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Maternal BMI with Respect to Infant Feeding Mode is Associated with Differential Composition and Functional Phenotypes of the Gut Microbiome of 4-month-old Infants

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SUMMARY Maternal body mass index (BMI) is well known to impact the development and composition of the infant gut microbiome, however there is a lack of understanding of the mechanisms behind this association. The present study aimed to explore these potential mechanisms by investigating how maternal BMI, with respect to infant feeding mode, affects the diversity and composition of the gut microbiomes of 4-month-old infants. Using the dataset generated by Dr. Kyung Rhee, we analyzed the effect of feeding mode on infant microbial diversity, across mothers of high BMI (> 30.0) and low BMI (≤30.0). We showed that infants from low BMI mothers have increased gut microbial diversity when formula-fed compared to breastfed. Furthermore, we demonstrated that gut microbial diversity in infants from high BMI mothers was unaffected by the choice of infant feeding mode. We also identified significant increases in the relative abundance of specific bacterial genera including *Bifidobacterium, Lactobacillus and Collinsella*, as well as notable changes to the microbial functional potential of formula-fed infants from low BMI mothers. Namely, we saw an increase in pathways associated with glycogen and tryptophan degradation. Our findings illustrate that the effect of feeding mode on infant microbial diversity and functionality may be modulated by maternal BMI class.

INTRODUCTION

Obesity is becoming an increasingly concerning threat to public health, with an estimated 1 billion people worldwide who are obese—650 million adults, 340 million adolescents, and 39 million children according to the WHO (1). Many known socioeconomic factors contribute to this phenomenon, however there is a gap in understanding of the individual-level factors that contribute to a person's BMI and weight management (1). The formative years of early childhood play an important role in the establishment of one's health span over their lifetime. Of particular importance is the development and maturation of the gut microbiota, which is known to have a significant impact on weight management by regulating energy absorption, influencing appetite, and impacting fat storage (2,3,4,5). The broader scientific literature has established that from conception through the first two years of life, the gut microbiota is highly variable and is particularly susceptible to change due to early life events such as infant mode of delivery, feeding mode, and antibiotic treatment (6). Interestingly, the broader literature has also established that maternal BMI plays an important role in shaping the infant gut microbiota (7), which is the association the present study explores.

Lin *et al.* have previously used this dataset to investigate the impact of mode of delivery and maternal BMI on the infant gut microbiota composition, and concluded that these factors had weak impacts on infant gut microbiota composition and weight gain (8). These findings were particularly interesting, as they seem contradictory to the well-established association between maternal BMI and infant gut microbiota composition in the broader literature (8). This unexpected contradiction was the inspiration for our study which explored the impact of maternal BMI with regards to the feeding mode of the infant, breastfeeding compared to formula feeding, instead of infant mode of delivery.

The motivation for exploring the impact of maternal BMI with regards to infant feeding mode on infant gut microbial diversity stems from Saben *et. al'*s research on human milk oligosaccharide (HMO) intake by infants (9). Notably, maternal obesity has been shown to

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lower the concentration of both fucosylated and sialylated HMOs, thus leading to reduced infant HMO intake (9). HMOs play an integral role in shaping the infant gut microbiota, as they are a carbohydrate source that is selectively used by certain bacterial taxa in the gut microbiota (10). Further, as maternal BMI has been shown to alter the composition of the human milk microbiota—another factor known to affect the development of the infant gut microbiota—we hypothesized that infant feeding mode contributes to the impact of BMI on infant gut microbiota composition and development (11,12,13).

Infant formula milk is one of the most complex manufactured foods in the world, with thousands of different formulations worldwide each containing an intricate balance of fats, proteins, carbohydrates, vitamins, and minerals to replicate breastmilk (14). While breastmilk is still considered the "gold standard" for supporting infant development, the composition of formula milk is evolving to closely mimic its breastmilk counterpart (15). However, the human milk microbiota and bioactive compounds present in breast milk often cannot be added to formula milk due to shelf life constraints (16). This lack of bioactive compounds in formula has been shown to contribute to differences in the composition of the developing infant's gut microbiota (17). Specifically, formula-fed infants have been shown to have lower levels of beneficial bacteria (17). This study aims to investigate how these differences between breastfed and formula-fed infants change across different maternal BMI classes.

The results of this study have important implications for society, as they can provide insights into the early life establishment of the gut microbiota, and potential associated health outcomes. Understanding how vertical transmission of certain bacterial taxa influences infant health may have the potential to inform preventative healthcare policies and recommendations (18). This could have profound impacts on improving the overall social-emotional wellness of young children (19), which would improve long-term health outcomes and reduce healthcare costs (20).

