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Determining the role of food enjoyment, responsiveness, antibiotic use and weight in infant gut microbiome diversity

Eui-Young Jung, Tony Liu, Shovon Das

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

SUMMARY The growing global epidemic of obesity has contributed to various health conditions costing healthcare systems billions of dollars. Previous research has investigated potential factors that could increase this risk in infants such as antibiotics, breastfeeding and mother's health. However, factors such as infant food responsiveness and enjoyment have not been investigated. This is important to investigate as it could potentially identify new factors behind infant obesity, hopefully opening new areas of research, early diagnosis, and possibly treatment. The main objective is to investigate the effect of food enjoyment, food responsiveness, antibiotic use, and weight on the microbiomes of infants. Our study found that infant food responsiveness/enjoyment and antibiotic usage did not alter the infant microbiomes. The microbiomes of healthy and obese infants were not statistically significant however underweight infants had a statistically higher frequency of Bacteroidetes compared to overweight infants. Our findings establish the foundation to study the role of Bacteroidetes and other factors impacting infant obesity and how future studies can study impacts of the phylum in childhood.

INTRODUCTION

Obesity is a global epidemic that affects virtually all ages and socioeconomic groups, as more countries have changed the structure of their diet as they become more developed. It affects virtually all ages and socioeconomic groups, as more countries change the structure of their diet as they become more developed (1). In the United States it affects about 42.4% of the adult population and has been increasing since 1999 (2). It is one of the largest preventable chronic diseases, costing the US healthcare system $147 - $210 billion per year because it results in multiple serious health conditions such as diabetes, heart disease and high blood pressure (3). Various methods and treatments used to combat this disease, such as diet plans and weight loss medication, have shown little success with frequent cases of relapse (3).

Recent studies have looked at potential factors that could increase the risk of infants/children becoming obese. A study done by Dawson-Hahn and Rhee (2019) suggested that increased exposure to antibiotics in an infant's first year had a greater growth trajectory over time and could contribute to the increasing prevalence of obesity among children (4). Another factor that has been seen to affect infant obesity is breastfeeding. A study by Yeung et al. (2017) concluded mothers who exclusively breastfed their children regardless of being obese or not for 4 months had slowed their weight gain rate at 1 year than those who used formula fed. They suggested that breastfeeding children could be a potential method to prevent early onset obesity. Another study has suggested that the gut microbiome composition could potentially play a role in obesity; research shows obese individuals had gut microbiomes containing more short chain fatty acid (SCFA) metabolizing microbes and bacteria in the phylum, Firmicutes and fewer Bacteriodetes bacteria (5). Researchers have proposed that these microbial signatures could be used to diagnose obesity and changing the microbiome to reflect the composition of those with a healthy weight range could help treat obesity (5). However, additional research is still needed to understand how the presence/absence of these microbes could be used to treat obesity.

Previous research has focused on the effects of certain factors on infant weight gain,

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Address correspondence to: https://jemi.microbiology.ubc.ca/

which includes antibiotics use, infant diet and the mother's health. A study investigating specific antibiotic treatments such as macrolide, clarithromycin, vancomycin, ciprofloxacin, and clindamycin had significant results where macrolide significantly decreased the phyla *Actinobacteria*, and *Firmicutes* and significantly increased *Proteobacteria* (6). Additional research is required to identify potential factors that could increase infant weight gain and prevent the risks of obesity early.

We will be using the infant dataset compiled by Dr. Rhee and her team from the Department of Pediatrics of the University of California to study the following three aims. The dataset was collected from the European Nucleotide Archive (7).

Our first research objective is to determine if there is a relationship between the infant's enjoyment or responsiveness towards food influences their microbiome. The infant dataset contains information on the infant's food responsiveness and enjoyment. We hypothesize that infants who have positive food responsiveness/enjoyment will have higher weight gain and we predict that there will be a significant difference in the microbiome of infants who enjoy/respond to food better than those who do not, as their behaviour may result in them consuming more food.

Our second research objective is to investigate how antibiotic usage affected infants. Based on the study done by Dr. Rhee (2019), we hypothesize that infants who use antibiotics will have increased growth rates as increased exposure to antibiotics could be modifying the gut microbiome resulting in infant obesity.

