5-2023

Determining the Effects of Maternal Adiposity on Preterm Neonatal Microbiome and Short Chain Fatty Acid Profiles

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An undergraduate thesis

submitted to

East Tennessee State University

In partial fulfillment

of the requirements for the

Honors College

University Honors Scholars Program

by

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May 2023

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ABSTRACT

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The gut microbiota and its metabolites have vast impacts on the human digestive system, immune system, and health outcomes. Short chain volatile fatty acids (SCVFAs) present in feces can be representative of the interactions of the microbiota present in the gut. Low microbiota diversity in the human gut is highly associated with obesity and adverse health outcomes. Furthermore, the maternal microbiome has a direct impact on neonatal microbiota through various pathways such as environment, skin flora, breast milk composition, and vaginal secretions. This study is aimed to further understand the associations between various factors (maternal adiposity, gestational time, length of life, delivery mode, and presence of diabetes) and neonatal microbiome and its metabolites, SCFA. Data (pre-pregnancy BMI, gestational time, length of life at time of sample collection, delivery mode, presence of diabetes, SCVFA profiles, fecal fermentation profiles, and 16s RNA sequences, n=75) was obtained from 75 mother-infant dyads. Qiagen CLC Genomics Workbench was used to process 16s RNA data, generate quantitative and qualitative measures of alpha and beta diversity, and generate an analysis of the composition of microbiomes for differential abundances. Multiple metrics were analyzed for alpha and beta diversity and no significant differences were found for acetic acid (A), propionic acid (P), butyric acid (B), or APB combined. Shannon diversity index, a measure of Alpha diversity, showed no significant difference between groups in each subset. BMI differences were significant for no c-section vs. c-section and Black vs. White race/ethnicity. There were no

significant differences found in PERMANOVA, a measure of beta diversity or found in differential abundances among the groups.

ACKNOWLEDGEMENTS

This undergraduate thesis would not have been possible without the continued support and guidance of several individuals. I would like to acknowledge Dr. Andy Clark for allowing me to work in his lab and teaching me things far beyond the scope of just this project. I would also like to acknowledge Dr. Sean Fox for so willingly being my second thesis reader. I would especially like to acknowledge Kristy Thomas for always taking time out of her day to help me better understand the data, lab procedures, computer programs, and how to write this thesis. Without her constant help and guidance, this paper would not have been possible. Lastly, I would like to thank the ETSU Honors College for the opportunity to delve into the world of research.

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CHAPTER 1. BACKGROUND

Purpose

The purpose of this study was to determine if maternal BMI has any effect on the health outcomes of the neonate. A study of fecal fermentation profiles and microbiome analysis allows for the understanding of neonatal microbiome and possibly elucidates a link between maternal health and neonatal health outcomes.

Research Questions

1. Is there a difference in the microbiome diversity of neonates that come from obese mothers and neonates that come from lean mothers?
2. Is there a difference in the SCFA profiles of infants receiving breast milk from mothers of differing BMI?

Hypotheses

1. We expect there will be a significant difference in microbiome diversity between neonates that are born to obese mothers and ones that are born to lean mothers.
2. We expect to see less diverse SCFA profiles in the infants receiving milk from obese mothers as shown by elevated Acetic, Propionic, and Butyric combined % compared to that from normal weight.

CHAPTER 2. REVIEW OF LITERATURE

Severity of Preterm Births

Preterm birth poses serious issues for public health in developed and undeveloped nations alike. Infants that are born prematurely are significantly more susceptible to disparate amounts of time spent in hospitals and clinics compared to full term infants, along with frequent respiratory, metabolic, neurological, and somatic ailments [1, 2, 3]. Preterm infants are about 6.8 times higher at risk of death than full term infants [4]. Among other diseases, they have higher risks of cardiovascular diseases, metabolic syndromes, and necrotizing enterocolitis [5, 6, 7]. Despite all of the ailments that are common in neonates, the leading cause of death is preterm birth itself at a rate of 35% [1, 8]. According to reports from the CDC on November 1, 2022, the preterm birth rate rose 4% from the 10.1% rate in 2021 to the 10.5% rate that is currently seen. Preterm births are also skewed according to race, as is seen in the higher 14.4% rate that is displayed by those of African descent [9]. Across the world, about 41,000 infants are born preterm each day [10]. According to the World Health Organization, survival rates of preterm births vastly differ throughout the world, specifically in low-income communities, where more than half of the preterm infants die due to lack of care. Roughly 60% of all preterm births happen in Africa and South Asia [11]. On average, low-income countries experience a rate of 12% of preterm births compared to the 9% those high-income countries experience [11, 12].

Causes of Preterm Births

Preterm births can come about in several different forms. The most common forms that Preterm birth presents itself as spontaneous labor with membranes that are still intact, premature rupture of membranes, and preterm labor induction or caesarean section. A common causes of

preterm birth follows pre-eclampsia or eclampsia, and intrauterine growth restrictions [13]. Other causes of preterm births belong to a broad syndrome that can be caused by various factors such as infection, cervical pathology, overdistention of the uterus, unregulated progesterone levels, vascular deformities, stress, and likely other unknown factors. These various factors can all act in tandem or separately to cause premature birth pathways through uterine contractions, cervical ripening, or membrane rupture [13, 14].

Effects of Mother's Nutrition on Neonate Health

Murine models have identified that the offspring of obese mothers are more susceptible to obesity with the potential mechanism of transfer of obesity from milk fdam milk to pup [15, 16, 17, 18, 19, 20, 21, 22, 23]. A human study also found a milk-related mechanism that transferred obesity from mother to child [24]. Maternal dysfunction and dysbiosis can genetically predispose offspring to obesity [25, 26, 27]. Childhood obesity predisposes individuals to hypertension, metabolic syndrome, fatty liver disease, dyslipidemia, and psychological disorders among other ailments [28, 29, 30]. Severe maternal malnutrition has been shown to be strongly correlated to low birth weight, neurological disorders, impaired physical growth, and mental retardation [31]. Babies born to obese mothers are at increased risk for congenital birth defects, pneumonia, and fetal macrosomia [31].

Effect of Maternal Nutrition on Maternal Health

The World Health Organization outlines several recommendations for women of childbearing age, including proper or appropriate diet and nutrition, weight management, physical activity, and physical and mental health. Obesity during pregnancy increases the risk for

adverse perinatal outcomes such as gestational diabetes mellitus or preeclampsia [32]. Overweight, pre-obese, and obese women are recommended to take in foods rich in vegetables, essential and polyunsaturated fats, and fiber-rich carbohydrate-containing foods [32]. Special attention should be given to the adequate intake of micronutrients such as iron, calcium, folate, vitamin D, and carotenoids in order to support pregnancy and eventual breastfeeding [32, 33]. Micronutrients such as vitamins and minerals support the development of a healthy neonate, as well as every stage of interaction between the mother and the fetus during gestation [33]. Appropriate amounts of folic acid intake, ideally started 5-6 months before conception with levels of about 1200 nmol/L, is beneficial in preventing neural tube defects within the first 28 days after conception [34]. Poor nutrition, overnutrition, and undernutrition have complex roles during pregnancy and can go on to effect birth outcomes such as preterm birth [35, 36]. In the United States, 32% of women of childbearing age are obese and 24% are overweight [37]. In recent years, pre-conception obesity has been on the rise, with an increase from 2013 to 2016 across all racial, educational and age groups [38]. Multiple studies have shown that obese women are at greater risk of giving birth prematurely [35, 39, 40]. Obese mothers are more likely to have prolonged hospital stays of more than 10 days [41]. Proper maternal nutrition consists of balance between energy and proteins, proper amounts of micronutrients of vitamins and minerals, especially folate, and fatty acids [35, 39, 42]. Adequate maternal nutrition plays a significant role in fetal growth and development and the incidence of preterm birth [43, 44, 45].