METHODS AND MATERIALS

Study System. The dataset used in this study was collected by Dr. Kyung Rhee from the Department of Pediatrics at the University of California. It was originally intended to analyze eating behavior phenotypes, such as poor satiety responses and food enjoyment, and potential predictors of these behaviors, such as maternal obesity, maternal eating behavior, and diet (21). The dataset consists of 82 infant-mother dyad stool samples collected at 0.5 months, 2 months, 4 months, 6 months, and 9 months of infant age. Within each dyad sample, there are 171 rich metadata categories described including infant feeding mode, mode of delivery, and maternal BMI. The specific microbial sequences analyzed for each sample were obtained using the 515fbc and 806r primers to isolate the V4 region of the 16s rRNA gene (22-23).

Metadata filtering and grouping. To focus our research specifically on the effect of maternal BMI and infant feeding mode, the metadata was filtered and re-formatted using the tidyverse (24) and dplyr (25), package in R version 4.1.2 (26,27). In this process, feeding mode, mode of delivery, and maternal BMI, the three metadata categories of interest for our specific research question, were retained. Following this initial filtering, a second filtering step was performed on the metadata to exclusively retain samples from 4-month-old infants (n = 45). This time point was chosen primarily based on a study performed by Rutayasire *et al.* which showed that the effect of infant mode of delivery as a confounding variable on the infant gut microbiota was reduced between the first 3-6 months of life (28). Furthermore, for the metadata provided, the 4-month-old timepoint included a sufficient sample size, which the later time points lacked. Lastly, as multiple time points were recorded for each sample, selecting one time point allowed the retention of a single sample for each infant for downstream analyses. To improve the statistical power of the study, the "combined" feed type (n = 6) and "formula" feed type (n = 11) were grouped within the "feed" metadata column. Lastly, all 4-month-old infant samples in which the data for our metadata categories of interest were not collected were excluded from the study. Following filtering, 34 samples of 4-month-old infants across all maternal BMIs were retained.

Preliminary data processing in QIIME2. Dataset processing was completed through the QIIME2 pipeline by importing and demultiplexing our dataset using a filtered manifest file,

which retained only the 34 samples of interest and corresponded to the samples retained in the filtered metadata file. Following demultiplexing, a quality control step was performed on our reads using the Divisive Amplicon Denoising Algorithm 2 (DADA2) (29). We selected a truncation length of 0-150bp and generated a representative sequences file and a feature table of amplicon sequence variants (ASVs). Retention of all 150 base pairs in all our reads, without trimming, was performed as every base pair position displayed a Phred quality score above 30.0. Taxonomic analysis was performed on our ASVs table by training the classifier using the SILVA 138 99% OTU database on the 515F/806R (V4) region of the 16S rRNA gene, and we further performed taxonomy-based filtering to remove mitochondrial and chloroplast sequences (22-23).

Diversity and Statistical Analyses. Alpha and beta diversity analyses were performed in R using the following packages: tidyverse (2.0.0) (25), dplyr (v1.1.3) (26), phyloseq (v1.44.0), vegan (v2.6-4), ggplot2 (v3.4.4), ggpubr (v0.6.0), picante (v1.8.2), ape (v5.7-1), gridExtra (v2.3), data.table (v1.14.8), ggh4x (v0.2.6) (30-39). Before running these analyses, each infant sample was assigned to either the "high" (>30) or "low" (≤30) maternal BMI category. The generated phyloseq object was filtered to remove any non-bacterial sequences, any ASVs with fewer than 5 total counts, and any samples with fewer than 100 reads, which resulted in a loss of two samples from the low maternal BMI category. The remaining 32 total reads in this object were rarefied to a sampling depth of 14456 and then subsetted into two separate phyloseq objects each corresponding to either the high or low maternal BMI class. The initial alpha diversity analysis was run using the combined BMI phyloseq object, and all other downstream alpha and beta diversity analyses were run using both subsetted phyloseq objects to compare the effect of infant feeding mode within each maternal BMI class. Alpha diversity analyses included Shannon's and Faith's Phylogenetic Diversity which were visualized as boxplots using the R package ggplot2 (32), and statistical significance was evaluated using linear regression models with a cutoff value of $p<0.05$ while controlling for mode of delivery. Beta diversity analyses included Bray-Curtis and Weighted UniFrac visualized as PCoA plots using ggplot2 (32), and statistical significance was evaluated using PERMANOVA with a cutoff value of $p<0.05$ while controlling for mode of delivery.

DESeq2 Analysis to Determine Differentially Abundant Taxa. Differential Expression Sequence (DESeq2) analysis was performed in R using the following packages: tidyverse, dplyr (24, 25), phyloseq (30), data.table (38) and DESeq2 (40). This analysis was run twice using each subsetted phyloseq object once, and the resulting plots were combined for easier visualization. Formula-fed infants were set as the reference group and visualization of the results was performed using ggplot2 (32). Results were displayed at the genus level with log2FoldChange > 2 and statistical significance measured at $p < 0.01$ while controlling for mode of delivery.