Another study investigated the dietary influence on gut microbiome found that when the diet is fat restricted, carbohydrate restricted, western diet reduced fat and western diet reduced carbohydrates had increased populations of Bacteroidetes and decreased populations of Firmicutes (8). This signifies that with healthier dietary influences Bacteroidetes increase and Firmicutes decrease in population highlighting an opposite effect of a healthier gut microbiome. By sorting infants based on their weight gain rate, we can determine if those with a higher weight gain rate will have similar microbiomes to obese indiviudals in previous studies. By identifying which infants have microbiomes containing an abundance of SCFA metabolizing microbes, Firmicutes and fewer Bacteroidetes microbes, if they have higher weight gain rates, this may indicate early obesity. Our third research objective is to examine the differences in a healthy weight and overweight infant's gut microbiome. We hypothesize that infants with higher weight gain rates will have more SCFA metabolizing microbes, *Firmicutes* and fewer *Bacteroidetes* microbes in the gut.

The research objectives established in this paper is foundational to the field of infant obesity as it investigates research objectives that have been barely studied in the literature. Beginning with the first research objective is studying the food responsiveness/enjoyment of infants. We believe that infants with a positive relationship with food responsiveness/enjoyment will have a significantly more diverse gut microbiome compared to infants who respond/enjoy food less. The next research objective studies how antibiotic usage affects infant weight, where higher weight infants have increased antibiotic usage. Our third research objective is comparing overweight and healthy weight infant gut microbiomes to determine the differences, where higher weight infants will have more SCFA metabolizing microbes. All of these research objectives are important areas of study regarding infant obesity as they build upon already existing research and identify new factors that require more research in what could potentially cause infant obesity.

METHODS AND MATERIALS

Dataset Description. We used a dataset created by Dr. Kyung Rhee and the Department of Paediatrics from the University of California, which included 309 gut microbiome samples for 82 infant-mother pairs spanning the first 12 months of life. In total, 171 fields of accompanying metadata, describing each subject's diet, health, and feeding behaviours, were translated from the Infant Feeding Practices II (IFP II) study by the CDC (9). The microbiome samples were provided as Illumina sequences for the V4 region of 16s rRNA, obtained using 515fbc and 806r primers and following the Earth Microbiome Protocol (10). This information is publicly available on the European Nucleotide Archive (7) and Qitta (11).

Preliminary Processing. Samples for the mother and those with missing data in our field of interest were filtered out and the resulting collection was generated into a manifest file for each of antibiotic intake, food enjoyment and food responsiveness, as well as normalized weight per infant length (wlz) at 2 weeks and 2, 4, 6, 9, and 12 months using R Using R ver. 4.0.3 (12) and R studio ver. 1.3.1093 (13). Antibiotic intake was recorded as yes/no while food enjoyment and responsiveness are in the form of a 1 to 5 Likert scale (ratings), with 1 meaning they enjoyed/responded to food the least. Infant weight was normalized to a statistical z-score and grouped into normal, underweight (lightest 5%), or overweight (heaviest 5%) categories. Reads were not truncated as all base positions were found to maintain a 25th percentile Phred score of over 30 across all reads. An alpha rarefaction depth of 23500 was used as this was found to maximize both the number of features and the number of reads retained. Resulting filtered sequences and matching metadata tables then imported into QIIME2 (14) for downstream analysis. In addition to data, the commonly used alpha value of 0.05 was selected for all statistical methods.

Core Diversity. To calculate the phylogenetic distances for computing core diversity metrics, three QIIME2 plugins were used. MAFFT first performed multiple sequence alignment on the imported sequences (15). Mask then filtered out poor alignments with high variability (16) before the final phylogenetic tree was generated with FastTree (17) and midpoint rooted with QIIME2's phylogeny module (13). QIIME2's core diversity command was then used to calculate both alpha and beta diversity metrics. From alpha diversity, Shannon, Faith's diversity, Pielou's evenness and observed features were exported to juxtapose abundance with phylogenetic distances. Significance for these metrics were determined using Kruskal-Wallis analysis from QIIME2's group significance command. Similarly, Bray-Curtis, Jaccard, unweighted and weighted UniFrac principal component analysis plots were exported for beta diversity and their significance were computed using overall and pairwise PERMANOVA, also from QIIME2's group significance command. The resulting visualizations were manually matched to the filtered metadata category to produce the final figures.

Taxonomic analysis. Sequences were classified using an sklearn, machine learning, classifier pre-trained on the Greengenes database for 515F/806R (V4) region of 16s rRNA (17, 18, 19). The classified sequences were then cross referenced with the metadata to obtain a taxonomic bar graph for each time point using qiime2's taxa-barplot command.

