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Probiotic use results in transient benefits due to changes in microbial community structure in the infant gut microbiome

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SUMMARY Probiotics are supplements containing various microorganisms capable of conferring health benefits to individuals that consume them. As such, they are widely used amongst children and adults, however, relatively little research has been done on their effect in infants. Studies on the effects of probiotic use have been conflicting, with there currently being no general consensus on whether they are truly beneficial or not. In this study, we sought to determine whether probiotic use has an effect on the infant gut microbiome, both overall and long-term. This study was conducted using various bioinformatics platforms to analyze a dataset containing information on 325 mother-infant dyads developed by Dr. Kyung Rhee. We examined the effect of both infant (direct) and mother (indirect) use of probiotics. While investigating the long-term effects of probiotic use, we looked at a cohort of subjects that had taken probiotics at the same time point to determine a potential before and after effect. We found that probiotic use can result in a reduction in the prevalence of amplicon sequence variants affiliated with harmful gut bacteria. However, we had also found through a time point analysis that this effect is not sustained after probiotic supplementation has ceased. These findings may provide some insight into whether probiotics should be provided to infants and possibly the intervals in which they should be provided.

INTRODUCTION

The role of probiotic use in influencing the human gut microbiome has been a major area of microbial research in recent decades (1, 2). Probiotics are defined as live microorganisms that are administered to potentially confer health benefits on the host (2). Previous studies have indicated that healthy gut microbiomes consisting of these beneficial organisms have been shown to help improve immune system function, maintain integrity of the gastrointestinal lining, and regulate gut motility (3). In a study conducted by Karczewski et al, they found that *Lactobacillus plantarum*, which is considered a "good bacterium", was involved in the maintenance of the epithelial barrier in the human gut against bacterial toxins and other gut bacteria; without its protection resulted in cases of inflammation and gastrointestinal complications (3, 4). Another study by Machiels et al found that a decrease in butyrate-producing probiotic species such as *Roseburia hominis* and *Raecalibacterium prausnitzii* is associated with ulcerative colitis, which demonstrates that these probiotics are critical in maintaining a healthy gut (5). Moreover, Derrien et al suggested that the *Bifidobacterium* species can aid the process of breaking down proteins and human milk oligosaccharides and tends to dominate during the third to fourteenth months of birth (6). Since these microbes found in the gut play an important role in regulating gut function, an active area of research currently investigates whether the gut microbial composition and diversity can be altered through the use of probiotics to potentially benefit the host (2).

As previous research seems to suggest that the colonization of the gut microbiome during infancy plays a key role in establishing susceptibility to diseases, altering the infant gut microbiome through the introduction of probiotics could be a strategy that confer host benefits (2). Despite research on probiotic use in infants has been done in the past few decades, there is no general consensus on whether it has a significant impact on the gut microbiome and its long-term use and consequences were not well understood (7). In a double-blind, randomized, placebo-controlled trial, Rutten et al found that despite a higher abundance of supplemented probiotic strains found in fecal samples during supplementation, these differences were minor and short-term, suggesting that probiotics have no significant impact on long-term gut

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microbiome composition (7). Another retrospective clinical study done by Quin et. al suggested that probiotic use did not lead to significantly better health outcomes in infants, and instead resulted in a higher frequency of oral, respiratory and gastrointestinal infections (2). Contrastingly, Niers et al found that a combination of probiotics helped prevent the incidence of eczema in high-risk children and that this preventative effect can be established with probiotics when taken during the first three months after birth (8). Given these conflicting findings, our study sought to further investigate the effect of infant and mother probiotic use on infant gut microbiome and examine the effects of before and after probiotic use in infants. Mother probiotic use was also examined since previous literature has indicated mixed evidence regarding their impacts on the infant gut microbiome (9).

In order to evaluate the impact of probiotic use of infant gut microbiota, we used a public dataset produced by Dr. Kyung Rhea (1). The dataset was generated with the primary aim of examining how the infant gut microbiome was impacted by feeding behaviours and how that could lead to infant obesity. Stool samples were collected from 325 mother-infant pairs from Michigan, United States, at 0.5 months, 2 months, 4 months, 6 months, 9 months, and 12 months in order to analyze their microbial diversity and composition. Factors such as antibiotic use and probiotic use were collected to determine their effect on the infant growth trajectory by tracking their height and weight (1). Despite collecting metadata relevant to probiotic use in infants, Rhee and colleagues did not investigate much in this area. Here we sought to expand upon the work of Rhee and colleagues to evaluate the impact of long-term probiotic use on infant gut microbiota and determine results in an area of research where there is no clear consensus. Our first aim is to determine the effect of infant and mother probiotic use on the infant gut microbiome, with infant use being direct use, and mother being indirect use of probiotics. Our second aim is to conduct a time-point analysis to determine whether before and after probiotic use at the 2-month time point would have an effect on the infant gut microbiome.