PICRUST2 Analysis to Determine Predicted Functionality. Microbiome-predicted functionality was analyzed using a PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2) analysis of gut microbial 16S rRNA genome sequences (41,42). HHMER and SEPP tools in PICRUSt2 were used to place ASVs into a phylogenetic tree according to the reference-based sequence similarity (41,42), and output metagenomic data was input into R and analyzed using the R ggpicrust2 package (43). Before analysis, metadata and metagenomic files were subsetted into low and high maternal BMI classes. To assess the potential impact of breastfeeding on pathway abundance, software integrated into ggpicrust2 (43) was used on MetaCyc pathways to provide corresponding p-values for statistical significance. The results were visualized using a grouped bar chart, allowing for the comparison of pathway abundances between infants who were breastfed and those who were formula-fed within each maternal BMI class.

Data availability. The raw sequences analyzed in this study are available through the European Nucleotide Archive. (Accession Number: PRJEB39437). Preliminary data processing scripts can be found in the Supplemental QIIME2 Script and the R diversity analyses, statistical analyses, and PiCRUST analysis scripts can be found in the

Supplemental R Script - all of which can be found at https://github.com/emilymoffat/MICB-475.git.

RESULTS

Formula feeding may increase the average richness of microbes in the gut microbiome of 4-month-old infants with a low maternal BMI. In our initial analysis, we examined the differences in alpha diversity scores of the gut microbiomes of infants either formula-fed or breastfed across all BMIs, followed by a microbial diversity analysis of infants from mothers with a low or high maternal BMI class (Supplemental Figure S1). No significant differences in infant gut microbial diversity were observed between infants belonging to mothers with a low vs. high maternal BMI class, however, we did observe significant differences in infant gut microbial diversity scores between different infant feeding modes across all BMIs. This prompted us to explore whether these significant differences remained present within the low and high maternal BMI classes, separately. Faith's Phylogenetic Diversity analysis revealed a significant increase in gut microbial diversity of 4-month-old low maternal BMI formula-fed infants (n = 2) compared to breastfed infants (n = 17). In contrast, there were no statistically significant differences in the gut microbial diversity of infants formula-fed (n = 6) compared to breastfed (n = 7) in the high maternal BMI class (Figure 1). The same pattern was observed when analyzing alpha diversity using Shannon's diversity metric (44). Namely, formula-fed infants in the low maternal BMI class had significantly enriched gut microbial diversity compared to breast-fed infants, and no statistical significance was observed when comparing gut microbial diversity across infant feeding modes from infants in the high maternal BMI class (Figure 1). Based on these findings, we reasoned that the significant difference we observed between infant feeding modes across all maternal BMIs may be associated with the significant differences in the feeding mode of infants belonging to the low maternal BMI class.

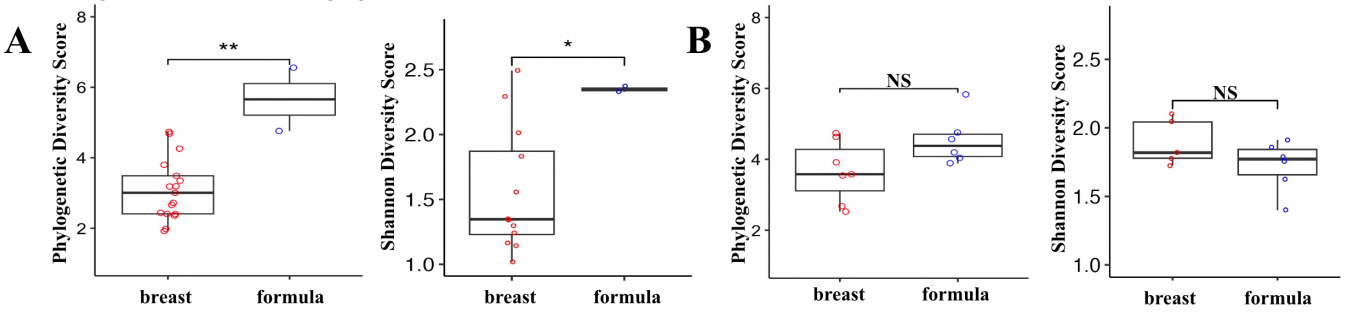

FIG. 1 4-month old formula-fed infants from mothers with a BMI < 30.0 exhibit higher alpha diversity than breastfed infants. Comparing 4 month old infants in either the low (A) or high (B) maternal BMI class who were either breast-fed or formula-fed. Linear regression models controlling for infant mode of delivery found significant differences in the low maternal BMI class using Faith's Phylogenetic Diversity (p=0.003) and Shannon's Diversity (p=0.048), while no significant differences were found in the high maternal BMI class (p>0.05).

