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Original Article

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A Pilot Study Exploring Temporal Development of Gut Microbiome/Metabolome in Breastfed Neonates during the First Week of Life

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ABSTRACT

Purpose: Exclusive breastfeeding promotes gut microbial compositions associated with lower rates of metabolic and autoimmune diseases. Its cessation is implicated in increased microbiome-metabolome discordance, suggesting a vulnerability to dietary changes. Formula supplementation is common within our low-income, ethnic-minority community. We studied exclusively breastfed (EBF) neonates' early microbiome-metabolome coupling in efforts to build foundational knowledge needed to target this inequality.
Methods: Maternal surveys and stool samples from seven EBF neonates at first transitional stool (0–24 hours), discharge (30–48 hours), and at first appointment (days 3–5) were collected. Survey included demographics, feeding method, medications, medical history and tobacco and alcohol use. Stool samples were processed for 16S rRNA gene sequencing and lipid analysis by gas chromatography-mass spectrometry. Alpha and beta diversity analyses and Procrustes randomization for associations were carried out.
Results: Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria were the most abundant taxa. Variation in microbiome composition was greater between individuals than within (p=0.001). Palmitic, oleic, stearic, and linoleic acids were the most abundant lipids. Variation in lipid composition was greater between individuals than within (p=0.040). Multivariate composition of the metabolome, but not microbiome, correlated with time (p=0.030). Total lipids, saturated lipids, and unsaturated lipids concentrations increased over time (p=0.012, p=0.008, p=0.023). Alpha diversity did not correlate with time (p=0.403). Microbiome composition was not associated with each samples' metabolome (p=0.450).
Conclusion: Neonate gut microbiomes were unique to each neonate; respective metabolome profiles demonstrated generalizable temporal developments. The overall variability suggests potential interplay between influences including maternal breastmilk composition, amount consumed and living environment.

Keywords: Infant; Breastfed; Formula-fed; Human milk; Formula milk; Gastrointestinal microbiome; Metabolome; Metabolite; Infant gut microbiome; Lipidomic

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Conflict of Interest
The authors have no financial conflicts of interest.

INTRODUCTION

As the prevalence of childhood obesity continues to grow, efficacious preventative measures are of utmost importance. The gut microbiome has been proposed to play a role in obesity [1,2]. Colonization of commensal gut bacteria during infancy is influenced by many factors including mode of delivery, antibiotic exposure and both maternal and infant nutrition. Breastfeeding has been implicated as a vital early influence on infant microbiome development. Advantageous differences in microbial composition between exclusively breastfed (EBF) infants and their non-EBF counterparts have been shown to persist throughout infancy and provide protection against pediatric illnesses [1,2]. Formula-fed children depict an altered microbial composition relative to EBF children. Formula feeding is believed to facilitate the colonization of microbial species that comprise the "obese phenotype", predisposing infants to metabolic syndromes [1-3]. Supplementation with formula during the first week of life reprograms normal weight trajectory, leading to a greater risk for obesity by the age of two years [1,2]. Breastfeeding is shown to prevent the "obese phenotype", with longer durations of breastfeeding associated with decreased growth acceleration rates, and thus, decreased likelihood of being obese or overweight [4,5].

Despite our growing understanding of early gut microbial changes, few studies have described the temporal development of the infant gut metabolome. Preliminary studies have revealed that alterations in the infantile gut metabolomic profile are correlated with their relative microbiome community composition [6-8]. Increased discordance in microbiome-metabolome coupling has been demonstrated later in infancy after the cessation of exclusive breastfeeding. As such, the interplay between gut microbiome community composition and gut metabolome appears to be particularly evident during, and influenced by, early infancy [6-8].

Despite increased understanding of the benefits, epidemiological studies have identified wide variations in exclusive breastfeeding rates between counties and hospitals [9]. As per Centers for Disease Control and Prevention, the rates of breastfeeding remain suboptimal, especially amongst African American women from low-income communities. Equally as alarming, 1 in 5 children, ages 2 to 6, within the low-income communities are obese. Reflective of these disparities, it was reported that children from our community were at greater risk for obesity than their national counterparts, especially at ages 3–5 (35% vs. 21% nationally). Hospital-driven initiatives and maternal educational efforts have attempted to address these inequalities within our socioeconomically disadvantaged community. Yet, despite intervention, a significant proportion of mothers intending to exclusively breastfeed report the implementation of formula feeding.

The suboptimal rates of exclusive breastfeeding and its association with childhood obesity illuminate a currently under-recognized disparity burdening this community and call for further intervention. Evidence regarding microbiome alterations and subsequent risk of childhood obesity and other detrimental health sequelae continues to mount; yet less has been established regarding the role of the gut metabolome. Limited studies have profiled microbiome-metabolome coupling and illustrated the alterations associated with infant feeding patterns within the first week of life from serial stool samples [7,10-12]. Currently, a dearth of evidence exists regarding the impact of infant feeding practices on the microbiome-metabolome relationship and its subsequent impact on health outcomes. As such, this observational cohort study of mother-infant dyads aims to characterize the development of the gut microbiome and metabolome during the first week of infancy.

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MATERIALS AND METHODS

Study design

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by our institution's Institutional Review Board (IRB) (Protocol # 18-060). Women delivering via vaginal birth at our institution from January 1st 2018 until December 31st 2019 were included in the study. Patients were screened for eligibility upon admission and were excluded if they were preterm (under 37 weeks), delivered via caesarian section, received antibiotics during their third trimester, or if the mother/newborn was HIV+. Mothers were informed of the opportunity for voluntary participation in this observational study. Informed consent was obtained from willing mothers, per guidelines set by the IRB. Newborn stool samples were collected at three consecutive time points: first transitional stool (day 1: 0–24 hours), day of discharge (day 2: 30–48 hours), and their first pediatric appointment (days 3–5 post-delivery). Baseline patient characteristics at enrollment are shown in **Table 1**.