Analysis of variance (ANOVA). Each time point was exported from qiime2 as csvs before importing into R as tables containing the hits per clade for each infant gut sample across 7 taxonomic levels and the original metadata. To determine the effect of infant weight on the abundance of Bacteroidetes and Firmicutes, each table was grouped by a precomputed weight category (wlz_new_bin) which identified each sample as either). Boxplots for each group were generated for each time point and an ANOVA F-test was used to determine the significance of differences observed at the 5% confidence level.

RESULTS

Food Enjoyment and Responsiveness did not impact microbiome diversity. After analyzing the alpha and beta diversity metrics we ran, we found that there was no significant difference of gut microbial diversity of infants who enjoyed or responded to their food more than those who did not. When looking at the alpha diversity metrics such as Shannon's diversity and Faith's phylogenetic diversity for food enjoyment/responsiveness, the p-value for Kruskal-Wallis tests were greater than 0.05 (Fig. 1,2). For the beta diversity metrics, between microbial communities in food responsiveness and food enjoyment, there were no significant differences as well (Fig. 3, Supplemental Fig. 2). All alpha and beta diversity metrics and their associated p-values or f-ratios for food enjoyment are in Table S2, S6 and responsiveness are in S1, S5.

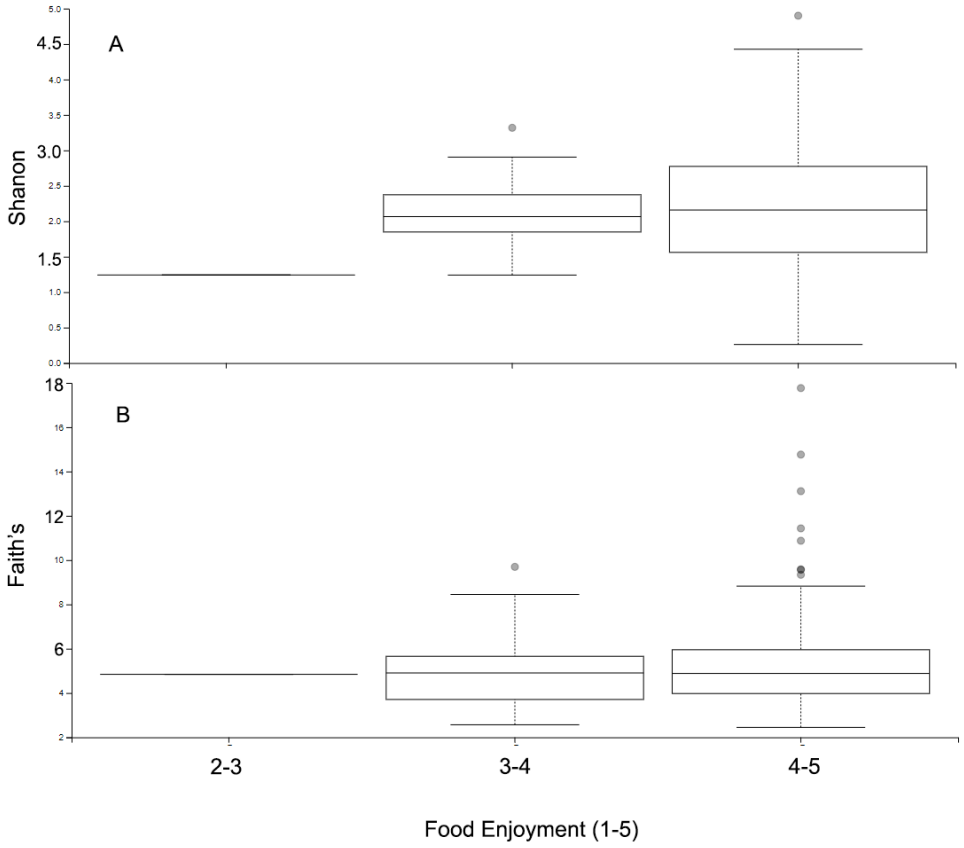

FIG. 1 Boxplots showing no significant difference in alpha diversity for infant gut microbiomes based on food enjoyment. (A) Shannon's and (B) Faith's phylogenetic diversity metrics are shown for food enjoyment. A 1 -5 Likert scale was used for both responsiveness and enjoyment. $p > 0.05$ for all alpha diversity metrics (Kruskal-Wallis pairwise test).