Infant feeding mode is correlated with changes to the bacterial phylogenetic relationships within the gut microbiome of 4-month-old infants. Following our alpha diversity analyses, the compositions of the 4-month-old infant gut microbiomes were compared across different infant feeding modes and maternal BMIs using Weighted UniFrac and Bray-Curtis metrics. When evaluating beta diversity across maternal BMI categories, neither Weighted UniFrac nor Bray-Curtis found significant differences between the composition of the gut microbiomes of infants from low (n = 19) vs. high (*n* = 13) BMI mothers (Figure 2). However, Weighted UniFrac analysis, which takes into account abundance and phylogenetic relationships between bacterial taxa (45), revealed a significant difference in the composition of the gut microbiomes of infants who were formula-fed (*n* = 8) vs. breastfed (*n* = 24). Interestingly, the Bray-Curtis analysis, which only takes bacterial abundance into account, found no significant difference in the composition of the gut microbiomes of infants who were formula-fed vs. breastfed (Supplemental Figure S2). Taking both of these results into account, the beta diversity data suggests that the gut microbiomes

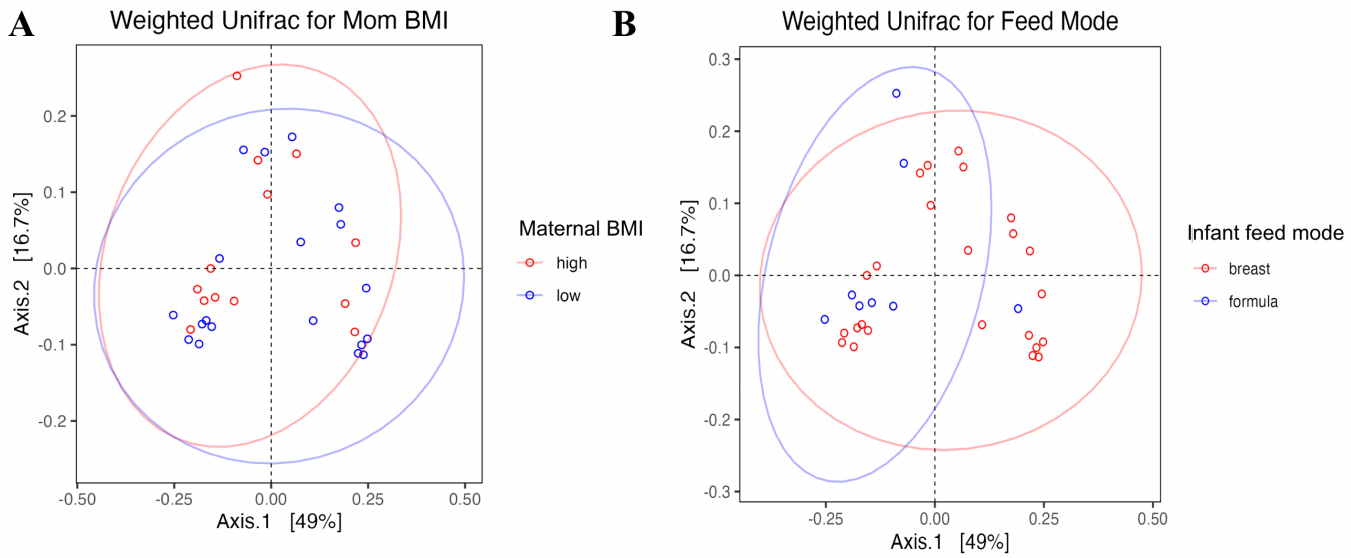

FIG. 2 4-month-old infants have differences in the phylogenetic distances between bacterial taxa across different feed modes. A) Significant differences (p = 0.049) in Weighted UniFrac metrics between 4-month-old infant gut microbiomes were found between infants who were breastfed vs. formula-fed. B) Weighted UniFrac metrics showed no significant difference (p = 0.393) between infants from high vs. low BMI mothers. The p-values were calculated using PERMANOVA while controlling for mode of delivery.

of infants who were formula-fed vs. breastfed were significantly different in terms of their phylogenetic distances, and to a lesser extent the abundances of certain taxa. These results are in alignment with the results from the alpha diversity analyses, which indicated that formula-fed infants from low BMI mothers have significantly increased microbial diversity when compared to infants who were breastfed. In short, beta diversity analyses recapitulated the previously observed correlation between infant feeding mode and differences in gut microbial diversity.

Many bacterial genera have differential abundances correlated with different infant feeding modes in the gut microbiomes of 4-month-old infants. DESeq2 analysis was used to investigate how the abundance of specific bacterial genera changed across different feeding modes in infants from mothers belonging to either a high or low BMI class (Figure 3). The analysis revealed that in both maternal BMI groups, the infants' feeding mode is correlated with significant changes in the abundances of many different bacterial genera. Upon first glance, there appear to be more differentially abundant genera in the high maternal BMI category, however, this is likely because the low maternal BMI category had a smaller sample size. Interestingly, the genera that are differentially abundant within each maternal BMI category are mostly distinct from each other, implying that the genera influenced by feeding modes may vary between BMI categories. Differentially abundant taxa of particular interest include *Lactobacillus*, *Bifidobacterium* and *Collinsella*, which had differential abundance across different feeding modes and are discussed in more detail later in this report. Overall, the DESeq2 analysis revealed many differentially abundant genera that are associated with different infant feeding modes across both maternal BMI categories, with different genera affected in each maternal BMI category.