Table 1. Mother demographics

Baseline characteristics of mothers	Value (N=7)
Age (yr)	
20–25	4 (57.1)
26–30	3 (42.9)
Body mass index (pre-pregnancy)	
18.5–24.9	1 (14.3)
25–29.9	3 (42.9)
30 and above	3 (42.9)
Ethnicity/race	
African American	4 (57.1)
Caucasian	2 (28.6)
Hispanic	1 (14.3)
Other	0 (0.0)
Highest education	
Less than high school	0 (0.0)
High school	4 (57.1)
Undergraduate	2 (28.6)
Graduate	0 (0.0)
Post-graduate	1 (14.3)
Annual household income ($)	
Up to 23,000	6 (85.7)
24,000–50,000	0 (0.0)
51,000–75,000	0 (0.0)
75,00–100,000	0 (0.0)
Above 100,000	1 (14.3)
Smoking	
Never smoked	3 (42.9)
Quit smoking	3 (42.9)
Continued smoking	1 (14.3)
Smoking during pregnancy	
Yes	1 (14.3)
No	6 (85.7)
Smoking after delivery	
Yes	0 (0.0)
No	7 (100.0)
Alcohol before pregnancy	
Yes	2 (28.6)
No	5 (71.4)

Values are presented as number (%).

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Study population

Data were collected from patients presenting to our institution. Our community is considered to be one of the poorest areas in the country. As per the Census data, the unemployment rate is double that of the entire country and job growth percentages are in the negative numbers. The median household income is less than half of the average household income for the entire country. Of the population under the age of 65, 12% do not have insurance, and 9% are disabled. About 38% of the population is non-Caucasian, and 9% of the residents are foreign-born. Thus, this community's demographic includes several elements that are reported to contribute to the suboptimal breastfeeding rates observed.

Sample and survey collection

Mothers were given detailed instructions about sample collection and were provided with a kit that included a labeled collection tube in a zip lock bag and gloves. Stool samples were collected from babies' diapers in Stool Nucleic Acid Collection and Preservation Tubes (Norgen Biotek Co.). The stabilizer allows easy storage and transport of samples to the laboratory and optimal preservation of gut microbiome composition [13]. It does not interfere with the fatty acid metabolome analysis. Upon receipt, samples were stored at –80°C for long-term storage and subsequent analysis. Mothers were also provided with survey questionnaires to return with each sample. Information regarding current feeding practices, medication use, changes in medical conditions, as well as tobacco and alcohol use was collected using these surveys. Surveys used are included as **Supplementary Fig. 1**.

16S rRNA gene sequencing

Total genomic bacterial DNA was extracted from fecal samples using the Qiagen DNeasy PowerSoil HTP extraction kit (Qiagen) according to the manufacturer's instructions. Marker genes in isolated DNA were PCR-amplified using GoTaq Master Mix (Promega). For high-throughput sequencing, 515 F (5'-GTGCCAGCMGCCGCGGTAA-3') 806 R (5'-GGACTACHVGGGTWTCTAAT-3') Golay barcoded primer pair targeting the V4 hypervariable region of the 16S rRNA gene, which is highly conserved and ideal for gut microbiome analysis, modified with a unique 12-base sequence identifier for each sample and the Illumina adapter were utilized as previously described [14]. The thermocycling program consisted of an initial step at 94°C for 3 minutes followed by 35 cycles (94°C for 45seconds, 55°C for 1 minutes, and 72°C for 1.5 minutes), and a final extension at 72°C for 10 minutes. PCR products were cleaned and normalized using a SequalPrep Normalization Kit (Cat. No. A1051001, ThermoFisher) following manufacturer's instructions. 16S rRNA gene sequencing and library preparation were performed on normalized pooled amplicons using the Illumina MiSeq System using a V2 300-cycle MiSeq reagent kit. Sequencing of the samples was conducted at the University's Next-Gen Sequencing core facility.

Fatty acid analysis

Fecal samples were analyzed by gas chromatography–mass spectrometry (GC-MS). Homogenized stool samples (100 mg aliquots) were used. Liquid/liquid extraction was performed to extract fatty acids and remove the nucleic acid preservative. A 250 μL aliquot of each extract was transferred to a clean analysis tube. The solvent was removed by evaporation under a stream of nitrogen. Internal standard solution was added to the dried sample extracts, quality controls (QCs), and calibration standards. The solvent was again removed by evaporation under nitrogen. The dried samples and QCs were subjected to methylation/transmethylation with methanol/sulfuric acid, resulting in the formation of the corresponding fatty acid methyl esters (FAMEs) of free fatty acids and conjugated

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fatty acids. The reaction mixture was neutralized and extracted with hexanes. An aliquot of the hexanes layer was injected onto a 7890A/5975C GC-MS system. Mass spectrometric analysis was performed in the single ion monitoring positive mode with electron ionization. Quantitation was performed using both linear and quadratic regression analysis generated from fortified calibration standards prepared immediately prior to each run. Raw data were collected and processed using Agilent MassHunter GC-MS Acquisition B.07.04.2260 and Agilent MassHunter Workstation Software Quantitative Analysis for GC-MS B.09.00/ Build 9.0.647.0. Thus, the total fecal content of 30 fatty acids after conversion into their corresponding FAMEs was measured. Concentrations are provided in weight-corrected μg/g of fecal dry mass. Values below the lower limit of quantification were treated as a concentration of 0.001 μg/mL.

Statistical analysis

Analysis of the data from 16S rRNA gene sequencing was performed using Quantitative Insights Into Microbial Ecology (QIIME) 2 2020.11 and Python-based packages (Python 3.6.12) with packages in accordance with the QIIME2 2020.11 environment. Sequences were de-multiplexed, then filtered and clustered into sub-operational taxonomic units (sOTUs) using QIIME 2 DADA2 [15]. Phylogenetic tree was created using SATé-enabled phylogenetic placement via QIIME 2 [16]. Taxonomy was assigned using a naïve-Bayes classifier based on the latest SILVA version 138 16S rRNA gene database (March 2021) via the QIIME 2 interface. Additional Python packages (SciPy, Statsmodels, Scikit-bio) were used for statistical tests on QIIME 2-generated data. Diversity analyses were performed in QIIME 2, rarefied to an even sampling depth of 18,100 [17].