Antibiotic use did not significantly impact microbial diversity of the infant gut microbiome. For all the alpha diversity metrics we ran (Shannon, Faith's, Pielou's evenness, observed features), we determined that there was no significant difference in gut microbiome diversity for infants who used and who did not use antibiotics (Supplementary Fig. 3, 4). We had $p > 0.05$ for all the Kruskal-Wallis tests for all the alpha diversity metrics, such as Shannon's diversity and Faith's phylogenetic diversity (Table S3). Additionally, there was no significant difference between microbial communities for the two groups, as shown by all the beta diversity metrics; there were no distinct microbial communities for all the beta diversity metrics such as Bray-Curtis and weighted unifrac metrics (Supplementary Fig. 5). All alpha/beta diversity metrics and their associated p-values can be found in Table S3, S7.

Microbiomes between obese and healthy weight infants were not statistically different but the frequency of Bacteroidetes was significantly lower in obese infants. There were no significant differences between the microbiomes of average, over and underweight infants at different time points however the frequency of Bacteroidetes was significantly lower in obese infants. Alpha and beta diversity metrics revealed that at different time points, there was no statistical significance between average, overweight and underweight infants (Supplementary Fig. 6, 7). However, an ANOVA test was ran to compare the frequency of Bacteroidetes at various time points. With a calculated F-value of 3.41 and an F-critical value of 2.67, the ANOVA test identified the frequency of Bacteroidetes to be statistically higher in obese and very obese infants compared to average at 6 months (Fig. 4). These results suggested that microbiomes of obese and healthy weight children were not statistically significant, and the frequency of Bacteroidetes is significantly higher in infants with unhealthy weights. We investigated the frequency of Firmicutes as well, but there were no significant differences in frequency among the three groups (Fig. 5).

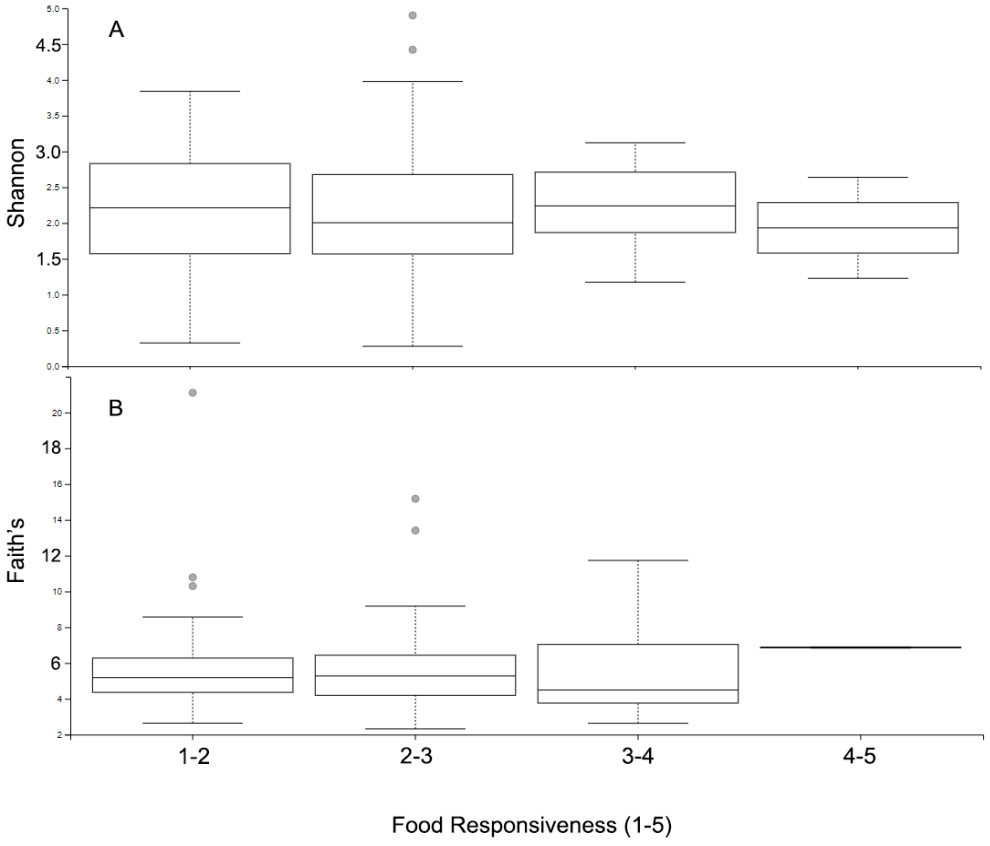

FIG. 2 Boxplots showing no significant difference in alpha diversity for infant gut microbiomes based on food responsiveness. (A) Shannon's and (B) Faith's phylogenetic diversity metrics are shown for food responsiveness. A 1 -5 Likert scale was used for both responsiveness and enjoyment. $p > 0.05$ for all alpha diversity metrics (Kruskal-Wallis pairwise test).