The formula-fed gut microbiomes of 4-month-old infants belonging to mothers in the low maternal BMI class are associated with significant differences in microbial functional potential. The functional potential of the gut microbiomes of infants breastfed and formula-fed in low and high maternal BMI groups was analyzed using PiCRUSt2 ASV alignment. 16s rRNA amplicon sequencing data was initially aligned to ASVs, before phylogenetic alignment and MetaCyc pathway predictions were analyzed. After performing a LinDA differential abundance analysis, we identified ten pathways with statistically significant differences in relative abundance between breastfed and formula-fed infants within the low maternal BMI class (Figure 4). We found that the predicted functionality of the gut microbiome of formula-fed infants from low BMI mothers was shown to be associated

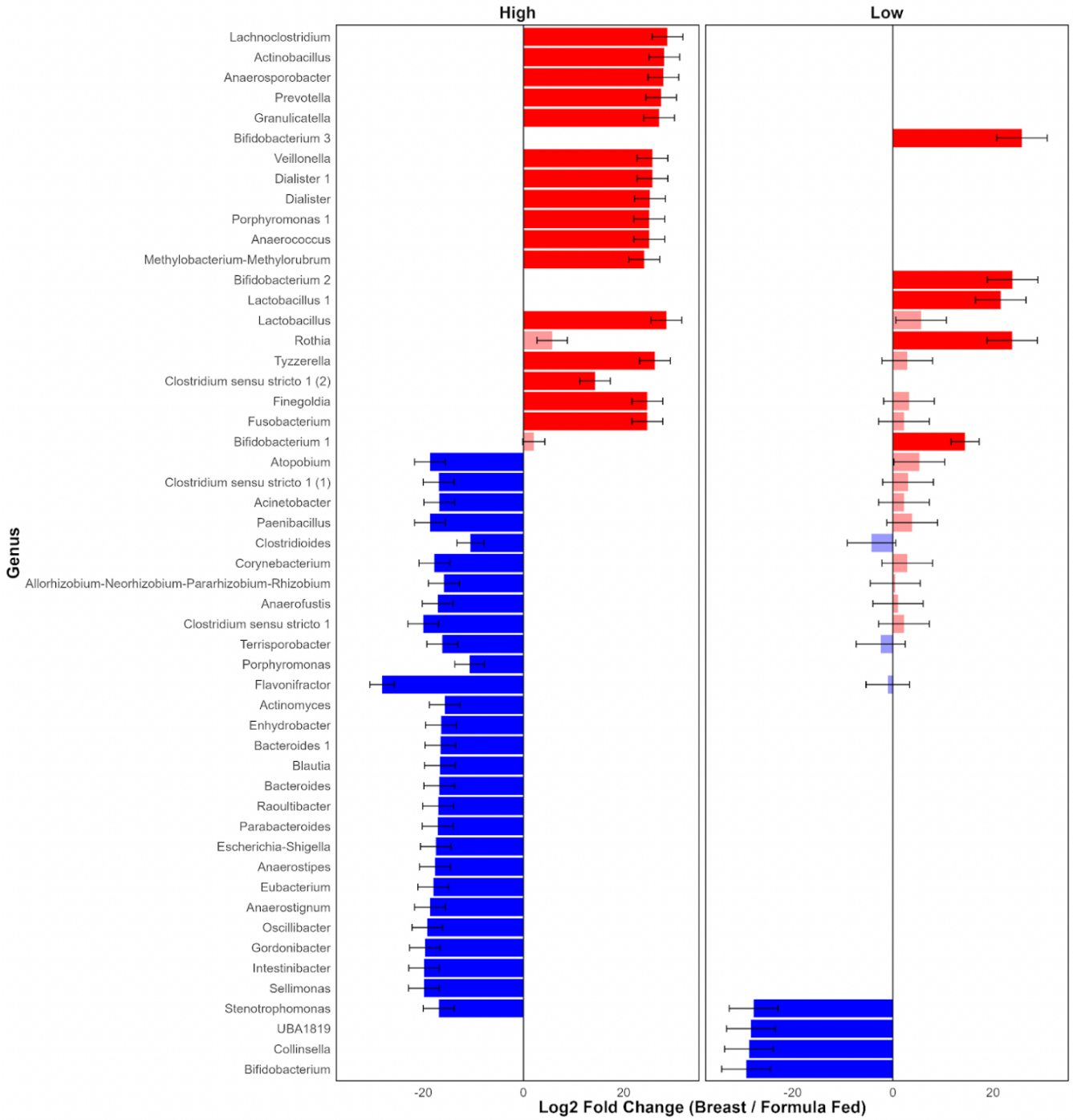

FIG. 3 4-month-old breastfed infants have differentially abundant genera relative to formula-fed infants in both high and low maternal BMI groups. Differentially abundant taxa with $p < 0.01$ and Log2FoldChange > 2 are shown as dark bars. Transparent bars show non-significant ($p < 0.01$) fold changes in genera with significant differential abundance. Gaps represent outputs of NA due to insufficient information for DESeq2 analysis. Increased abundance is shown in red for breastfed and blue for formula-fed infants.