For alpha diversity, relationships between Faith's phylogenetic diversity (Faith's PD) and participant metadata were assessed using linear mixed effects models with a random effect for participant ID. Due to the sample size and skewed nature of the Faith's PD values, the rank of Faith's PD was used as the outcome variable in the mixed effect models, rather than the raw values. Correlations between community composition (beta diversity) and participant metadata were analyzed (stratified at each timepoint) using mantel tests with metadata converted to Euclidean distance matrices, and group differences were analyzed using permutational MANOVA (PERMANOVA) stratified at each time point. Correlations between specific lipids and time were assessed using linear mixed effects models (implemented via statsmodels 0.13.1) with a random effect for participant (formula: $\log_{10}$lipid ~ timepoint+(1|participant ID), where (1|participant ID) indicates a random effect for participant ID), and a Benjamini-Hochberg false discovery rate (FDR) correction was applied.

Principal coordinates analysis (PCoA) was performed using the Python package Scikit-bio on Bray-Curtis distance matrices constructed from both the microbiome sOTU table and metabolome data. A Procrustes randomization test (PROTEST) was then performed on PCoA coordinates for each sample. As described by Peres-Neto and Jackson [18], a PROTEST was performed by permuting the MDS coordinates and performing a Procrustes test on the reordered coordinates 10,000 times, and the *p*-value was calculated based on the portion of randomized Procrustes tests with resulting m^2 (Gower's statistic) scores lower than the Procrustes m^2 score of the observed (unpermuted) datasets. Code for analyses, as well as link to available data, can be found at https://github.com/sterrettJD/Breastfed-Neonates.

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RESULTS

Demographics

Patient demographics (**Table 1**) with respect to education level and household income reflected the socio-economically disadvantaged population surrounding our institution. Additionally, self-reported maternal tobacco and alcohol use prior to pregnancy was noted. No complications during pregnancy were documented for any participating mothers. None of the participating mothers had high cholesterol. None of the participants used probiotics during pregnancy or post-natal. Mothers did not use antibiotics at least in the last trimester. Average gestational age of the babies was 39.3 weeks (37.43–40.28) and average birth weight was 6.74 pounds (5.96–8.75). Five out of seven neonates were females and all neonates were EBF at all time points included.

Gut microbiome analysis

Boxplot presented in **Fig. 1** shows Faith's PD over time. There was no significant correlation of phylogenetic diversity with time, per a linear mixed effects model on the rank of Faith's PD with a random effect for participant (p=0.403). Additionally, the relationship of Faith's PD with the neonates' sex, length, weight, and gestational age, as well as mothers' body mass index (BMI), education, ethnicity, smoking during pregnancy, and antibiotic usage was assessed using linear mixed effects models. There were no significant relationships between these variables and Faith's PD (p>0.050). A stacked bar plot (**Fig. 2**) shows that Firmicutes, Proteobacteria, Bacteroidota and Actinobacteriota were the most abundant taxa across the samples.

With weighted and unweighted UniFrac, as well as Bray-Curtis distances, a PERMANOVA revealed that the variation between individuals' microbiome composition was greater than the variation within individuals (weighted UniFrac pseudo-F=2.834, p=0.001; unweighted UniFrac pseudo-F=1.596, p=0.008; Bray-Curtis pseudo-F=2.918, p=0.001). A Mantel test using Spearman correlation revealed that time point did not significantly correlate with microbiome beta diversity, though p-values for all distance metrics used were consistently approaching significance (weighted UniFrac r=0.129, p=0.060; unweighted UniFrac r=0.129,

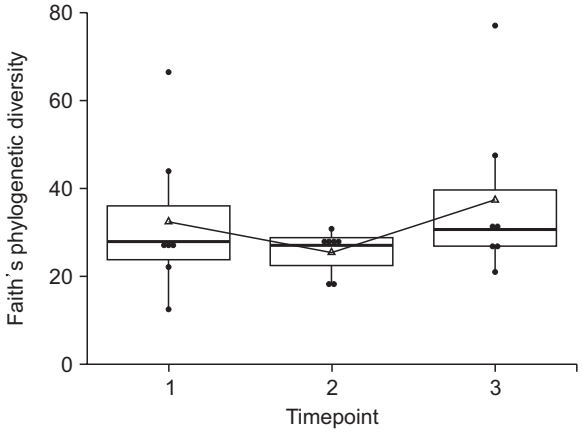

Fig. 1. Faith's phylogenetic diversity over time. Boxplot shows Faith's phylogenetic diversity over time. Each dot represents one sample, whereas the triangles represent the mean Faith's phylogenetic diversity at that time point. Time points: 1: first transitional stool (day 1: 0–24 hours), 2: day of discharge (day 2: 30–48 hours), and 3: their first pediatric appointment (days 3–5 post-delivery).

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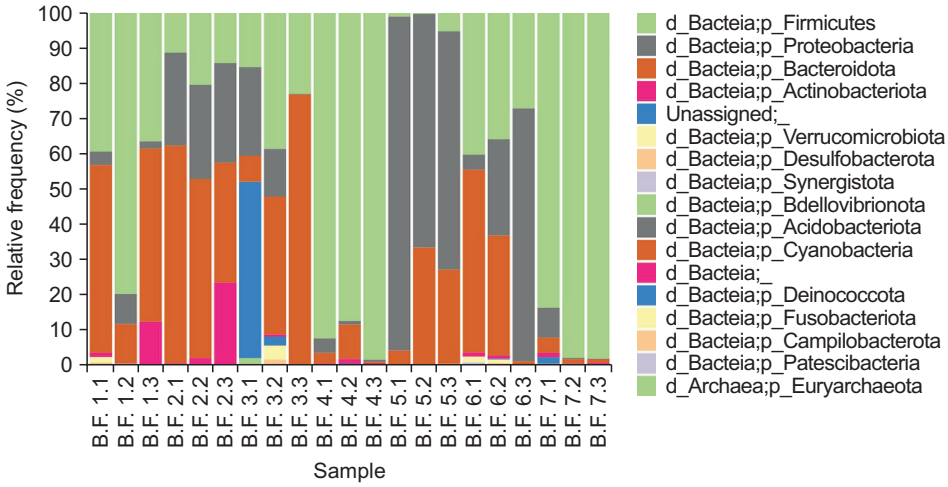

Fig. 2. Taxa bar plot. Taxa bar plot shows relative abundances of phyla in each sample. Participant number and timepoint are denoted as "B.F. [Participant].[Timepoint]" along the x axis. From top to bottom, the legend and stacked bars are ordered according to descending average relative abundance of each phylum.
B.F.: breastfed.

p=0.060; Bray-Curtis *r*=0.129, *p*=0.051). Two PCoA plots (**Fig. 3A, C**) show ordinations of microbiome weighted UniFrac distance colored by participant and by time point, respectively.