Effect of Maternal Health on Human Breast Milk and Microbiome

Human breast milk (HBM) and maternal microbiome are influenced be several factors such as body mass index (BMI), antibiotic use, health, age, and time and duration of lactation [46, 47,

48, 49, 50, 51, 52]. Neonate microbiome is influenced most heavily by the mother through modes such as mode of birth, breastfeeding, bottle feeding, lactation stage, HBM composition (which differs in healthy and obese mothers), and environment [46, 51, 53]. Various studies have reported differences in HBM between healthy and obese mothers [54, 55, 56]. Oligosaccharides in human milk consist of a diverse group of carbohydrates that are generally undigested by the host and serve as prebiotics for the microbiome [57]. The prebiotic content of HBM is correlated with the maternal gut microbiota which exhibits a marked decrease in diversity in obese mothers when compared to normal weight mothers [50, 58, 59, 60]. Short-chain volatile fatty acids (SCVFA) and HBM are integral in infant development and growth and are byproducts of bacteria in the gut that that ferment human milk oligosaccharides [61]. Studies show that SCVFA concentration and type play a role in energy metabolism due to an association between lower body weight and SCVFA distribution [61]. Various studies have indicated that human milk short chain fatty acids play a beneficial role in weight gain and adiposity during infancy [61] and that low amounts of SCVFA increase the risk of infant adiposity [62]. It is possible that SCVFA are present in HBM, as a consequence of maternal microbiome, which can eventually affect the health and weight of the neonate [61, 62].

Preterm Infant Nutrition

Maternal HBM has been proven to be most beneficial when it is taken within the first few days of birth in terms of decreasing rates of infection, morbidity, and mortality [63]. Maternal HBM as well as donor milk have proven to decrease intestinal permeability, increase gut maturation, and reduce the risk of necrotizing enterocolitis [64, 65, 66, 67, 68]. Common issues for infant nutrition present as early termination of breastfeeding and delays in lactogenesis due to factors

such as maternal age, illness, primiparity, elevated BMI, gestational diabetes, and delivery by caesarean section [40, 69, 70]. HBM has proven to affect the infant gut microbiome which results in increased immune factors and eventually the health outcomes of the infant [51, 71]. It is still unclear how much of an effect epigenetics plays in infant health outcomes.

Microbiome Diversity Measurements

Alpha diversity is a measure that describes the amount of diversity within an organism, commonly taking into account the different number of species that can be observed. Observed taxa, also referred to as observed OTU's, is the easiest measure of alpha diversity to calculate as it is simply the sum of the number of OTU's or taxa present in a given sample [72]. The **Shannon Index** (H) is a tool that is useful for estimating species richness and evenness and is calculated as:

$$H = -\sum_{i=1}^{R} p_i \, ln(p_i)$$

Where R is the total number of OTU's present and p_i is the proportion of each species in the sample [73].

Beta diversity is a useful tool for measuring the diversity between an organism and the whole population . The Bray-Curtis dissimilarity is a function that takes the abundance of microbes present in both samples into account. Dissimilarity is measured by the difference in the composition of the microbiomes of two separate samples. Bray-Curtis dissimilarity finds the dissimilarity, D, between two groups, A and B, by the following formula:

$$D_{A,B} = \frac{\sum_{i=1}^{S} |n_{Ai} - n_{Bi}|}{n_{A+} + n_{B+}}$$

Where n_{Ai} is the abundance of OTU, or i, in sample A, and similarly for sample B. The n_{A+} and n_{B+} terms are the total species abundances in sample A and B, respectively. Jaccard distance measures dissimilarity by taking into account the presence of microbes in one or both samples (A and B) and is calculated by the following [74]:

$$Jaccard_{A,B} = 1 - \frac{|A \cap B|}{|A \cup B|}$$

Unifrac considers the phylogenetic relationships between microbes that are present in the two samples. Unifrac can be weighted or unweighted where unweighted can be calculated by dividing the sum of unshared branch lengths by the sum of all branch lengths. Weighted unifrac considers abundance of microbes into account and is calculated by the following equation:

$$Weighted\ Unifrac = \sum_{i}^{n} b_i * \left| \frac{A_i}{A_T} - \frac{B_i}{B_T} \right|$$

Where n is the total number of branches on the tree, b_i equals the length of branch i, A_i and B_i are abundances in samples A and B, respectively, and A_T and B_T are the total numbers of sequences in samples A and B [75].

Principle coordinate analysis (PCoA) converts distance data into a three-dimensional map. The generated plot allows for the visualization of the distance data to quickly understand the similarity and dissimilarity of data sets. The three axes, PCo1, PCo2, and PCo3 represent the dimension of the axis [76].

CHAPTER 3. METHODS

Freeze-drying for Fecal Fermentation Profiles

Labeled 1.5 micro centrifuge tubes containing stool sample(s) were placed in a 600 ml LABCONCO freeze dry flask and lid was placed on LABCONCO FreeZone 2.5 freeze dryer

using stainless steel adapters. The samples ran for at least 12 hours on 0.077 mBar at -50 degrees Celsius. Once thoroughly dry, samples were ready for SCFA extraction.