DISCUSSION

Our study aimed to investigate the potential variables such as food enjoyment/responsiveness and antibiotic use, that could alter the gut microbial diversity in infants and cause obesity. However, using the dataset compiled by Dr. Rhee, these variables do not significantly impact gut microbial diversity, contrary to previous studies (4, 6). We also investigated the abundance of specific bacterial phyla and how their presence/absence could show signs of early obesity.

Food Responsiveness/Enjoyment. There is a lack of knowledge surrounding how behaviour can influence the gut microbial diversity in humans. Other studies have found that gut microbes do have influence on their hosts' behaviour through modulating their hormone levels, neural mechanisms, and host receptor expression (20, 21, 22). However, our alpha and beta diversity metrics show that food responsiveness and enjoyment levels do not influence gut microbial diversity in infants (Fig 1-3, Supplemental Fig. 1, 2). This means our results do not support our hypothesis that infants who enjoyed/responded to food more would have a higher gut microbial diversity. Although we could not find any research in the literature that specifically investigated the impact of food responsiveness/enjoyment on gut microbial diversity, our results contradict other studies that suggested that eating behaviour impacts gut microbial diversity (20, 21, 22). The existence of the vagus nerve connecting the enteric nervous system in the gut to the base of the brain in the medulla has encouraged researchers to conduct many studies suggesting the nerve can regulate eating behaviour and weight (23). However, many of these studies focus on adults and mice, not infants. Adults can express their eating behaviour more easily since they have more control over what they eat and have access to a larger variety of foods compared to infants, who must eat what they are given and have a more limited diet. This could be why there was no significant differences in microbial diversity in infants, as their lack of variety and control over their diet could have a larger impact on microbial diversity than their eating behaviours. Results from mice studies may not be the same when conducted in infants and

should be used as a starting point for future studies done with human subjects. For these reasons and others, which are explained later in the study limitations section, could be why our results contradict other studies in the literature.