with an increased abundance of multiple metabolic pathways, most notably, 1,5 anhydro-fructose degradation and L-tryptophan degradation (Figure 4). Furthermore, there were no statistically significant differences in pathway abundance between breastfed and formula-fed infants for the high-maternal BMI class when using the same statistical software.

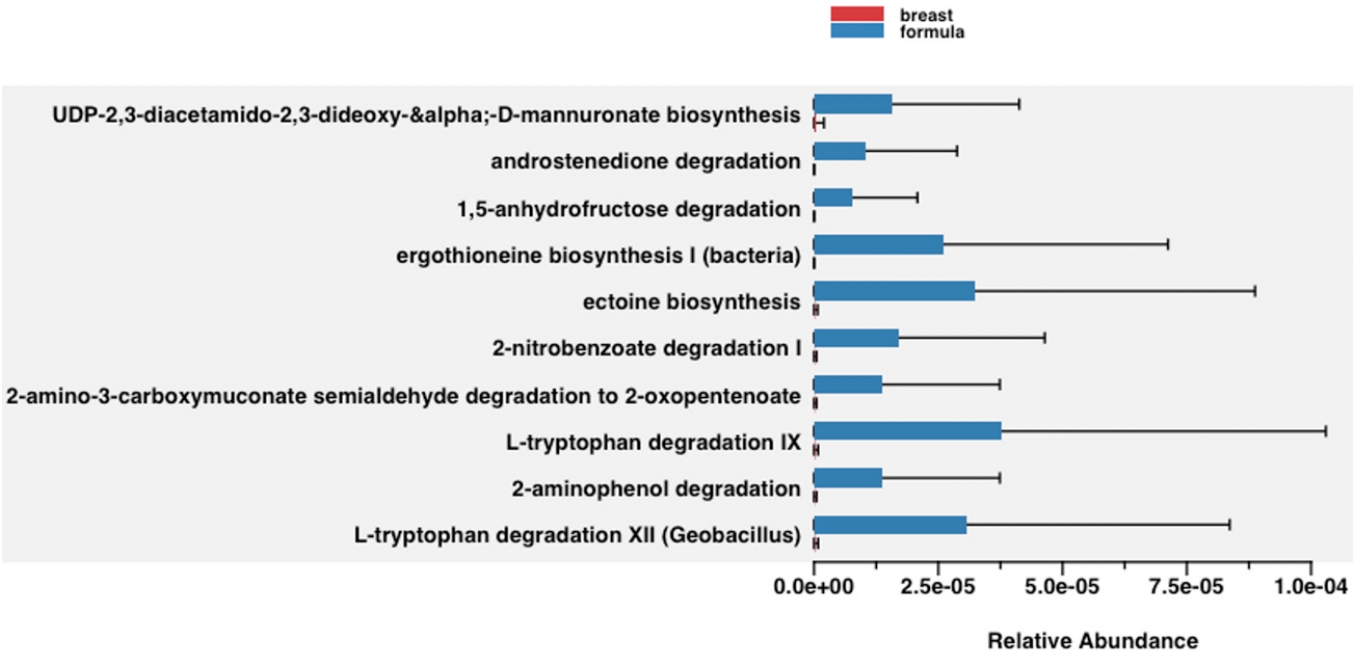

FIG. 4 Functional Potential Analysis Identified 10 differentially abundant pathways in 4-month-old infants breastfed versus formula-fed in the low maternal BMI class. Bar graphs indicate MetaCyc pathway relative abundances in the low-maternal BMI group, with illustrated error bars, as confirmed through LinDA data processing ($p<0.05$). No statistically significant differences in pathway abundances were observed between feed type in the high-maternal BMI class, and no graph was produced.

DISCUSSION

This study aimed to investigate how maternal BMI influences the gut microbiome of 4-month-old infants with respect to the infant feeding mode. Overall, we observed an association between low maternal BMI formula-fed (LB-FF) infants and higher microbial diversity. This result aligns with broader literature which indicates that formula-fed infants have a more diverse gut microbiota compared to breastfed infants at 4 months of age (17).