Short Chain Fatty Acid Extraction and Analysis

SCFA extractions were performed using a procedure developed by Schwiertz et al. that was modified [76]. One mL of the SCFA extraction solution, containing Oxalic acid (0.1 mol/L) and Sodium Azide (40 mmol/L) was added to 80mg of freeze-dried stool sample in a 16 x 100 mm disposable glass culture tube. The tube was capped and vortexed for 30 seconds. The tube was placed on a horizontal shaker for 1 hour. The tube was centrifuged in a ThermoScientific™ Sorvall Biofuge Primo Centrifuge D-37520 Osterode (Germany)in a ThermoScientific™ 4x100mL Swinging Bucket Rotor (part #75007591) at 4000 rpm for 20 minutes. After centrifuging, the supernatant was removed and placed in a 1.5mL polypropylene Fisherbrand micro-centrifuge tube. The solution was re-centrifuged in a ThermoScientific™ 24-place Fixed-Angle Rotor (part #75007593) at 12,000 rpm for 15 minutes. Again, the supernatant was removed and placed in a new 1.5 mL micro-centrifuge tube. The solution was re-centrifuged at 12,000 rpm for 15 minutes. Finally, the supernatant was removed, placed in a 2 mL amber vial, and was stored at -80◦C until being analyzed using a Shimadzu GC2010 gas chromatograph equipped with a SigmaAldrich ZB-Wax Plus capillary column (part # 7HG-G013-11). Samples were run using a method adapted from Schaefer et al [77]. The method included injecting 1 µL of solution with an SPL1 temperature of 75◦C. The initial column temperature was 50◦C, held for 2 minutes, which rose at a rate of 15 degrees/minute until reaching 140◦C with a hold of 5 minutes, followed by a rise at rate of 10 degrees/minute until reaching 160◦C with a hold of 3 minutes and a rise of 10 degrees/minute until reaching 175◦C with a hold of 3 minutes. The

flame ionization detector temperature was 180◦C, and the end time of the run was 24 minutes. Samples were run in duplicate, and values for each participant were averaged.

16s rRNA Isolation and Quantification

Microbiome analysis was performed on all stool samples. Powersoil DNA isolation kit was used to isolate DNA from the portion of the fecal samples stored at -80◦C. Approximately 250mg of stool sample was added to the Qiagen-supplied PowerBead tube and vortexed. 60 µL of solution C1 (Qiagen lysing agent) was added, the tube was briefly vortexed manually, and then the tubes were secured to an adapter to be vortexed at maximum speed for 10 minutes. The tubes were then centrifuged at 1000 x g for 30 seconds, and the supernatant was transferred to a 2 mL collection tube. 250 µL of solution C2 (Qiagen precipitating agent) was added to the collection tubes, which were then vortexed for 5 seconds and then incubated on ice for 5 minutes. Tubes were then centrifuged for 60 seconds at 10,000 x g, and 750 µL of supernatant was transferred to clean 2 mL collection tubes. 200 µL of solution C3 (Qiagen precipitating agent) was added, and the tubes were briefly vortexed manually and incubated on ice for 5 minutes again. Following incubation, the tubes were centrifuged at 10,000 x g for 60 seconds, 750 uL of supernatant was added to 2 mL collection tubes, 1200 µL solution C4 was added, and tubes were vortexed for 5 seconds. 675 µL of this solution was loaded into a Qiagen kit spin column and centrifuged at 10,000 x g for one minute three times with flow through being discarded between centrifugations. 500 µL of solution C5 was added to the spin column, which was centrifuged at 10,000 x g for 30 seconds and one minute with flow through discarded between centrifugations. The spin columns were placed into clean 2 mL collection tubes, 100 µL nuclease-free water was added to the center of the spin column's white filter membranes, and the samples were allowed to incubate at room temp for 5 minutes. Tubes were centrifuged for 30 seconds at 10,000 x g to

elute the DNA, spin columns were discarded, and DNA from the samples were qualified and quantified via ThermoScientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer.

Library Preparation and 16s rRNA Sequencing

DNA was fragmented and tagged with a 615f/806r adapted sequence before polymerase chain reaction amplification. The primers were 341F and 785R. Amplicon sequencing of the 16S rRNA region was performed at the University of Tennessee Genomics Core Laboratory, following their standard operating procedures of a two-step polymerase chain reaction (PCR). The V4 region of the 16S rRNA gene was amplified from the extracted DNA using primers 341F and 785R [78]. modified with adapters for Illumina MiSeq sequencing. The initial PCR consisted of 2 × KAPA HiFi HotStart ReadyMix Taq (Roche, Indianapolis, Indiana, United States), 1.5 μM each primer, and 2.5 μL DNA. The reaction consisted of 3 min at 95°C, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. Successful PCR amplification was confirmed via gel electrophoresis on a 2% agarose gel. The PCR product was then purified using 20 uL of AMPure XP beads (Agencourt, Beverly, MA, United States) and eluting in 50 uL of Tris-HCl. Nextera XT indexes (Illumina) were then added to the PCR products by use of a second, reduced cycle PCR, such that each sample had a unique combination of forward and reverse indexes. This reduced reaction consisted of 3 min at 95°C, followed by 8 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. The products were purified again using 56 uL of AMPure XP beads, with a final elution in 25 uL Tris-HCl. Samples were quantified on a NanoDrop spectrophotometer (Fisher Scientific) and pooled to approximately equal concentrations. Final product sizes and concentrations were confirmed on an Agilent Bioanalyzer (Santa Clara, CA, United States) using the standard sensitivity kit. The final library was then diluted to 5 pM, and

combined with 20% of a 10 pM PhiX library control, and run paired-end 275 bp on a v3, 600 cycle flow cell of an Illumina MiSeq sequencer at the University of Tennessee Genomics Core.

Microbiome Analysis

Operational Taxonomic Unit (OTU) clustering and taxonomic analyses were performed using CLC Genomics Workbench v. 23 and CLC Microbial Genomics Module v. 2.5 (Qiagen, Hilden, Germany). Sequences were first trimmed and merged and then were clustered into OTUs at 97% sequence similarity level using the OTU clustering tool. The most abundant sequences were selected as representative of each cluster and then assigned to a taxonomy level using CLC Microbial Genomics default values and the Greengenes Database 13_8 release. Lowdepth samples (less than 5000 sequences per sample) were removed from the analysis. OTUs were then aligned by multiple sequence comparison using log-expectation (MUSCLE) and used to construct a "maximum likelihood phylogenetic tree", followed by alpha diversity analysis. Total number and Shannon entropy alpha diversity indexes were calculated using the Abundance Analysis tool. Bray-Curtis and the weighted Unifrac metric were used to calculate inter-sample diversity (beta-diversity). The Differential Abundance Analysis tool was utilized to determine OTUs with significant differences in abundance via Across groups (ANOVA-like) comparisons between the groups, then OTUs with and FDR (False Discovery Rate) of ≤ 0 were used to create a heat map.

Microbiome Statistical Analysis

A permutational multivariate analysis of variance (PERMANOVA) test was applied to data to detect differences between the groups. The level of accepted statistical significance was $p < 0.05$.

CHAPTER 4. RESULTS

Alpha Diversity

Maternal BMI

Alpha diversity differences were not statistically significant ($p>0.05$) between maternal pre-pregnancy BMI groups of unknown BMI, normal weight, overweight, and obese (Figure 1).