In assessing and contributing to prior research, we hypothesize that mother and infant probiotic use has no significant impact on the infant gut microbiome diversity, but may have an impact on microbial abundance based on previous literature (2). Moreover, based on previous research done by Rutten et al, we hypothesize that there may be minor changes in infant gut microbiome composition and diversity during the probiotic supplementation period, however, these changes are not long-lasting once probiotic use terminates (6). We predict that there will be a higher relative abundance of *Bifidobacterium* species since this genus tends to dominate during an infant's third to fourteenth months, however, this could change significantly as a result of probiotic use (6). Our findings will contribute to the ongoing discussion of conflicting research regarding the effect of probiotics on the infant microbiome.

METHODS AND MATERIALS

Dataset/Dataset Description. The dataset used for this analysis was generated by Dr. Kyung Rhee and her team at the University of California San Diego and is available on the European Nucleotide Archive (ENA) Browser under the accession no. ERP122953 (1). The sequences were taken from the V4 region of 16s rRNA gene, which is highly used to distinguish bacterial species and strain and obtained following the Earth Microbiome Protocol 9 (10). They collected various metadata categories including several anthropometric measurements, levels of appetite and food enjoyment measured on a Likert scale, infant method of delivery, the occurrence of negative health conditions, antibiotic use, and nutritional supplement use amongst many other categories. We primarily focused our study on the categories pertaining to infant and mother probiotic use.

Preliminary Processing/Early data analysis with QIIME. The raw data from Dr. Rhee's dataset underwent preliminary processing measures. This was done in order for the dataset to be analyzed through the bioinformatics platform QIIME2 (11). Raw sequences underwent a process of demultiplexing, during which they were clustered based on barcodes, which were subsequently removed. The demultiplexed sequences were imported into QIIME2 for visualization to find quality scores and determine if truncation of sequences was required to

remove low-quality reads. A threshold minimum Phred score of 30 was used as a determinant of quality to establish truncation length (12). As values above 30 were observed for all 150 sequence bases, all 150 were preserved for quality control/denoising using the DADA2 QIIME2 plugin to generate amplicon sequencing variants (ASVs) (13).

Based on the DADA2 and ASV statistics that were visualized using QIIME2, to generate an alpha rarefaction curve, different maximum sampling depths were chosen for our various study aims. The rationale for selecting varying sampling depths was to retain as many individuals as possible that reported the appropriate type of probiotic use for the associated study aim. In assessing infant (or direct) probiotic use, a maximum sampling depth of 492 was used, but for mother (or indirect) probiotic use, a depth of 1518 was used. When assessing individuals before and after probiotic use as per our second study aim, a depth of 15513 was used. As a result, different sampling depths were chosen for our aims based on the differently formed alpha-rarefaction plots; a depth of 70 when assessing direct probiotic use, 195 for mother/indirect probiotic use and 1900 for assessing the cohort of samples used for our time point analysis. For diversity analyses of these different aims, a phylogenetic tree was generated.

For the purpose of our study, mother microbiome samples were filtered out from the metadata. This was done by subsetting the data based on the "life_stage" metadata category. Additionally, samples in which information on direct and indirect probiotic use was not collected to allow for only analysis of infant microbiome samples where probiotics either were or were not administered. This was accomplished by removing values reporting "not collected" under the "probioic_inf" and "probiotic_mom" metadata categories.

TABLE. 1 Overview of subjects that had taken probiotics and the time points at which they had taken them. To determine schedules of probiotic intake of individuals within the dataset, this table was generated. Individuals with similar patterns of probiotic intake were grouped together for further analysis as those that only took probiotics at the same 2-month time point (3 individuals, IDs 70001, 70014, and 70027, highlighted in grey). Asterisk (*) indicates probiotic use and "NC" indicates data was not collected for that subject at that time point. Empty cells indicate the subject reported no probiotic use.

INFANT ID	TIMEPOINT (MONTHS)					
	0.5	2	4	6	9	12
70001		*				NC
70010	*	*	*	NC	NC	NC
70014		*			NC	NC
70017					*	
70027		*	NC	NC	NC	NC
70031	*	*				
70043			*	*		
70071	*	*	*	NC	NC	NC
70092		*	*			

Timepoint Analysis - Grouping infants based on probiotic intake schedules. To investigate our second aim of long-term probiotic use, we sought to complete a time point analysis using individuals that had provided samples to Dr. Rhee's dataset multiple times. In order to select which subjects to use for a time point analysis, Table 1 was created to better visualize all the infants that had probiotic use at some point. Individuals were determined using the "anonymized_name" metadata category and referred to based on their returned value. Values for the "age_category" metadata category were 0.5, 2, 4, 6, 9, and 12 months. We identified three individuals with a similar probiotic intake schedule over the course of the time points. Individuals were referred to by their values in "anonymized_name". 70001, 70014, and 70027 all had reported direct probiotic use at only one time point, 2 months, and had either reported no use or did not have data collected for the other time points. Given this cohort of individuals that exhibited single probiotic use, we focused our time point analysis on determining changes in the infant gut microbiome before and use probiotic use,

focusing on the 0.5-month time point as before probiotic use, and the 4-, 6-, 9-, 12-month time points for after. Data for this cohort was filtered for analysis using QIIME2 and Rstudio as described above.