Initial alpha and beta diversity analyses pointed to an association between formula feeding and higher infant gut microbial diversity in infants born to mothers with a low maternal BMI (≤30.0). Conversely, the infant mode of feeding did not significantly modulate gut microbial diversity in infants born to mothers with a high maternal BMI (>30.0). Taken together, these results seem to suggest that the choice of infant mode of feeding may only significantly modulate the infant gut microbiome if the mother's BMI is below, or equal to 30.0. It's important to understand the impact of infant feeding mode and maternal BMI on the development of the infant gut microbiome, as microbial curation in early colonization is important in reducing the presence of pathogenic bacteria, which can aid in mitigating downstream negative effects on infant gut functionality (17).

Human breast milk is known to be rich in HMOs, which have been shown to impact the development and composition of the infant's gut microbiota (9). Specifically, breastfeeding has been correlated with lowered infant gut microbial diversity due to the presence of HMOs (46). Being the third most abundant component of milk (47), the high concentration of HMOs in breast milk allows for preferential selection of beneficial bacterial taxa that can perform oligosaccharide degradation. *Lactobacillus* and *Bifidobacterium* are two such genera that have been shown to selectively colonize the gut of breastfed infants due to their ability to degrade HMOs (48,49). Notably, the DESeq2 results reflect this association, as *Lactobacillus* and select *Bifidobacterium* genera are more abundant in breastfed infants. Furthermore, prior research has shown that breast milk from mothers with a BMI above 30.0 have significantly reduced HMO content compared to mothers with a BMI below, or equal to 30.0 (9). It follows that high maternal BMI breastfed (HB-BF) infants will have reduced HMO intake compared to LB-BF infants (9). Therefore, we postulate that the reduced infant microbial diversity

observed in LB-BF infants relative to HB-BF could be correlated to higher HMO intake in LB-BF infants. The results from the DESeq2 analysis corroborate this hypothesis, in that while the increased abundance of *Lactobacillus* and select *Bifidobacterium* are present in both breastfed BMI groups, they are dramatically more represented in the LB-BF infants.

In contrast, HMOs are completely absent in formula milk, thus reducing the selective pressure for colonization of bacteria capable of oligosaccharide degradation (48), which potentially explains the higher infant microbial diversity observed in formula-fed infants across all maternal BMIs. Due to government regulations and storage half-life, certain bioactive components that selectively modulate the gastrointestinal tract are unable to be added to formula (50). As a result of these restrictions, formula milk is predominated with stable components including proteins and high dietary fiber, and it lacks bioactive components including HMOs and the human milk microbiota (16). This increase in protein content (51), and lack of HMOs relative to breastmilk, have been shown to drive increased alpha diversity in formula-fed infants and promote a shift towards an adult-like microbiome at a faster rate than breast-fed infants (52).

To understand how the differential abundance of genera across different infant feeding modes, as shown in the DESeq2 analysis, impacts the predicted functionality of the gut microbiota, we compared differentially abundant pathways in breastfed vs. formula-fed infants using MetaCyc pathway analysis. Using this data, we aimed to establish a connection between the observed increase in the overabundant functional pathways in LB-FF infants and the changes seen in bacterial abundances. While there were several potential links between these data, the results taken together seem to highlight an important correlation: the increase in abundance of the genus *Collinsella* and enhanced 1,5 anhydro-fructose degradation, which were both seen in LB-FF infants. The genus *Collinsella* is known to be associated with increased levels of circulating insulin (53), a hormone whose upregulation triggers glycogen production (54). Glycogen serves as an important substrate that is synthesized from glucose (55). LB-FF infants demonstrated a significantly increased abundance of 1-5 anhydro-fructose degradation, an intermediate produced through the degradation of glycogen (56). We postulate that the increased abundance of *Collinsella*, and associated glycogenesis-stimulating insulin, may correlate to the enhanced degradation of 1,5 anhydro-fructose due to substrate accumulation. The health implications of this association are broad as an overabundance of *Collinsella* in the gut microbiome has been linked to an increased risk of developing insulin resistance (53). A potential intervention to mitigate this risk could be to modulate the nutrient intake of pregnant women to ensure sufficient fiber intake, as low dietary fiber has been shown to promote the growth of *Collinsella* in pregnant women (53).

In the LB-FF category, L-tryptophan kynurenine degradative pathways were shown to be significantly more abundant. Tryptophan metabolism is important in the host, as tryptophan serves as an essential precursor for serotonin biosynthesis (57). The bacterial degradation of L-tryptophan has been shown to secrete a wide range of metabolic byproducts from the kynurenine pathway, which may have significant impacts on the host (57). Specifically, it has been hypothesized that an overabundance of bacteria expressing the kynurenine pathway of L-tryptophan degradation may offset host tryptophan metabolism in the gut (57). Interestingly, prior studies have shown that the gut-brain axis can be modulated by the degree of GI-based tryptophan degradation (57). The kynurenine pathway intermediates produced during bacterial L-tryptophan degradation can act as modulators of brain activity by interacting with the central nervous system axis (57). Specifically, the health implications of upregulation of this pathway are broad as heightened kynurenine metabolite synthesis has been shown to be associated with Parkinson's Disease, and Huntington's disease (58). If this imbalanced L-tryptophan degradation is clinically proven to have negative effects on the infant host, this could prompt an avenue for early microbial intervention. Some plausible strategies include the utilization of antibiotics to reshape the gut microbiome to reduce the presence of bacteria expressing these metabolic pathways, or the use of probiotics to encourage other microbial species to outcompete the bacteria performing L-tryptophan degradation.