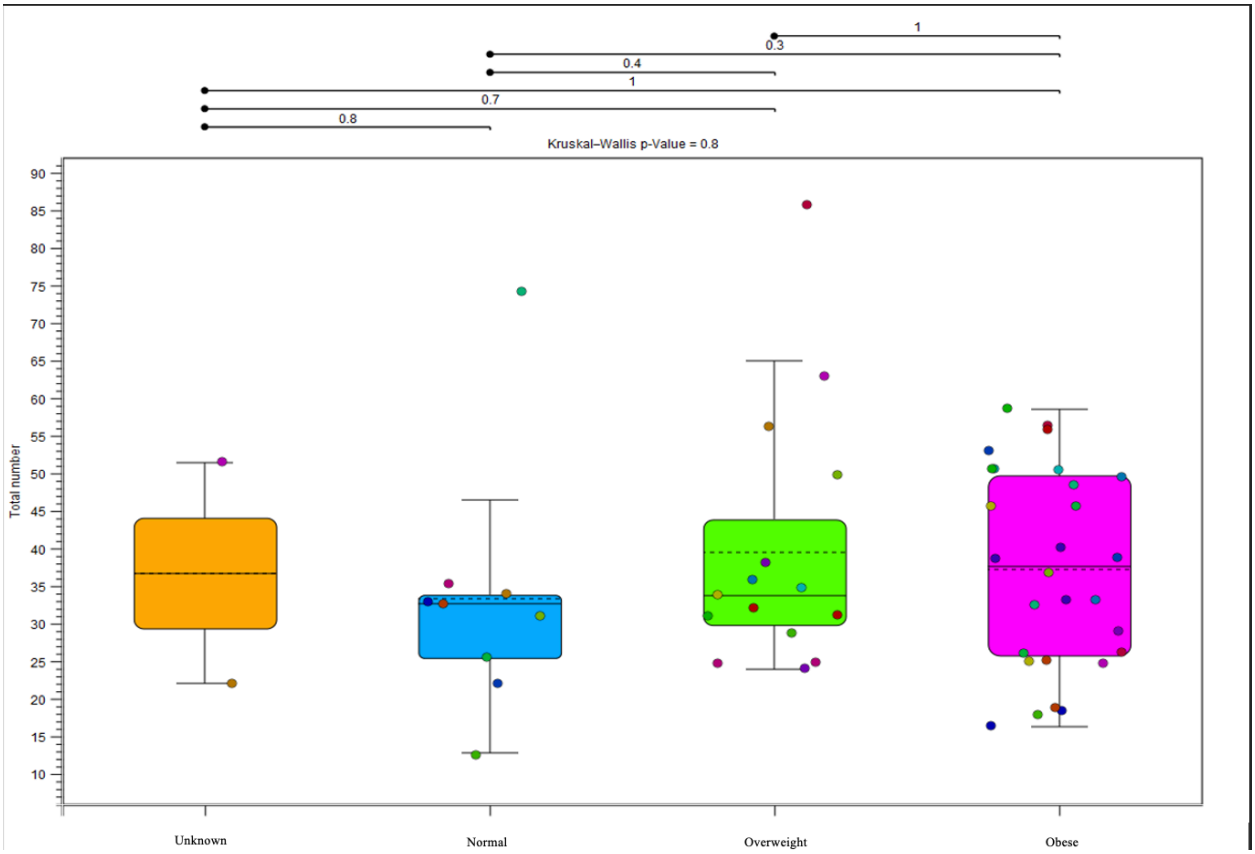

Figure 1.Box plot diagram for total number alpha diversity of BMI classification (x-axis) plotted against total number (y-axis) for unknown BMI classification (n=2), 1) normal weight (n=12), 2) overweight (n=21), and 3) obese (n=40). Kruskal-Wallis test for p-value=0.8

Alpha diversity showed no statistical significance ($p>0.05$) between maternal pre-pregnancy BMI (ppBMI) groups % acetic acid vs. ppBMI, % propionic acid vs. ppBMI, % butyric acid vs. ppBMI, % APB combined vs. ppBMI, or for Shannon's diversity index vs. ppBMI (Figure 2 and 3).

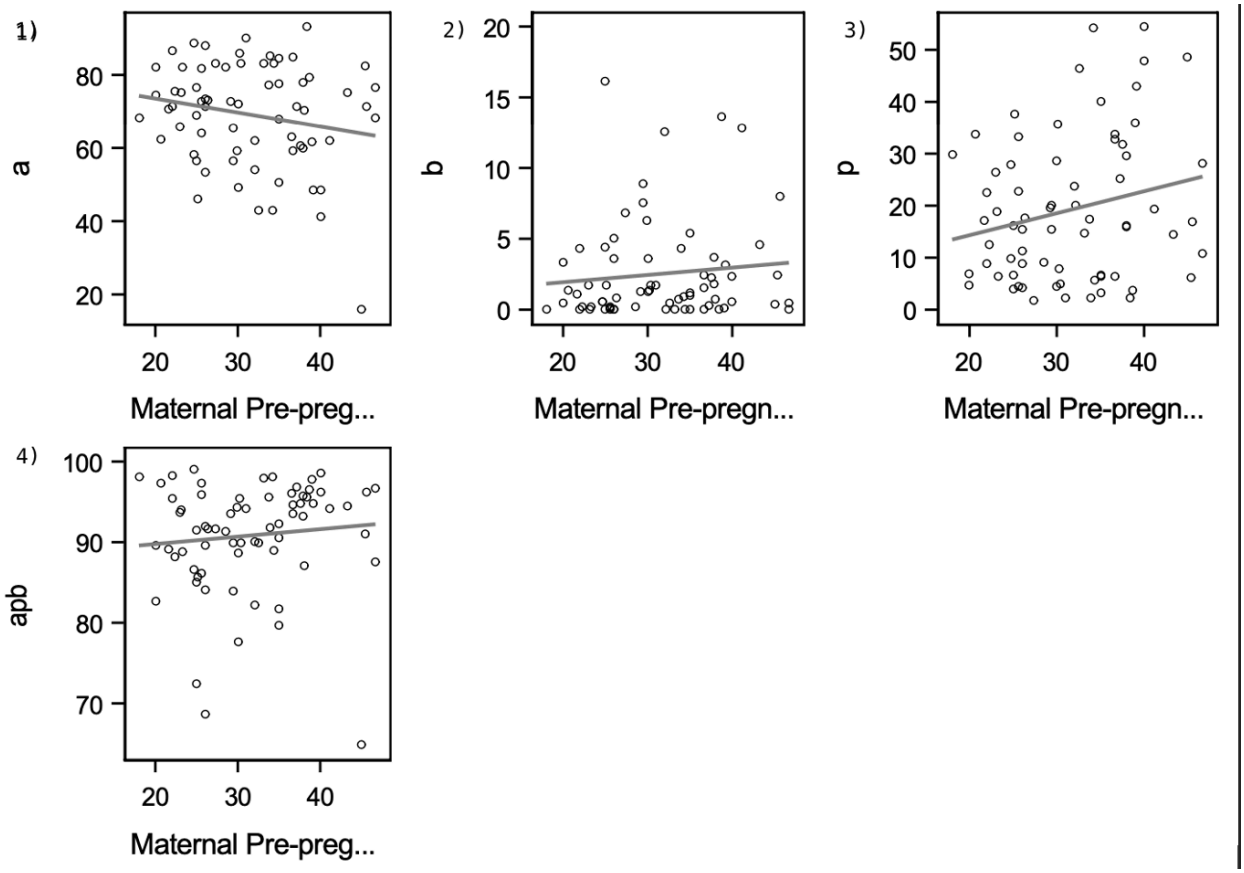

Figure 2. Maternal pre-pregnancy BMI (kg/ht m^2) (x-axes) plotted against 1) % acetic acid, 2) % butyric acid, 3) % propionic acid, and 4) % APB combined (y-axes)