As mentioned above, at each time point, the associations between beta diversity and infant sex, length and weight at birth, gestational age, as well as mothers' height, weight, BMI, education, ethnicity, and smoking during pregnancy were tested. At time point 1 (day of discharge-day 2: 30–48 hours), ethnicity was significantly associated with weighted UniFrac and Bray Curtis distances but not unweighted UniFrac (weighted UniFrac pseudo-*F*=2.76, *p*=0.030; unweighted UniFrac pseudo-*F*=0.824, *p*=0.710; Bray-Curtis pseudo-*F*=1.401, *p*=0.030; data not shown). Additionally, at time point 0 (first transitional stool-day 1: 0–24 hours), mothers' weight (but not BMI) was associated with microbiome composition (weighted UniFrac *r*=0.560, *p*=0.020; unweighted UniFrac *r*=0.560, *p*=0.020; Bray-Curtis *r*=0.560, *p*=0.030; data not shown).

Gut metabolome analysis

Fig. 4 shows the concentrations of lipids in a stacked bar plot. Overall, palmitic, oleic, stearic, and linoleic acids were the most abundant lipids across our samples (based on average concentration). Similar to the microbiome, variation in lipid composition between individuals was greater than the variation within individuals (Bray-Curtis PERMANOVA pseudo-*F*=2.1, *p*=0.040). Additionally, a Mantel test with Spearman correlation revealed that metabolome beta diversity correlated with time (*r*=0.165, *p*=0.030). Two PCoA plots (**Fig. 3B, D**) show ordinations of lipid Bray-Curtis distance colored by participant and by time point, respectively.

Fig. 5 shows the increase in saturated and unsaturated lipids over time. Linear mixed effects models (with a random effect for participant) revealed that over the time period studied, the concentrations of total lipids, saturated lipids, and unsaturated lipids increased. Total lipids had a baseline concentration of 246 μg per gram of fecal dry matter (μg/g FDM) and increased an average of 284 μg/g FDM each time point (*p*=0.012); saturated lipids had a baseline concentration of 240 μg/g FDM and increased an average of 143 μg/g FDM (*p*=0.008);

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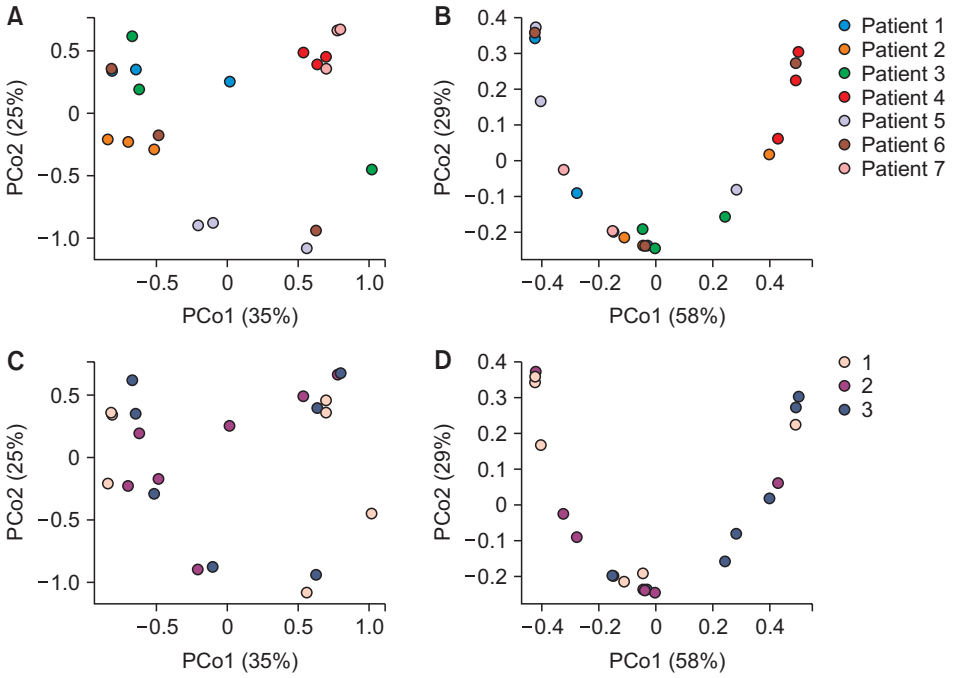

Fig. 3. Ordinations of microbiome and metabolome showing associations with participant and time point. (A, C) show weighted UniFrac microbiome PCoA plots; (B, D) show Bray-Curtis metabolome PCoA plots. (A, B) are colored by participant, whereas (C, D) are colored by time point. Each point represents one sample, and distance between points represents their dissimilarity. (A) shows grouping of microbiome composition by participant ID. PCoA, principal coordinates analysis. Time points: 1: first transitional stool (day 1: 0–24 hours), 2: day of discharge (day 2: 30–48 hours), and 3: their first pediatric appointment (days 3–5 post-delivery).
PCoA: principal coordinates analysis.