Core diversity. After the different sampling depths for our various aims were established, core diversity metrics were determined using under various alpha and beta diversity metrics. Alpha diversity was assessed using QIIME2 for both our aims using Faith's Phylogenetic Diversity (Faith's PD) and Pielou's Evenness, allowing for the evaluation of diversity with both sample abundance and phylogenetic distances considered (14, 15, 16). Beta diversity was also assessed, with Principal Coordinate Analysis (PCoA) plots and box-and-whisker being generated to visualize phylogenetic distances across samples within a cohort. Kruskal-Wallis pairwise tests and PERMANOVA statistical tests were performed on alpha and beta diversity metrics results on QIIME2 to determine significance using p-values and corrected p-values (q-scores).

Differential and Relative Abundance. For both aims, differential abundance analyses were conducted to determine if certain genera were more abundant in one associated cohort or the other. For our first aim, this analysis was conducted to determine genera that were either more abundant in individuals that did report direct probiotic use and those that reported no direct probiotic use and the same was repeated for indirect use. This was also done for our second aim, comparing across the various time points available given our cohort of similar probiotic intake. Relative abundance analyses were then conducted on the genera that were found to be differentially abundant for further investigation. Both differential and relative abundance analyses and plots were conducted and generated using R (v.4.1.1) and Rstudio (2021.09.0), with the use of the *tidyverse*, *vegan*, *phyloseq*, *DESeq2*, and *ggplot2* packages (17, 18, 19, 20, 21, 22).

RESULTS

Direct and indirect use of probiotics has no effect on the diversity of the infant gut microbiome. We calculated alpha and beta diversity metrics in infant populations that had either yes or no direct probiotic use. This was labelled as direct use of probiotics. There were 175 samples of which no direct probiotic use was reported and 16 of which direct probiotic use was reported. Across the tested metrics, our analyses did not yield any significant results, with Kruskal-Wallis q-values for Faith's PD and evenness and PERMANOVA p-values for weighted and unweighted UniFrac above the significance threshold of 0.05. This indicates that direct probiotic use has no effect on the diversity within the infant gut microbiome.

Alpha and beta diversity metrics were also conducted for infant populations whose mothers either did or did not administer probiotics. This was labelled as indirect use of probiotics. Similar to direct probiotic use, the majority of samples had an associated report of no probiotics being used (n = 99) with a smaller amount reporting probiotic use (n = 16). Analysis of alpha and beta diversity metrics comparing indirect use also did not yield any significant results with none of the aforementioned p- and q-values being smaller than 0.05. Figure 1 displays beta diversity analysis via weighted UniFrac distances between points representing individuals in regard to the presence and absence of probiotic use. There is no evidence of clustering amongst points, suggesting that both direct and indirect probiotic use have no significant effect on the infant gut microbiome.

***Escherichia-Shigella* genera was more abundant in infants that did not take probiotics.** As part of our study aims to determine changes to the infant gut microbiome caused by the use of probiotics, we sought to assess the potential impact of probiotics on the composition of the infant gut microbiome. In determining this, a differential abundance analysis was run. Figure 2 displays results for differential abundance analyses in both direct (A) and indirect (B) use of probiotics. It was found that in both cases, only the "no" probiotic use cohorts had differentially abundant genera compared to the "yes" cohorts. Six genera were found to be more prevalent in the cohort that reported no direct probiotic use; *Stenotrophomonas*, *Clostridioides*, *Akkermansia*, *Erysipelatoclostridium*, *Haemophilus* and