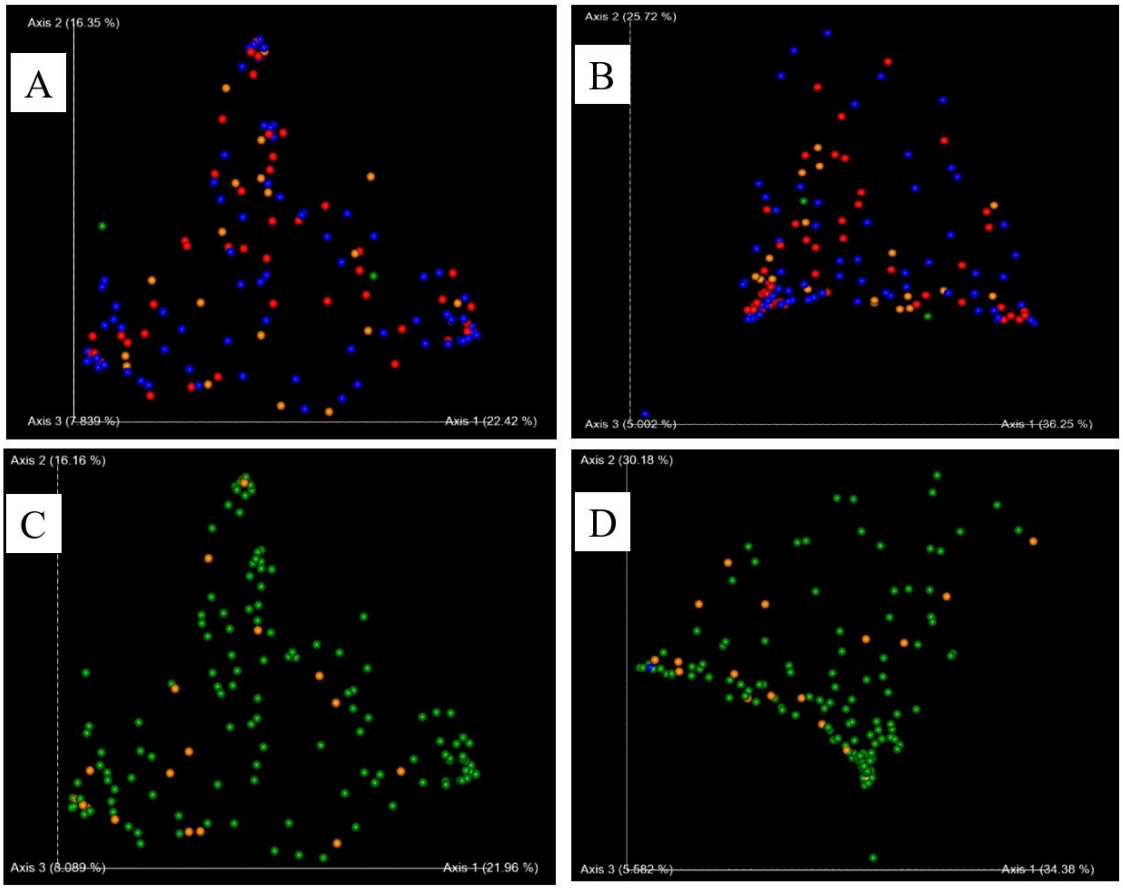

FIG. 3 PCoA plots showing that there are no distinct microbial communities in infant gut microbiomes based on food responsiveness/enjoyment. Bray-Curtis and weighted UniFrac distances are shown for (A, B) food responsiveness and (C, D) enjoyment, respectively. A 1 -5 Likert scale was used for both responsiveness and enjoyment.

Antibiotic Use. Our alpha and beta diversity analyses reveal that antibiotic use does not significantly impact gut microbial diversity in infants and our hypothesis was not supported (Supplemental Fig. 3-5). Previous studies show antibiotic usage does lower gut microbial diversity due to some antibiotics selecting for certain microbial species (24,25). However, these studies contradict our findings, as the microbial diversity showed no significant changes between the two groups. There was a lack of information on how much antibiotics the infants used, what type of antibiotics and how recently they were used, as the dataset only mentions if the infants did or did not use antibiotics. These factors could potentially influence the results. Some antibiotics have been shown to impact microbial diversity more than others; a study shows antibiotics such as azithromycin affect the microbiome composition significantly while others like amoxicillin and cotrimoxazole do not cause significant changes to the gut microbiome (24). Another study shows that gut microbial populations can recover to their normal values 2-4 weeks after treatment, showing that the timing of antibiotic treatments and sampling of the gut microbiota could affect whether changes in microbial diversity are seen (26). These studies also controlled the amount of antibiotics the subjects used, which could also impact the results. While our results show that antibiotic usage does not significantly impact gut microbial diversity in infants, additional information about how, when and which antibiotics were used must be known to better explain these results.

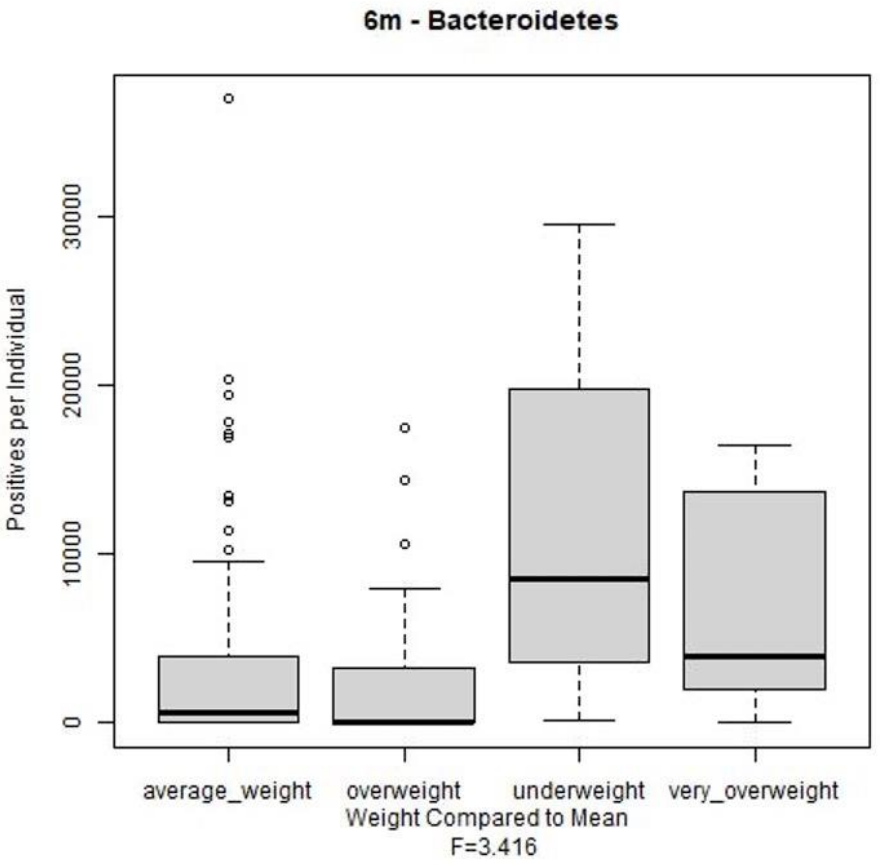

FIG. 4 Box plot showing more hits (positives per individual) for Bacteroidetes in underweight (bottom 5th percentile) infants at 6 months. Weight-per-length was used to group infants into four groups. With an F-value > 2.67, the differences are significant.