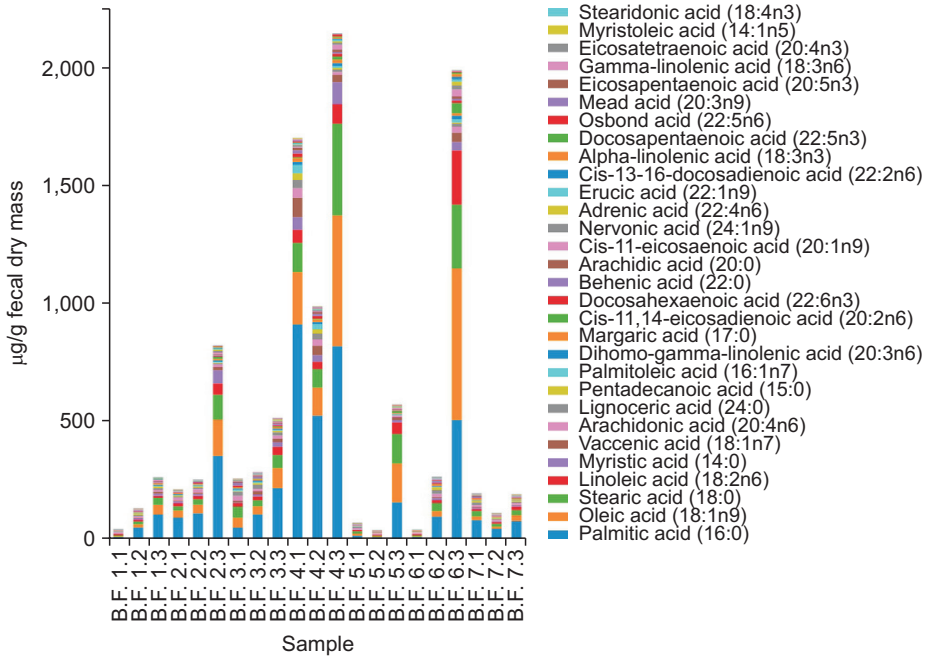

Fig. 4. Stacked bar plot showing the concentrations of detected lipids in fecal samples. From bottom to top, the legend and the stacked bars are ordered according to descending average concentration of each lipid. Each bar represents one sample, and the total height of the bar represents the total concentration of all detected lipids.
B.F.: breastfed.

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unsaturated lipids had a baseline concentration of 122 μg/g FDM and increased an average of 141 μg/g FDM (p=0.023) (**Fig. 5**). Out of the 30 detected lipids, linear mixed effects models on log10-transformed lipid concentrations (with a random effect for participant) identified 18 lipids that significantly increased over time and 1 lipid that decreased over time, after applying a Benjamini-Hochberg FDR correction. Of these, myristic, oleic, and cis-11-eicosaenoic acids had the strongest positive trends, and myristoleic acid had the strongest negative trend. **Fig. 6** shows the models' β values for each lipid, as well as their significance level. **Fig. 7** shows the concentrations of these lipids at each time point. A Procrustes randomization test revealed that the microbiome composition of each sample did not significantly associate with that sample's respective metabolome (Procrustes disparity=0.910, p=0.450) (**Fig. 8**).

DISCUSSION

Of the several factors implicated in shaping the early infant gastrointestinal microbiome, feeding practice is among the most influential [19-22]. This observational cohort study aimed to elucidate the role of early infant feeding practices in shaping the gut microbiome-metabolome profile of serially collected infant stool samples. Analysis of serially collected neonate stool samples revealed unique microbiome and metabolome profiles in each neonate that developed with time. Consistent with prior literature, the most prominent bacterial taxa among our neonate stool samples across all evaluated time points included Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria [12,22-24]. More than half of the phylum Actinobacteria is composed of *Bifidobacterium*, and transmission during delivery serves as the initial source for *Bifidobacterium infantis* colonization in the infant gut. Breastmilk further facilitates colonization and preferentially favors the prevalence of *Bifidobacterium* species by providing human milk oligosaccharides (HMOs), which are metabolized by *Bifidobacterium* species [12,22-24]. The gastrointestinal tract of exclusively breastfed infants has been shown to express an increased abundance of glucose, fructose and other metabolites of complex carbohydrate fermentation pathways compared to their formula-fed counterparts [25-28].

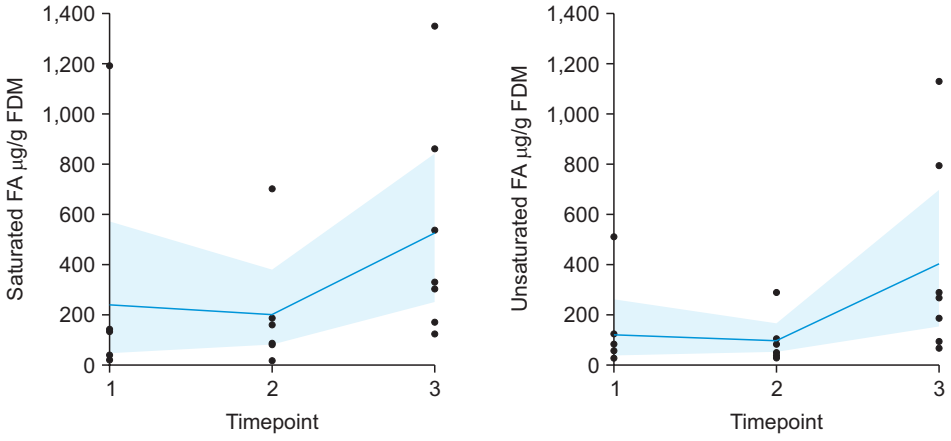

Fig. 5. Line plots showing total concentrations of saturated and unsaturated fatty acids. The left panel shows the total concentration of saturated fatty acids averaged at each time point, whereas the right panel shows the total concentration of unsaturated fatty acids averaged at each time point. Time points: 1: first transitional stool (day 1: 0–24 hours), 2: day of discharge (day 2: 30–48 hours), and 3: their first pediatric appointment (days 3–5 post-delivery).
FA: fatty acid, FDM: fecal dry matter.