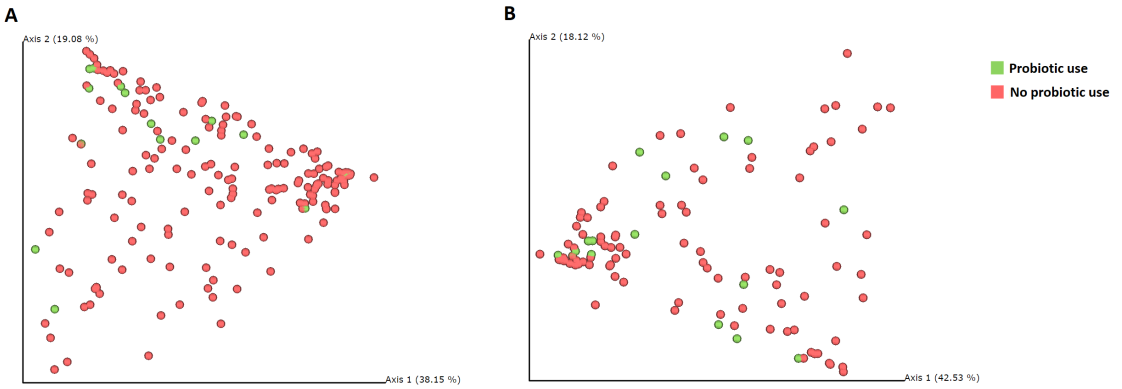

FIG. 1 Infant gut microbial diversity is not affected by direct or indirect probiotic use. Weighted UniFrac distances shown for both direct (A) and indirect (B) probiotic use exhibit no clear clustering of points indicating no effect on beta diversity within the infant gut microbiome. Points in green represent individuals that had used probiotics and points in red represent individuals that reported no probiotic use. PCoA plots were generated using the Qiime2 plugin Emperor.

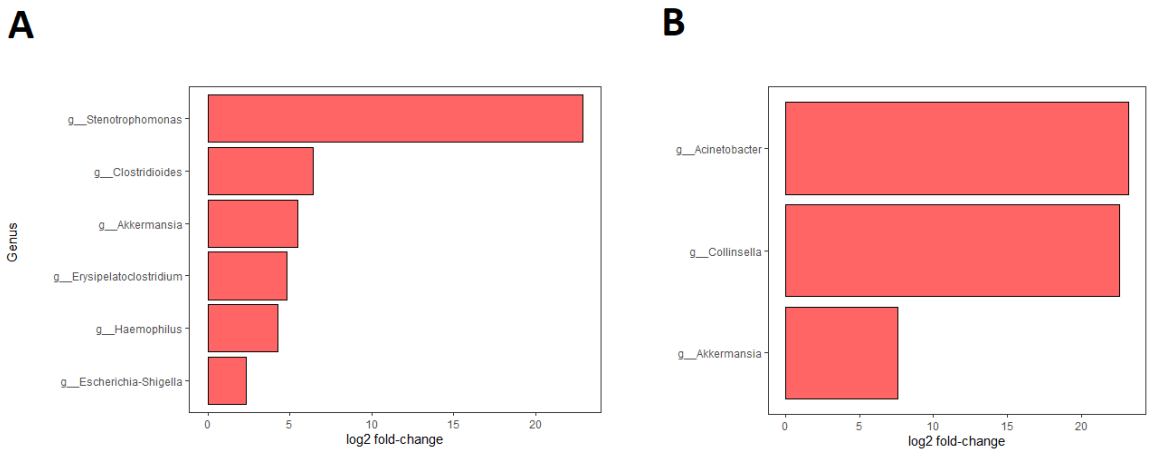

FIG. 2 Cohorts that reported no probiotic use exhibited greater abundance in various genera. Differential abundance analysis indicates various genera shown in the plots were more abundant in cohorts reporting no probiotic use when assessing direct use (A) and indirect use (B). Red bars indicate genera that were more abundant in individuals that reported no probiotic use. No genera were found to be more abundant in individuals that did report probiotic use. Differential abundance plots were generated using RStudio.

Escherichia-Shigella. Comparatively, only three genera were found to be differentially abundant when analyzing indirect probiotic use — *Acinetobacter, Collinsella,* and *Akkermansia*. The only genus found to be overlapping in both direct and indirect analyses was *Akkermansia*.

However, relative abundance analysis found the majority of these results to be due to groups of outliers (**Supp. Figure 3**). The only genus found to be differentially abundant was *Escherichia-Shigella* in direct probiotic use (**Figure 3**). All the genera found to be differentially abundant in the indirect use cohort were found to be due to groups of outliers (**Supp. Figure 3**).

Diversity does not change before and after probiotic use in the three selected infants. Similar analyses of alpha and beta diversity metrics were run for the three selected infants to compare before and after probiotic use at the 2-month time point. The anonymized IDs for these individuals were 70001, 70014, and 70027. Each individual had a different number of