Microbial Diversity in Obese and Healthy Weight Infants. The alpha and beta diversity metrics we generated shows that there were no significant differences in diversity found between infants who were average/underweight and those who were overweight (Supplemental Fig. 6-7). Despite these results, we decided to look further into the abundance of certain bacteria phyla, such as Firmicutes and Bacteroidetes since previous studies show that these specific phyla can determine between a healthy weight person's microbiota and an overweight one (5, 7). Our taxonomic bar graph analysis shows that the relative frequency of *Bacteroidetes* is significantly less in overweight infants than in average/underweight infants (Supplemental Fig. 8). Further analysis in R showed that the frequency of Bacteroidetes is statistically higher in underweight infants compared to average, overweight and very overweight infants at 6 months (Fig. 4). This result stayed consistent throughout all the time points collected in the dataset. These results are like ones found in other studies that specifically looked at children; they found a positive association between abundance of various Bacteroidetes phyla and obesity in children (27, 28). However, there were no significant differences in the relative frequency of Firmicutes between overweight and average/underweight infants (Fig. 5). This contradicts previous research that shows that obese infants tend to have a lower abundance of Firimuctes than in healthy weight infants (5). Although there is a large body of research supporting that there is a lower abundance of Firmicutes in obese infants than in healthy weight ones, there are other articles that contradict this. A study done by Bergstrom et al. showed that the clear difference in abundance of Firmicutes and Bacteroidetes disappeared after 18 months, which is like the results that we found, where there was no significant difference in microbial diversity in all three groups (29). Other bacteria such as Actinobacteria and Bifidobacterium have been identified as having some relation to obesity as well as being species-specific (30, 31). This means not all the bacteria in the phylum/genus increase or decrease based on the weight of the subject. These conflicting results in the literature and our own study as well as the potential for other bacteria playing a role in obesity and weight

gain suggests that these results are not conclusive and additional research is needed to identify what gut microbial species play a role in obesity, if any.