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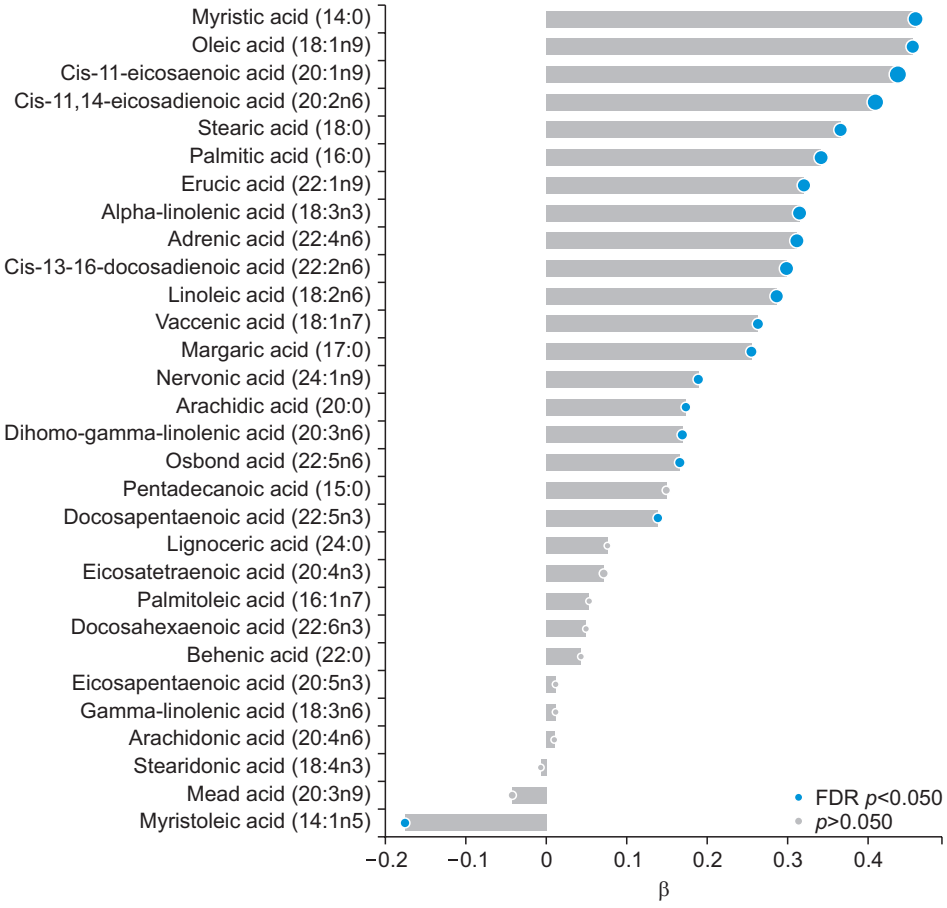

Fig. 6. Bar plot showing correlations between lipids and time. The x axis represents the coefficient of timepoint in a linear mixed effects regression, and the color at the end of each bar represents significance level of the association. A regression was run for each lipid with the formula $log_{10}(lipid) \sim timepoint + (1|Participant\ ID)$, where $(1|Participant\ ID)$ represents a random effect for participant ID. Positive values on the x axis indicate an increase in lipids over time, and a β of 1 would indicate that a 1 timepoint increase corresponds to a 10-fold increase in lipid concentration.
FDR: false discovery rate correction (Benjamini-Hochberg).

In addition to compositional variations, infant feeding practices have also been associated with differences in microbiota alpha diversity. While increased richness is commonly cited as most advantageous in adults, it is believed that high evenness is particularly favorable in infants for development of the gastrointestinal tract and immune system. Exclusively breastfed infants demonstrate increased gastrointestinal microbial evenness compared to their formula-fed counterparts, who express greater richness [12,22,23,29]. It has been postulated that the microbiota evenness demonstrated by exclusively breastfed infants is related to the increased relative abundance of *Bifidobacterium* (preferentially favored by the continuous supply of HMOs provided by breastmilk) and inhibition of pathogen binding and overgrowth by HMOs [22,29]. Conversely, the elevated richness in formula-fed infants has been attributed to the relative increased abundance of Firmicutes species. Studies have shown that infants fed a mixed diet of both breastmilk and formula demonstrated alpha diversity more similar to that of exclusively formula-fed infants [22]. The influence of infant diet on microbial development has been further suggested as higher alpha diversity has been noted in infants fed soy-based formulas compared to traditional milk-based formulas in that

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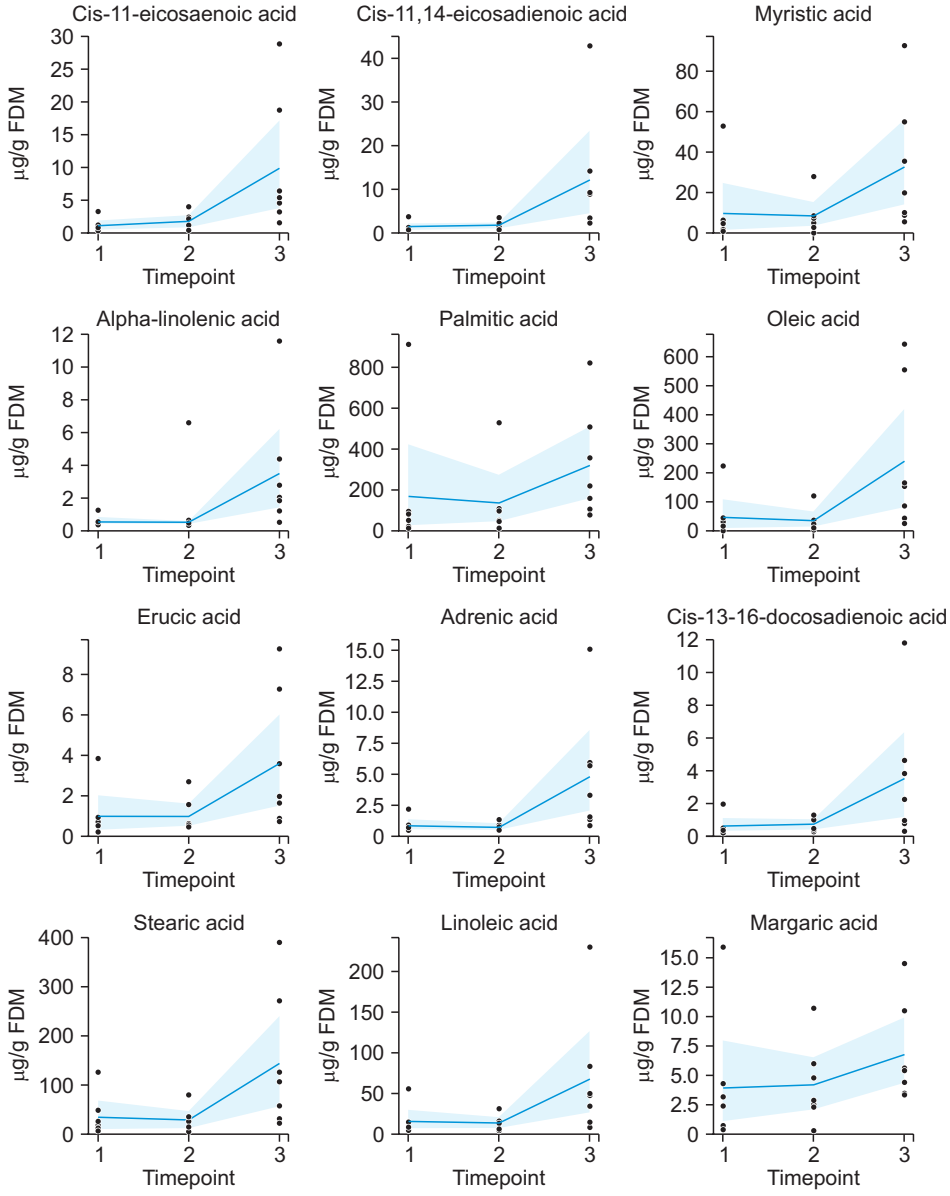