Limitations The main limitation of this study is the small sample size and uneven sample distribution. Overall, there were more breastfed infants compared to formula-fed infants for

both the low and high maternal BMI classes, with the low maternal BMI formula-fed infants only having $n = 2$. This reduced sample size drastically reduces our ability to generate extrapolative results, and increases the risk of type II error development. Additionally, we grouped infants with a "combined" feeding mode with formula-fed infants, which limits our capacity to make conclusions about exclusively formula-fed infants. Furthermore, due to the constraint on sample size, our data was divided into two groups based on "high" (>30) or "low" (≤30) maternal BMI. Effectively, this means the conclusions we draw about maternal BMI are limited solely to obese and non-obese mothers, with a lack of data about intermediate BMI categories—for example, underweight, normal weight, overweight, and obese, and how they affect infant gut microbial diversity.

While it offset other potential sources of error as previously described, our decision to exclusively study 4-month-old infants limited our capacity to draw conclusions about the patterns of microbial diversity during different developmental periods of the infant's life. Additionally, all the infant and mother dyads were geographically situated in Michigan, USA, which limits our ability to extrapolate our results to global populations. Specifically, there may be regional influences that impact infant microbial diversity differently, thus having the potential to yield results that are not representative of general populations.

As mentioned in our discussion, we speculate that some of the observed differences between breastfed and formula-fed infants and across different maternal BMIs could be correlated with differences in HMO concentrations in breast milk. However, since the concentration of HMOs in the mother's breast milk was not measured in this study, we can not conclusively determine whether the observed differences in microbial diversity measures can be attributed to the effect of the selective pressures created by HMOs.

Conclusions This study aimed to explore the effect of maternal BMI with respect to infant feeding mode on the infant gut microbiome. We identified a significant increase in microbial diversity amongst formula-fed infants compared to breastfed infants, particularly within the low-maternal BMI class, and we postulate that breastmilk HMOs play a key role in influencing these differences. Furthermore, taxonomic analyses revealed a significant differential abundance of many genera across different feeding modes in both BMI categories, including *Lactobacillus, Bifidobacterium,* and *Collinsella.* Analysis of the metagenomic data revealed significant differences in the predicted functionality of the microbiome between infants formula-fed and breastfed in the low maternal BMI class, which may be linked to observed differences in bacteria engaging in these pathways. Results from this study provide a basis for further research to explore the maternal factors contributing to the infant gut microbial composition and development, and could inform strategies for managing infant health outcomes and early childhood obesity.

Future Directions To further validate the role of maternal BMI with respect to infant feeding mode on microbial diversity, a similar investigation with a larger sample size could be conducted to confirm the patterns observed. A larger sample size would allow for a secondary investigation which subsets the BMI classes into more precise groups. This could identify specific BMI classes that influence the effect of feeding mode on infant microbial diversity with more precision. Additionally, a further study expanding the cultural and regional diversity of the infant-mother dyad samples could enable a broader extrapolation of the data to the general public.

Furthermore, a longitudinal study investigating how the influence of maternal BMI with respect to feeding mode affects infant microbial diversity and development over the infant's early life would be valuable. This approach could prompt future researchers to investigate infant health outcomes that may be associated with gut microbial diversity and composition as impacted by maternal BMI and feeding mode. Additionally, given that HMO concentrations in breast milk were not recorded in our dataset, a correlational analysis tracking infant HMO intake to infant microbial diversity could be explored.

Lastly, it would be interesting to further investigate the observed correlation between infant feeding modes and differential abundance of the genus *Collinsella.* Given that the existing literature supports a link between the increased abundance of *Collinsella* bacteria and increasing insulin circulation, further explorations of these associations could provide

insight into the effect of the infant gut microbial composition in driving specific health outcomes, such as insulin resistance and related challenges.

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CONTRIBUTIONS

Nicole Cormack: Materials and Methods, Alpha Diversity Metrics and Results, PICRUST2 Metrics and Results, Discussion, Limitations Revisions, References, General Review.
Madeleine Dunsmore: Abstract, Materials and Methods, Results, Alpha Diversity Metrics, Discussion, General Review.
Hayley Emery: DESeq2 Metrics, Study limitations, Conclusions, Future Directions, References, General Review.
Emily Moffat: Abstract, Introduction, DESeq2 Methods, Beta Diversity and DESeq2 Metrics, Results, Discussion, General Review.

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