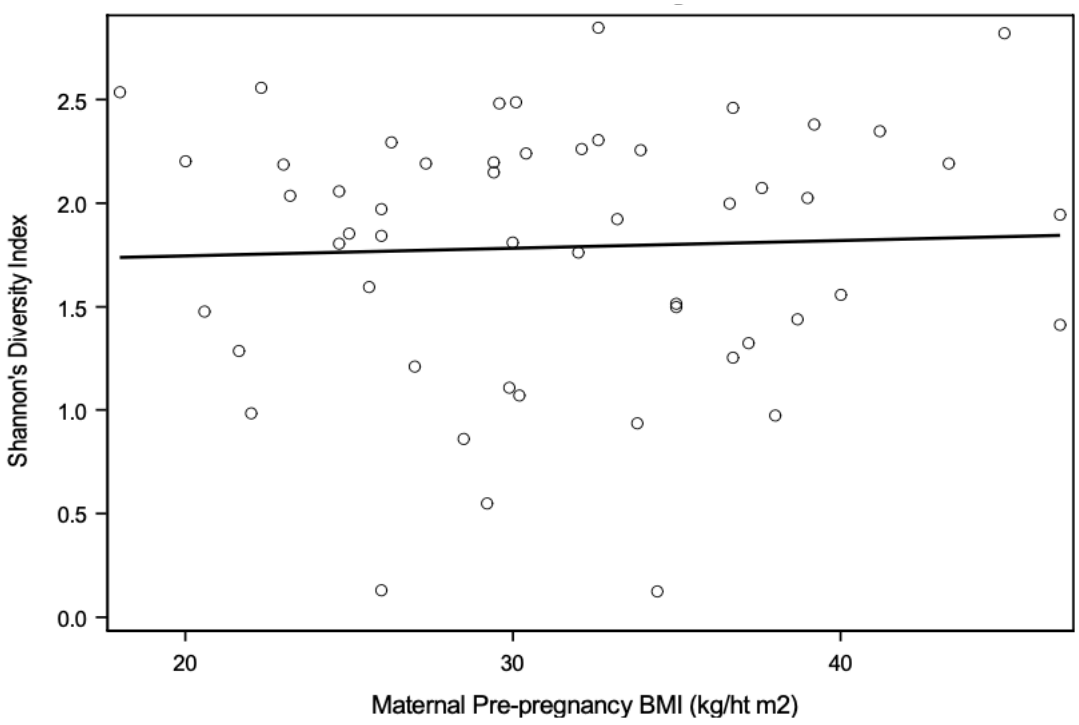

Figure 3. Maternal pre-pregnancy BMI plotted against the Shannon Diversity Index for each sample

Gestation Time

Alpha diversity differences were not statistically significant ($p>0.05$) between gestational times of <28 weeks, 28-32 weeks, and 33-36 weeks (Figure 4).

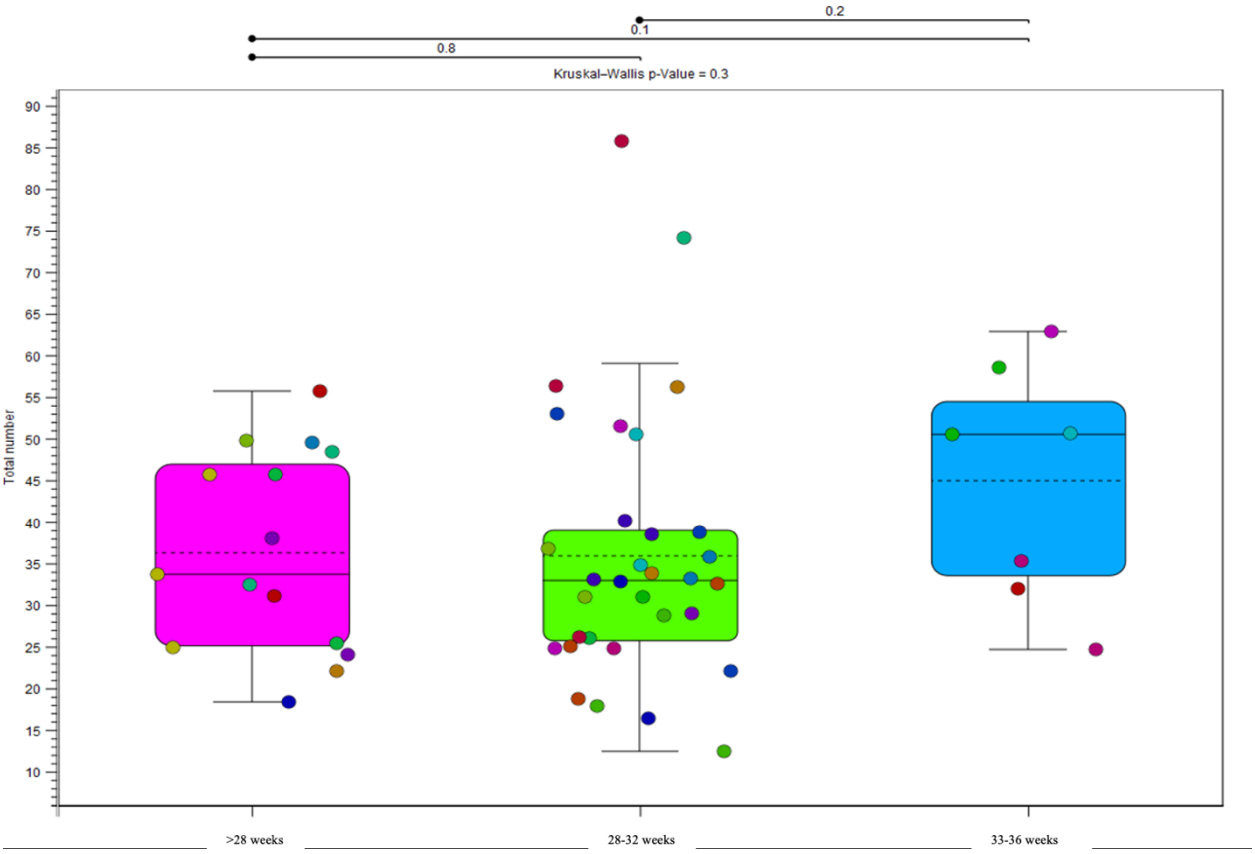

Figure 4. Box plot diagram of total number alpha diversity of gestational time (x-axis) plotted against total number (y-axis) for 1) >28-week gestational time (n=17), 2) 28-32 week gestational time (n=47), and 3) 33-36 week gestational time (n=10). Kruskal-Wallis test for p-value=0.3

Alpha diversity of the SCVFA had no statistical significance ($p>0.05$) between gestational time groups % acetic acid vs. gestational time, % propionic acid vs. gestational time, % butyric acid vs. gestational time, % APB combined vs. gestational time, or for Shannon's diversity index vs. gestational time (Figure 5 and 6).