Fig. 7. Scatter and line plots show concentrations of lipids over time. Lipids that were significantly correlated with timepoint are shown in each panel. Each point represents the concentration of the lipid in one sample, and lines show the average concentration of that lipid across all samples at each time point. Time points: 1: first transitional stool (day 1: 0–24 hours), 2: day of discharge (day 2: 30–48 hours), and 3: their first pediatric appointment (days 3–5 post-delivery). FDM: fecal dry matter.

(continued to the next page)

the BF infants had the lowest alpha diversity, infants that received soy-based formula had the highest alpha diversity and infants receiving dairy-based milk formula had intermediate alpha diversity [12]. In our analyses of phylogenetic richness, neither infantile (i.e., sex, length, weight, gestational age) nor maternal (i.e., BMI, education, ethnicity, smoking, and antibiotic usage) variables showed significant relationships with alpha diversity. Thus, we observed that the microbiome alpha diversity did not correlate with any other factors examined through the duration of this study. The microbiome has been characterized by high levels of inter-individual variability in previous longitudinal studies [30,31]. Consistent with prior studies, our analysis found that the variation between individuals' microbiome

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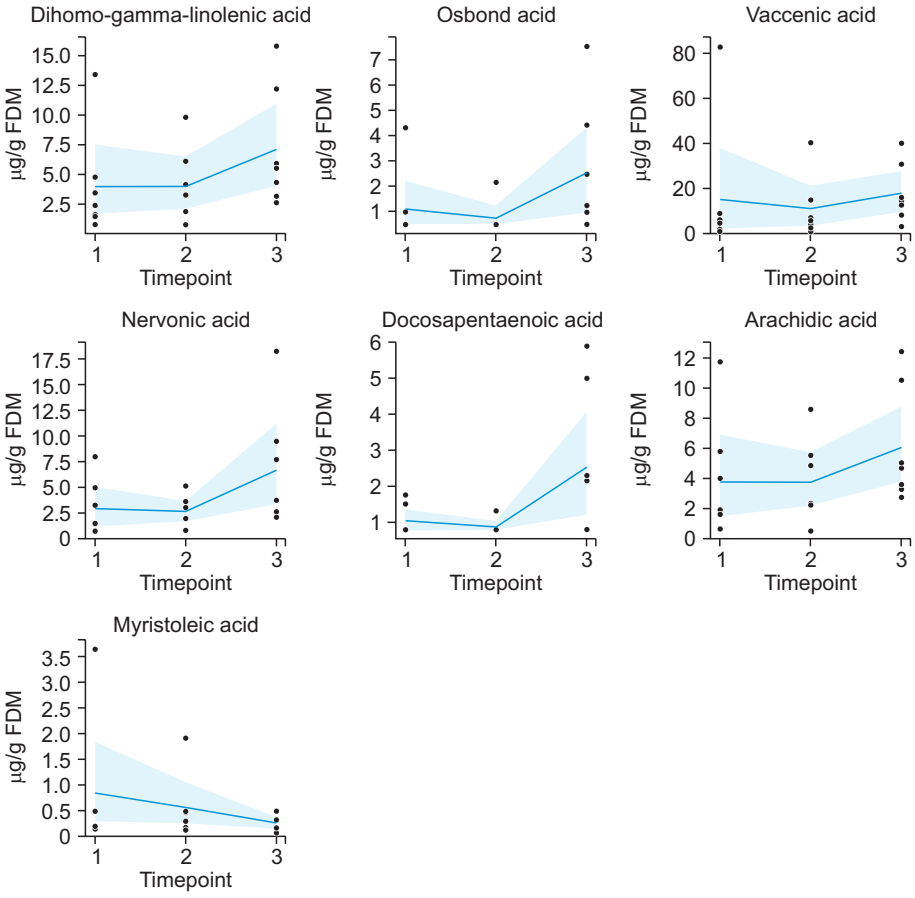

Fig. 7. (Continued) Scatter and line plots show concentrations of lipids over time. Lipids that were significantly correlated with timepoint are shown in each panel. Each point represents the concentration of the lipid in one sample, and lines show the average concentration of that lipid across all samples at each time point. Time points: 1: first transitional stool (day 1: 0–24 hours), 2: day of discharge (day 2: 30–48 hours), and 3: their first pediatric appointment (days 3–5 post-delivery).
FDM: fecal dry matter.

composition was greater than the variation within individuals. This provides evidence that each neonate possessed a distinctly unique microbiome, which could be a result of mothers' milk having a unique composition of HMOs and lipids. The HMO and lipid composition in turn may be highly influenced by lifestyle factors such as diet [23,24,32,33].

Furthermore, our analysis revealed that there was not a significant correlation of beta diversity with time. However, given the small sample size of our study, high dimensionality of the data, and high variation between individuals, this may not accurately depict the development of the infant gut, and other studies during this period of life with higher power should be used to characterize the development of microbiome composition more accurately. Correlations of beta diversity with time consistently resulted in $0.050<p\leq0.060$ across multiple metrics; thus, failure to consider that the microbiome composition is developing over this period given our understanding of the underlying biology and the limitations of our data would be flawed. Only two of the studied factors, maternal weight and ethnicity, were found to be significantly associated with infant microbiome composition, consistent with a recent report [32].