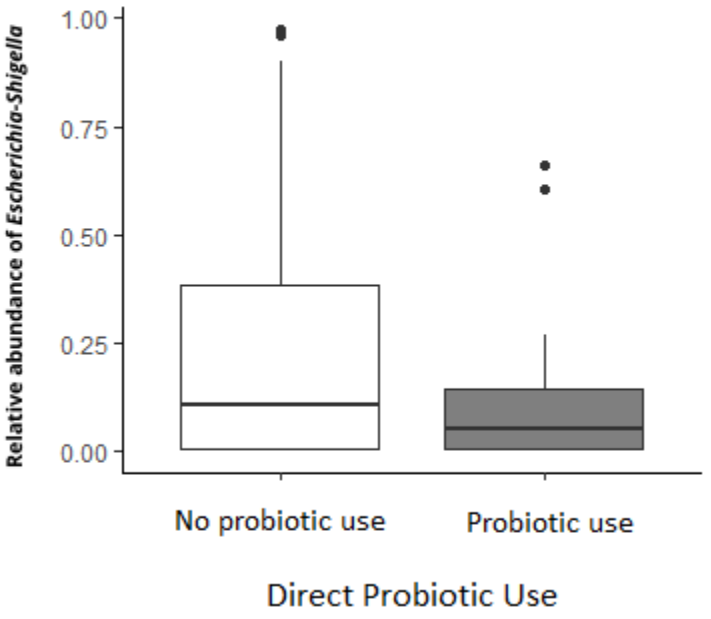

FIG. 3 Relative abundance analysis confirms a greater abundance of *Escherichia-Shigella* genera in individuals reporting no direct probiotic use. Relative abundance analysis was conducted amongst the genera found to be differentially abundant when comparing the presence and absence of probiotic use. The *Escherichia-Shigella* genus denominated by the greengenes database was the only one confirmed to be differentially abundant when disregarding outliers across direct and indirect probiotic use differential abundance analysis. Insignificant relative abundance plots are included in the supplemental section. Relative abundance plots were generated using RStudio.

samples collected, with 70027 only having samples from the 0.5- and 2-month time points, indicating no record of the presence or absence of probiotic use after the 2-month stage. Alpha and beta diversity analyses did not yield any significant results for these infants when comparing across timepoints. Kruskal-Wallis q-values for Faith's PD and evenness and PERMANOVA p-values for weighted and unweighted UniFrac were above the significance threshold of 0.05, suggesting that there is no significant difference in diversity in the infant gut microbiome before and after probiotic use.

Different genera are abundant in the 0.5-month time point and the 6-month time point, before and after direct probiotic use. Differential abundance analysis was conducted for the three selected infants to determine changes in infant gut microbiome composition before and after direct probiotic use at the 2-month time point. Each time point comparison (that was available given the recorded samples) was run with differential abundance analysis. Only two time points comparisons yielded significant results indicating particular genera being more abundant at a particular time point before and after probiotic use. Figure 4A displays the differential abundance analysis of the 0.5-month and 6 month time point. Two genera, *clostridioides* and *akkermansia* were found to be more abundant in the 6-month time point, while 1 genus, *bacteroides* was found to be more abundant in the 0.5-month time point. When comparing the 0.5-month time point with the 9-month time point (**Figure 4B**), two genera, *bifidobacterium* and *clostridium sensu stricto 1* are found to be more abundant at 0.5 months. No overlapping differentially abundant genera was found between the 6-month and 9-month stages, indicating changes occurring in the infant gut microbiome composition. Subsequent relative abundance analysis, shown in figure 5, confirmed that the genera were differentially abundant across the aforementioned timepoints, and were not a result of extreme outliers as previous with the relative abundance analyses for our first aim of direct/indirect probiotic use (**Supp. Figure 3**).

DISCUSSION

There are conflicting reports in the literature about the effectiveness of probiotics in changing the composition of the infant gut microbiome. Some studies have found that probiotic use can be helpful in preventing negative health conditions (7), while other studies have found probiotic use causes only very minor changes in the infant gut microbiome

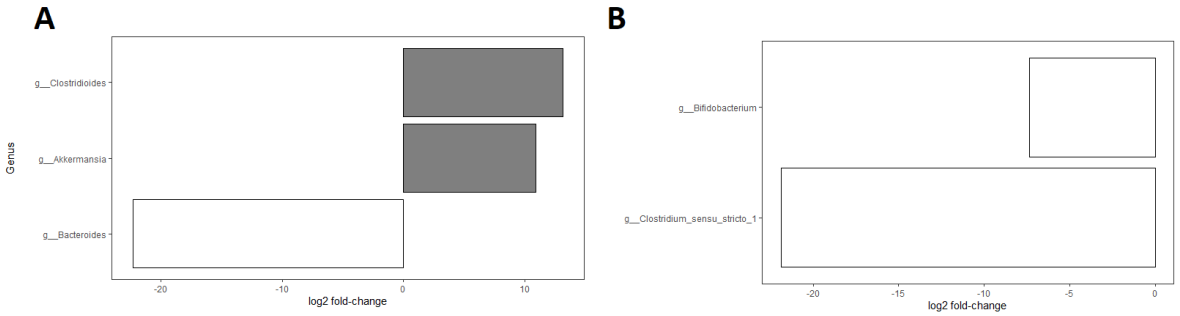

FIG. 4 Certain genera are differentially abundant across timepoints before and after probiotic use. Differential abundance analysis on subjects that had taken probiotics at the 2-month time point found various genera of bacteria are more abundant either before or after probiotic use. A) Differential abundance comparing the 0.5-month (before probiotics) with the 6-month (after probiotic use) time point. B) analysis comparing the 0.5-month time point with the 9-month time point. Pink bars represent genera more abundant in samples taken during the 0.5-month time point. Purple bars indicate genera more abundant in samples taken during the 6-month time point. Differential abundance plots were generated using RStudio.