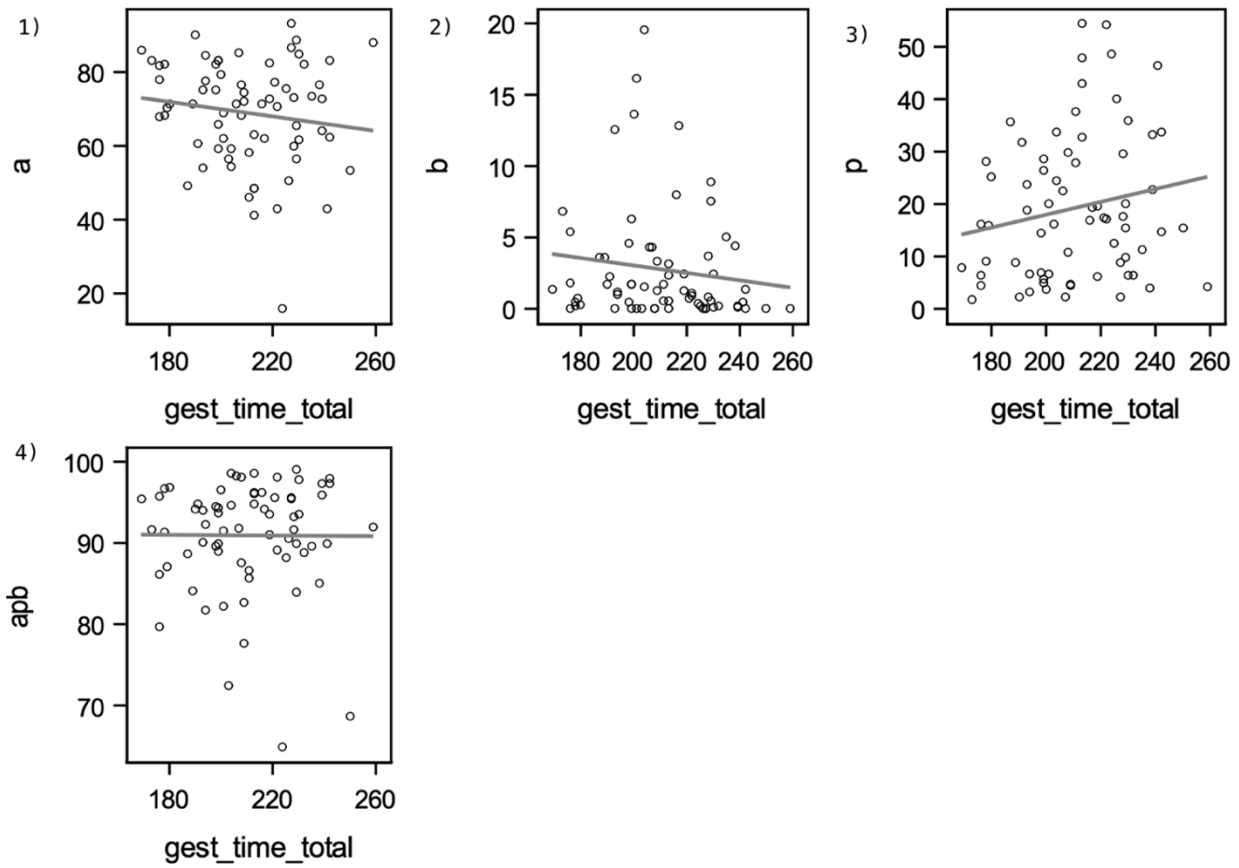

Figure 5. Gestational time (days) (x-axes) plotted against 1) % acetic acid, 2) % butyric acid, 3) % propionic acid, and 4) % APB combined (y-axes)

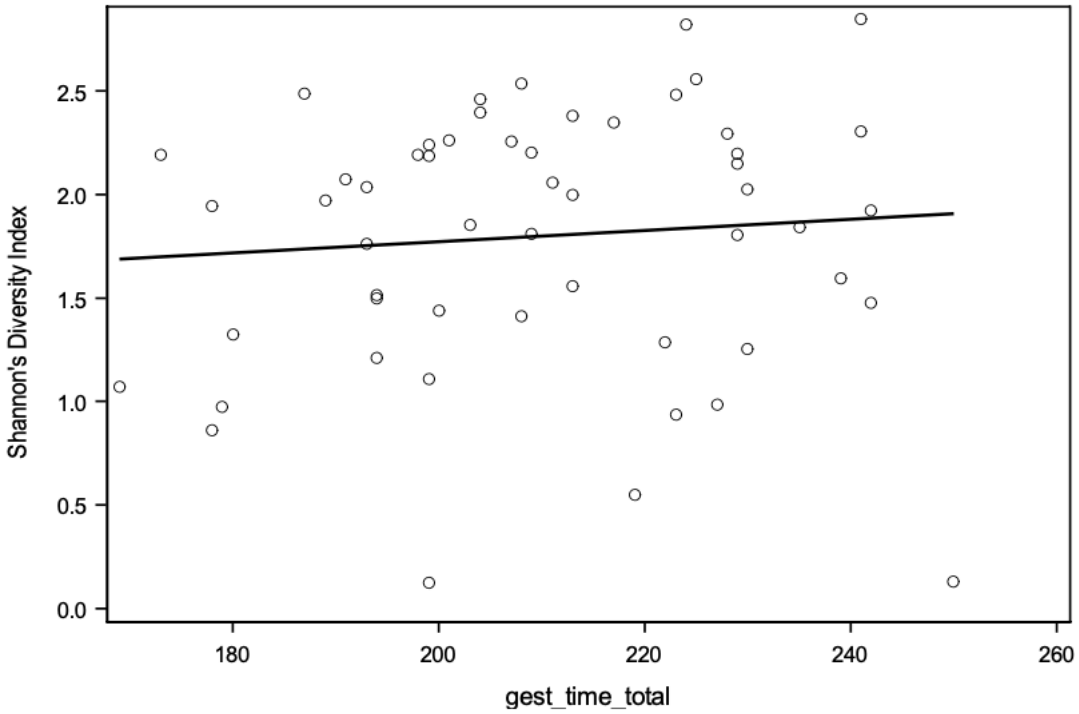

Figure 6. Total gestational time in days plotted against the Shannon Diversity Index for each sample

Day of Sample Collection

Alpha diversity differences were not statistically significant ($p>0.05$) for day of sample collection for 1-7 days of life, 8-14 days of life, 15-21 days of life, 22-28 days of life, and >28 days of life. (Figure 7).