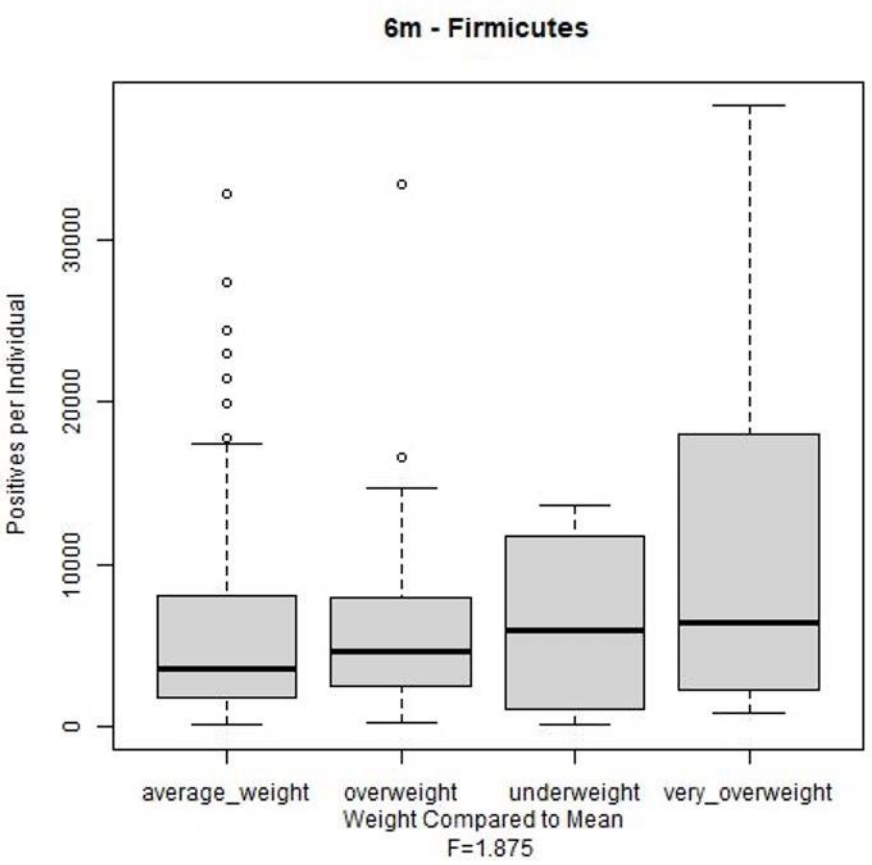

FIG. 5 Box plot showing no significant differences in Firmicutes between all weight classes at 6 months. Weight-per-length was used to group infants into four groups. With a F-value < 2.67, the differences are not significant.

Limitations This study had several limitations, including the lack of samples for certain metadata categories we were looking at, the subjective scale used and the lack of information about how/what the dataset collected. For some categories such as food responsiveness/enjoyment, only one sample was used for responsiveness/enjoyment levels less than 3, so we had to group different levels together. This limited our ability to identify possible outliers on each scale level. Also, despite the dataset surveying 82 infants, a large portion of them did not have the data we were looking for, which also limited our sample size greatly for some metadata categories.

Another limitation was the subjective scale that was used to measure food enjoyment/responsiveness. There was no information given on what each point the scale meant so it is difficult to know exactly what was being measured. The subjectivity of this data also makes it difficult to replicate for future studies that look at eating behaviours and gut microbiota diversity, so a more objective scale that measures food enjoyment/responsiveness may be required.

There was no detailed information on certain metadata categories such as antibiotics that made it difficult to make conclusions about the results we found. Without knowing which antibiotics, the infants took, how much and when, we cannot make definitive conclusions about antibiotics usage and microbial diversity. If we had this information for all the categories in the dataset, we could compare these results to similar studies that had similar conditions/controls and draw clear conclusions.

Another limitation of this study is the longer-term effects of these treatments are not investigated. Since data was collected within the infant's first year of life, we do not know if the effects of the factors we were looking at resulted in obesity later in life.

Conclusions In summary, this study aimed to investigate the impact of food responsiveness/enjoyment, antibiotic usage, and weight on microbiome diversity. Based on

the p-value standards, the alpha and beta diversity metrics for food responsiveness, food enjoyment, antibiotic usage, and weight all yielded no statistically significant differences. However, using the ANOVA results, the frequency of *Bacteroidetes* is significantly higher in underweight compared to overweight infants.

Based on these conclusions, the field of infant obesity and gut microbiota has a further understanding that factors such as an understanding that food enjoyment/responsiveness does not play any role in infant obesity. Considering the study limitations on antibiotic usage, and how potentially those limitations affected our results, further studies can be done to investigate antibiotic usage in obese infants within consideration of reducing the limitations from this study. Lastly, the frequency of the Bacteroidetes phylum can be further investigated, specifically which species contributed to the statistical difference between underweight and overweight infants. These foundational blocks are what will push this field of study forward.

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Future Directions While holistic analysis of gut microbiome diversity against infant food responsiveness yielded no significant correlations, there may have been differences within certain clades that were drowned out. This was the case when investigating weight vs abundance after all. Future analyses that drill down to individual taxa may reveal correlations too fine to have been seen here.

Besides taxa, metabolism naturally forms clustered networks of microbes based on the nutrient being processed. Instead of looking at food responsiveness, attitudes towards foods with differing nutritional compositions may correlate to differences in abundance for certain metabolic networks. It would thus be interesting to look for differences in the context of metabolic pathways.

Outside of this dataset, the IFP II study also included a 6-year follow-up so it would be interesting to see if the discovered differences in Bacteroidetes abundance with respect to infant weight gain continued into childhood. As other studies reveal the predictive power of microbiome composition (32), it is then natural to hypothesize Bacteroidetes abundance being an early predictor for obesity. Furthermore, as evidence is presented the link between health and microbiomes is bidirectional (33), it would become necessary to explore the therapeutic effects of boosting Bacteroidetes in infants to give them a healthier start in life.

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CONTRIBUTIONS

The co-authorship between Eui-Young Jung, Tony Liu, and Shovon Das should be considered equally. Shovon worked on the abstract, introduction, scripts, results, and discussion on the first objective, conclusion, and acknowledgements. Eui-Young worked on the introduction, methods, scripts, results on the second objective, discussion on the second and third objective, study limitations, and responses to review. Tony Liu worked on the methods, scripts, results on the third objective, and future directions.

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