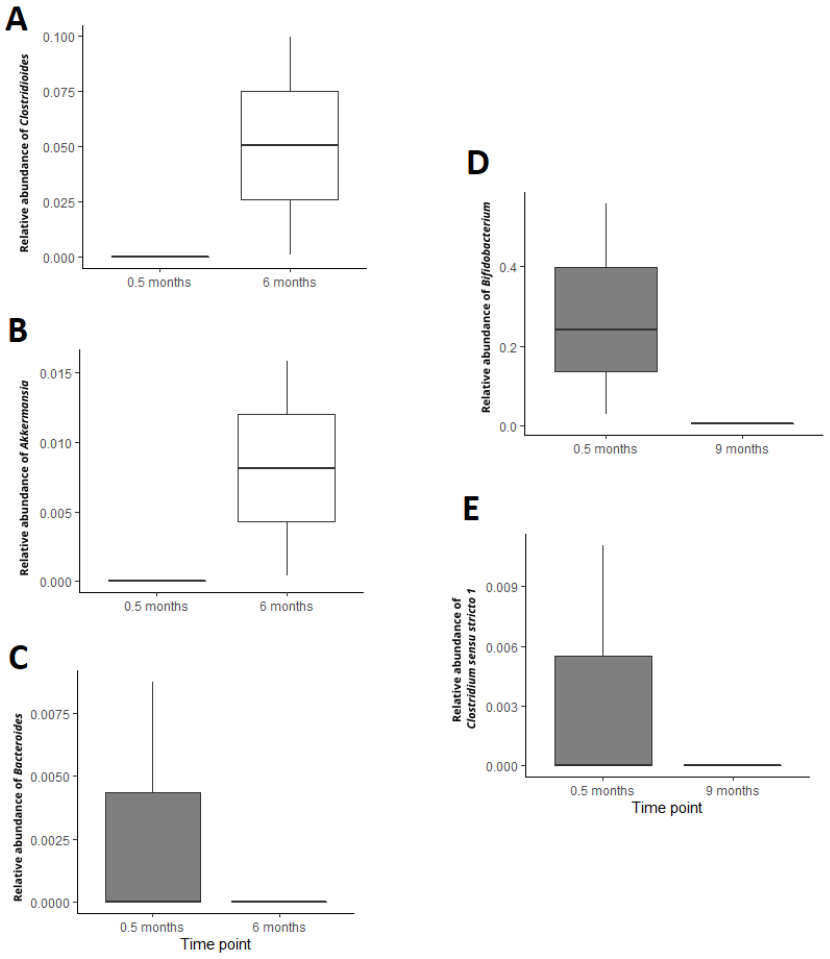

FIG. 5 Relative abundance analysis confirms various genera are differentially abundant in time points before and after probiotic use. Relative abundance plots were generated for genera found to be differentially abundant across time points before and after probiotic use. Panels **A-C** display relative abundance plots for genera across the 0.5- and 6-month time points while panels **D** and **E** compare across the 0.5- and 9-month time points. The genera analyzed per panel are as listed: **A**) *Clostridioides*, **B**) *Akkermansia*, **C**) *Bacteroides*, **D**) *Bifidobacterium*, **E**) *Clostridium sensu stricto 1*. Relative abundance plots were generated using RStudio.

composition and only on a short-term basis (6). There have also been studies conducted that show that probiotic use may actually be harmful to infants due to their use correlating with a higher frequency of infection (2). In this study, we aim to investigate their effectiveness further by reproducing these experiments with a different cohort. This was done by conducting diversity and differential abundance analysis to compare probiotic use and no

probiotic use in both mom (indirect) and infant (direct). As well, we compared the gut microbiome of the infants before and after direct probiotic use to observe any long-term effects. Based on previous studies we hypothesized that infant and mother probiotic use would not have an effect on the diversity of the infant gut microbiome, but it may have an effect on the prevalence of specific taxonomic groups. The findings of this study support this hypothesis, as it appears that neither infant nor mother use of probiotics shows a major change in the alpha or beta diversity of the infant gut microbiome. Pielou's evenness and Faith's phylogenetic diversity (PD) diversity (**Supp. Figure 1**) metrics did not show any differences in alpha diversity between the gut microbiomes of infants that took probiotics and those who did not. A study conducted in 2018 by Quin et al. performed a similar analysis by examining Pielou's Evenness, Shannon's diversity index, Faith's PD, and observed species richness for both mother and infant probiotic use, and also did not note any differences in diversity between these two groups, which supports our findings (2). Weighted UniFrac principal coordinate analysis plots (PCoA) (**Figure 1**) did not show any clustering of the samples based on probiotic use, indicating no impact on beta diversity. Additionally, PERMANOVA tests (**Supp. Figure 2**) did not reveal any significant differences in diversity between the probiotic and no probiotic use groups. Similar beta diversity results were found for both direct and indirect probiotic use. These findings are supported by the Quin et al. paper, which did not find any significant differences in beta diversity between probiotic use and no probiotic use groups, except for at one week of age (2). We were unable to assess some of these findings as the dataset used for this study did not collect microbiome data at a one-week time point.