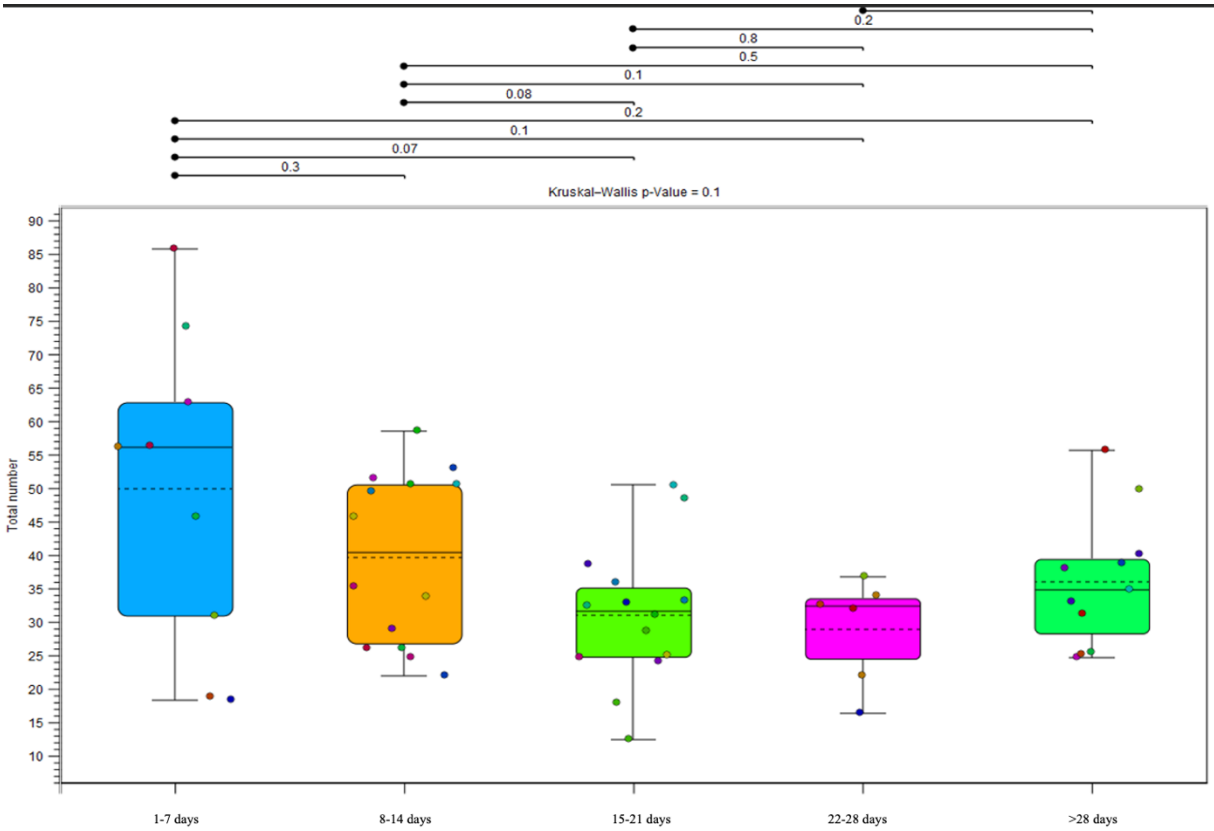

Figure 7. Box plot diagram of total number alpha diversity of day of sample collection (x-axis) plotted against total number (y-axis) for 1) 1-7 days of life (n= 17), 2) 8-14 days of life (n=17), 3) 15-21 days of life (n=17), 4) 22-28 days of life (n=8), and 5) >28 days of life (n=16). Kruskal-Wallis test for p-value = 0.1

Alpha diversity of the SCVFA had no statistical significance ($p>0.05$) between day of sample collection groups % acetic acid vs. day of collection, % propionic acid vs. day of collection, % butyric acid vs. day of collection, % APB combined vs. day of collection, or for Shannon's diversity index vs. day of collection (Figure 8 and 9).

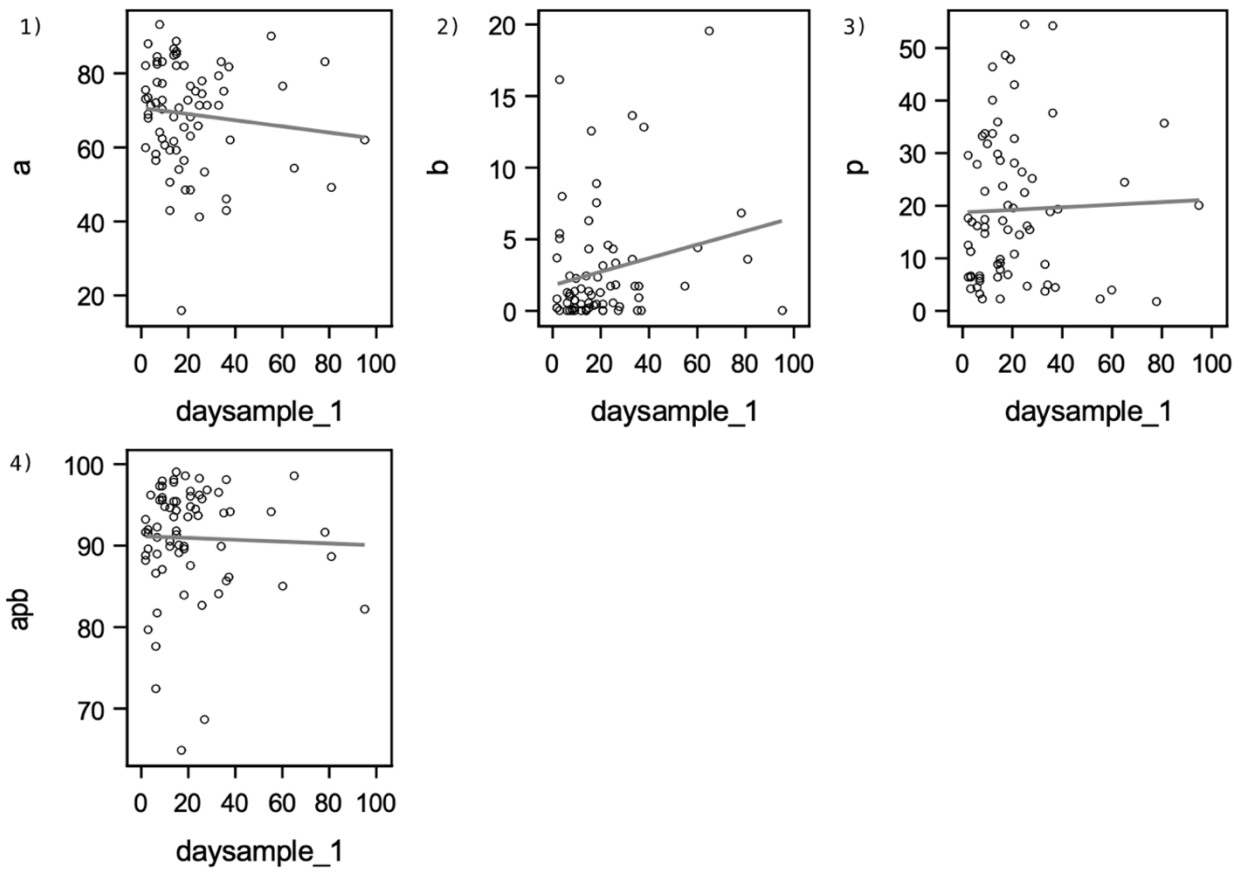

Figure 8. Day of sample collection (x-axes) plotted against 1) % acetic acid, 2) % butyric acid, 3) % propionic acid, and 4) % APB combined (y-axes)

