight, and infant diarrhea.

Conclusions In summary, this study investigated the effect of probiotic use on the diversity and composition of the infant gut microbiome. All infants taking probiotics were either breastfed or combined-fed and between the ages of 0.5 to 4 months. The observed significant differences in alpha and beta diversity metrics that are sensitive to rare taxa suggest that supplementation may decrease the abundance of low abundant taxa. Upon further investigation of taxonomy, differential abundance analyses reveal a decrease in Clostridium sensu stricto 1, Collinsella, Acinetobacter, and Erysipelatochlostridium in the supplementing versus non-supplementing cohort. Since previous studies have linked an overabundance of these genera to health risks, and the former 3 genera are reportedly more abundant in breastfed infants, compared to formula-fed infants, our study suggests that probiotic supplementation may benefit breastfed infants especially despite the current focus of probiotic supplementation for formula-fed infants.

Future Directions In the future, Dr. Rhee's dataset could be further used to investigate the infants longitudinally, in order to analyze probiotic intake and its relationship to the gut microbiome over time. As our study only examined the youngest time point, there may be further temporal changes that exist that relate the infant gut microbiome to probiotic supplementation.

In our study, there were no significant differences found in the diversity of the gut microbiome between infants taking probiotics directly versus indirectly. Indirect supplementation was likely similar to direct supplementation due to the recently discovered entero-mammary pathway (13-15). In the future, Dr. Rhee's dataset could be used to further examine this phenomenon by comparing the maternal gut microbiome to that of their infant's when breastfeeding versus formula-feeding, since their microbiomes are expected to be more similar when breastfeeding.

In Dr. Rhee's dataset, all infants taking probiotics were also breastfed, or combined-fed, so we could not examine the relationship between probiotics and mode of feeding (breast versus formula). In the future, a more targeted and larger data collection of breastfed and

formula-fed infants who are taking versus not taking probiotics could be used to investigate this relationship.

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CONTRIBUTIONS

Co-authorship should be considered equal for this manuscript. S.K. worked on the Abstract and Introduction. D.G. and H.H. worked on the References. A.G. worked on the Abstract and formatting of figures. All authors contributed to the Methods and Materials, Results, and Discussion sections, as well as data analysis in the scripts, and overall revision of the manuscript.

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