Although probiotic use did not appear to impact the diversity of the infant gut microbiome, differences were noted when conducting differential abundance analysis (**Figure 2**). This again supports our hypothesis. When comparing infant probiotic use to no probiotic use, multiple bacterial genera were more abundant in the group that did not use probiotics. However, relative abundance analysis showed that only *Escherichia-Shigella* was significantly different between the two direct use groups (**Figure 3**). The appearance of the other bacterium in the differential abundance plot was likely informed by outliers, resulting in the false discovery rate. *Escherichia-Shigella* are potential enteric pathogens and can be harmful to the host (23), therefore its higher abundance in the no probiotic use group aligns with the claim of probiotics decreasing the number of harmful bacteria. Of note, other studies consistently report increased abundance of *Bifidobacterium* in the microbiomes of infants who take probiotics (24, 25), however, this bacterium was not identified in our differential abundance analysis. This may be due to the fact that the infants in this study were not given probiotics that included *Bifidobacterium*. However, details of what was in the probiotic supplements given to the infants were not included in this dataset, so it is difficult to make any conclusions about this.

To observe the long-term effects of probiotic use, we decided to group the infants based on similar probiotic dosing schedules which allowed us to observe the infant gut microbiomes before and after probiotic use. There were no significant differences in alpha and beta diversity between the 0.5-month time point and any other time point post probiotic use at 2 months. This indicates that whatever effects probiotics have on microbiome diversity, it does not persist after discontinuing their use. This conclusion is supported by a study done in 2015 by Rutten at al. which found no long-lasting differences in the gut microbiota composition after supplementation had ended (6), again supporting our hypothesis.

In contrast, differential abundance analysis showed that certain bacterial genera were more represented at specific time points (**Figure 4**). The significance of these results was confirmed by relative abundance analysis (**Figure 5**). Specifically, when comparing bacterial abundance between the 0.5-month time point and the 6-month time point, *Bacteroides* was found to be significantly more abundant before probiotic use at 0.5 months. In contrast, *Clostridioides* and *Akkermansia* are more abundant at the 6-month time point. *Akkermansia* is considered to be a promising probiotic that is inversely associated with obesity, diabetes, inflammation, and metabolic disorders (26). However, *Clostridioides* has been associated with susceptibility to chronic disease later in childhood (27). Therefore, although it was mentioned above that probiotics decreased the abundance of harmful bacteria, this same effect is not seen after discontinuing their use. Differential abundance analysis of the infant microbiome at 0.5 months and 9 months identified two bacterial species to be abundant in the

former group: *Bifidobacterium* and *Clostridium Sensu Stricto 1*. *Bifidobacterium* is considered to exert positive health benefits on their host (28), and *Clostridium Sensu Stricto 1* has been shown to play a key role in modulating gut homeostasis (29). Therefore, there appears to actually be a decrease in the amount of beneficial bacteria after probiotic use. This again lends support to the theory that the beneficial effects of probiotics are no longer seen after ending the supplementation period.

An important note, however, these differential abundance results may be due to dietary changes in the infants. It is recommended by the CDC that solid food be introduced to infants around 6 months (30), and beginning solid food has been shown to have a significant effect on the infant gut microbiome (31). Therefore, it is possible that the differences in bacterial abundance seen between 0.5 months and 6 months and 9 months are due to this switch to solid food instead of probiotic use. This is further supported by the fact that no differences in bacterial abundance were seen between the 0.5-month and 4-month groups, which were both time points when the infants were most likely still being breastfed or bottle-fed.

Limitations One of the major limitations of this study is the extremely limited sample size regarding the factor of interest; there were only nine infants who took probiotics, with many taken at different times throughout the data collection period. Due to this fact, our sampling depths had to be smaller in order to retain all our samples, resulting in the loss of features that could have had an impact on our results. Moreover, for our second aim examining the changes in gut microbiome in infants before and after probiotic use at the 2-month time point, there were only three infants who had the same probiotic intake regimen. One of which, Infant 70027, did not have data collected for probiotic use at 4 months and onwards. The limited number of samples would decrease the statistical power and could introduce bias. There was also a lack of appropriate quantitative metrics in this study. As only ASVs were generated from this dataset, our analysis could not represent cellular abundance. Consequently, it was difficult to compare the results of our study against the literature since it references abundance for analyses.