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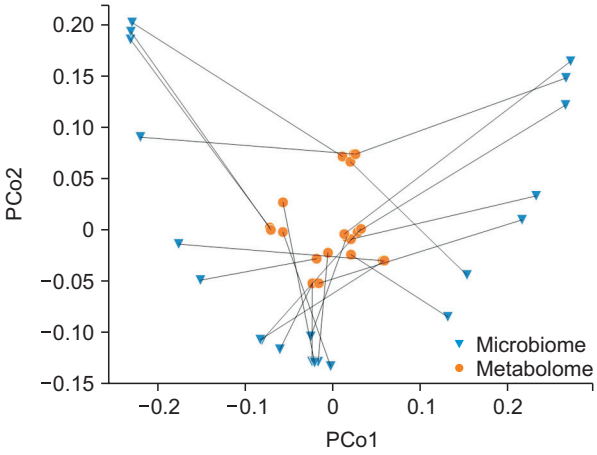

Fig. 8. Procrustes-transformed Bray-Curtis PCoA of microbiome (blue) and metabolome (orange). Microbiome points were plotted as their original PCoA coordinates, whereas metabolome coordinates were transformed using the Procrustes function to their best fit over the microbiome coordinates. Each point represents one sample, and the distance between points belonging to the same dataset represents the dissimilarity between those samples. Lines were drawn between metabolome and microbiome points corresponding to the same sample. A Procrustes randomization test found that participants' metabolomes did not significantly match up with their microbiomes (Procrustes disparity=0.910, p=0.450).
PCoA: principal coordinates analysis.

High levels of metabolome variability between infants have been established in recent literature investigating both the entirety of the infant metabolome as well as specific metabolites, including short-chain fatty acids [12,26,30,34]. This is supported by our data, which found higher variation between individuals than within individuals. Furthermore, our observed metabolome variation demonstrated temporal development across consecutive time points, identifying a development of the metabolomic profile over the first week of life.

Various sterol lipids, glycerophospholipids and fatty acids additionally account for metabolites discriminating between infants of various feeding modalities [25-27,35]. We observed palmitic acid, oleic acid, stearic acid and linoleic acid to be the predominant lipids comprising the infant stool fatty acid profiles. Upon analysis, the total lipid composition of our infant fecal samples did significantly change over time. When assessing changes to individuals' lipids over time, we found that the myristic acid, oleic acid, cis-11-eicosaenoic acid, and cis-11,14-eicosadienoic acid had the strongest increases over time. Myristic acid, which makes up roughly 8% of the milk fat in human milk [36,37], has been shown to moderately increase in concentration throughout early breastfeeding. Oleic acid is the most abundant monounsaturated fatty acid in human milk, making up roughly 40% of the milk fat [38]. Though it is found in lower concentration than the other lipids, cis-11,14 eicosadienoic acid is in its highest concentration in colostrum, then decreases as breast milk transitions toward mature milk [38]. Additionally, cis-11,14 eicosadienoic acid is not found in infant formulas [38] and may be a missed compound of breast milk for the gut lipid profile's development in infanthood. Cis-11-eicosaenoic acid is less studied in early human breast milk, but research in piglets revealed that it is higher in concentration during the first week of life in control piglets, compared to piglets given a fecal microbiome transplant from mature pigs [39]. Further work is needed to study if there is a role of Cis-11-eicosaenoic acid in the developing microbiome that is not as present in the mature microbiome. The strongest decrease in a single lipid over time was seen in myristoleic acid, which makes up less than 0.1% of human milk [38].

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Metabolomic profiles have been linked to feeding modality and have been shown to change across time in prior studies [26,27]. A double-blind trial of infants randomized to standardized formula or formula supplemented with *Bifidobacterium*, compared to exclusively breastfed control, discovered significant metabolite differences between exclusively breastfed and formula-fed infant cohorts beginning at one month that were maintained until one year [12,25]. Other studies have also demonstrated that breastfeeding alters the metabolic profile until about one year of age, at which point it has been postulated that the predominance of a solid food diet is responsible for the convergence. The analysis of infant fecal metabolite profiles may suggest that the overall variability demonstrated by each individual infant may reflect the interplay between genetic backgrounds and external influences, including the composition of maternal breast milk, amount consumed, and living environment [12,35].

Evidence suggests a dynamic relationship between the gut microbiome and metabolome, particularly during early infancy. This correlation is thought to be driven by infant feeding practice, as metabolites associated with various feeding modalities contribute to the subsequent taxa distribution of the infants' gastrointestinal microbiome [6,7]. We did not find that the microbiome composition of each fecal sample significantly associated with the respective metabolome of that sample. While literature suggests an association between taxonomic composition and metabolite concentrations, the functional redundancy and multidimensional nature of the microbiome makes it difficult to draw perfect connections on the multivariate level. Additionally, many studies, including the present study, that use 16S rRNA gene sequencing for microbiome analysis only uncover genus-level resolution with no information on functional capabilities or active transcription. This information may be provided by metagenomics and metatranscriptomics, respectively. Thus, the bidirectional dynamic relationship of the microbiome-metabolome necessitates further investigation [6,7].

Our recruitment efforts were severely hindered due to the coronavirus disease 2019 pandemic, resulting in a smaller sample size, which is a limitation of this pilot study. Recruitment was limited to patients with pediatric appointments scheduled at our institution. As mentioned above, due to the small sample size, definite conclusions cannot be drawn regarding the contributing factors. Despite this limitation, our pilot study introduces new insight and found important observations that will help further direct research on this topic. Future studies with a larger sample size, and multiple cohorts analyzing gut microbial community and gut metabolome changes with various external factors (i.e., formula fed, introducing formula to exclusively breastfed, solid food, maternal diet, etc.) will provide insight into gut microbiome/metabolome development and its translated effects on human health.

SUPPLEMENTARY MATERIAL

Supplementary Fig. 1
Surveys

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