In addition, the type of probiotics administered to the mothers and infants were not provided in the dataset. It is possible that an infant took probiotics of one specific species while another took a combination of probiotic species. Thus, having this information is crucial as it could help us better determine whether the probiotics administered would influence the infant gut microbiome when conducting taxonomic analysis. In the peer-reviewed article by Rutten et al, they found a transient increase in the abundance of the supplemented probiotic in the infant gut microbiome (6). It certainly would be interesting to look into the different probiotic species administered to mothers and infants if this data were provided, and how those would influence the infant gut microbiome composition.

Another limitation that could potentially influence the interpretation of our data is whether or not solid foods were introduced into the infant's diet during the 6-month time point and onwards. According to the Centers for Disease Control and Prevention, solid foods are highly recommended for infants after 6 months (28). No data regarding the consumption of solid foods in infants was collected in Dr. Rhee's dataset. Even among those who consumed solid foods, the type of solid foods consumed can also vary quite drastically among infants. All of these factors could potentially influence the infant gut microbiome composition and diversity, thus, we cannot make definitive conclusions about our results.

Conclusions As was predicted based on previous studies, mother and infant probiotic use showed no effect on the diversity of the infant gut microbiome. However, there was a lower abundance of the harmful bacterium *Escherischia-Shigella* in the probiotic use group, indicating that probiotic use may alter the microbial community structure by limiting colonization of certain harmful microorganisms. Given that the time point analysis did not show a sustained decrease of harmful bacteria and instead showed a decrease of beneficial bacteria, any benefits to the gut microbiome provided by probiotic use do not appear to be sustained after supplementation has ended.

Future Directions In order to address the concerns regarding limited sample size to better answer our experimental aims, similar analyses can be done in future studies with an increased number of infant and mother pairs. The data generated as a result of that should be more representative and help us better determine whether probiotic use in mothers and infants would impact the infant gut microbial composition and diversity. Additionally, if possible, the same probiotic dose schedule can be implemented for more infants, for example, having more than three infants taking probiotics for only the 2-month time point with no probiotic use prior to and after 2 months. This can potentially help us better predict the changes in infant microbiome before and after probiotic use.

With regards to the type of probiotics administered to mothers and infants, one species of probiotics should be administered to limit the experimental variables. This allows for a more accurate examination of one specific probiotic species on the infant gut microbiome. Alternatively, two different types of probiotics can be administered to different infants, but those with the same type of probiotic intake should later be grouped into the same category for data analysis, separate from the other group. This can allow us to more efficiently examine two species of probiotics within the same experimental time frame.

Another approach could be to examine the long-term use of probiotics with a wider time period, while also noting whether or not solid foods were introduced to the infants' diet. We can then categorize these infants into two distinct groups with either having solid foods introduced into their diet or not. This more comprehensive longitudinal study can allow us to better interpret the effects of probiotics on the infant gut microbiome, and determine whether there are short-term increases in the supplemented probiotic as observed by Rutten et al (6).

Moreover, the current ASV dataset provides very limited information on the mechanisms of action with respect to probiotic use on the infant gut microbiome. A possible way to examine this more closely is to assess the actual abundance profiles. Another approach could be to supplement the data with functional information through the use of whole genome shotgun sequencing. These methods could allow us to more accurately determine the mechanistic effects of probiotics on the infant gut microbiome.

In addition to assessing the effect of probiotics on the infant gut microbiota, Dr. Rhee's dataset also collected metadata categories that can be interesting to examine. Due to conflicting evidence in the literature regarding the effectiveness of probiotics, some studies suggested that they were beneficial, while others claim that they were a risk factor for harmful bacteria (2, 6, 7). By conducting correlation analysis, we can determine whether probiotic use is associated with certain negative health outcomes such as the occurrence of diarrhea, colic infection, fever, cough, and eczema. Given the findings of increased frequency of mucosal infections in infants treated with probiotics in Quin et al's 2018 research article, we can predict that there could be a positive correlation between probiotic use and negative health outcomes collected in the dataset (2). Since this dataset already contains incidences of outcomes that were not examined previously, determining this correlation can help progress current research regarding the association between probiotic use and negative health outcomes and help make better-informed decisions on recommending probiotic use in infants for potential health benefits.

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CONTRIBUTIONS

Rafid Haq was responsible for conducting alpha and beta analyses as well as differential and relative abundance analyses on QIIME2 and Rstudio. Rafid generated all the figures and supplemental figures as well as their captions and wrote the Methods & Materials and Results section.

Sachini Jayasinghe conducted literature searches and assessed data for results. Sachini wrote the vast majority of the Discussion section and contributed to the Methods & Materials section. Sachini wrote the

Conclusion section of the Discussion and was responsible for establishing the aims of the study and generated table 1.

Gary Yen conducted literature searches and wrote the Introduction section. Gary also wrote the Limitations and Future Directions sections of the Discussion, Acknowledgements section, and contributed to the Methods & Materials and Results sections.

All authors were responsible for overseeing and editing all the sections of the paper and gathering references.

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