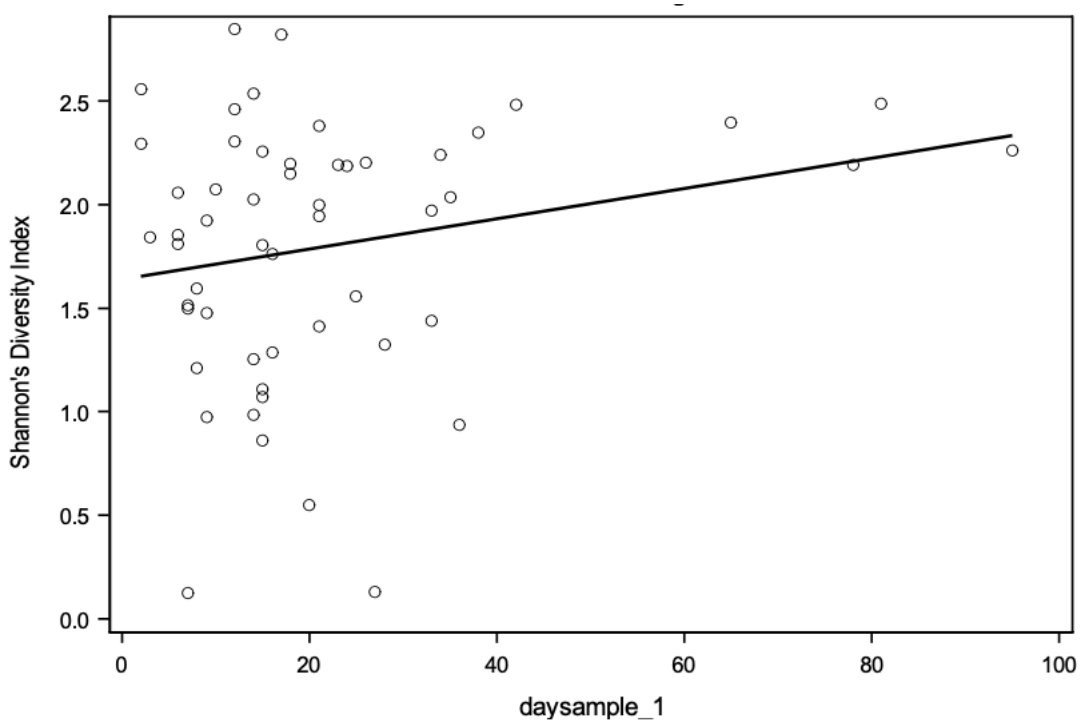

Figure 9. Day of life of sample collection plotted against the Shannon Diversity Index for each sample

Delivery Mode

Alpha diversity differences were not statistically significant ($p>0.05$) between delivery modes no-c-section and c-section (Figure 10).

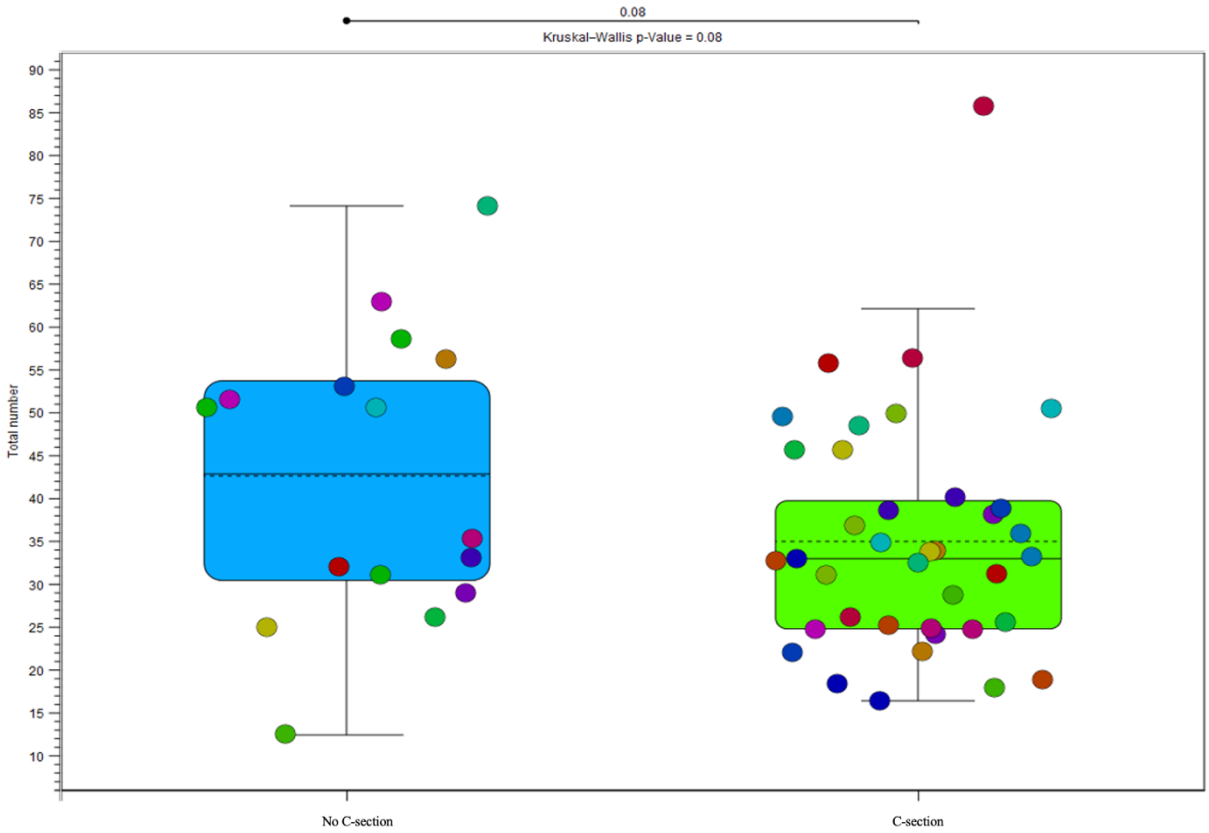

Figure 10. Box plot diagram of total number alpha diversity for day of sample collection (x-axis) plotted against total number (y-axis) for 1) no c-section (n= 27) and 2) c-section (n=48). Kruskal-Wallis test for p-value= 0.08

The alpha diversity for BMI of the delivery mode groups no C-section and C-section showed statistical significance (p=0.0499) (Figure 11).

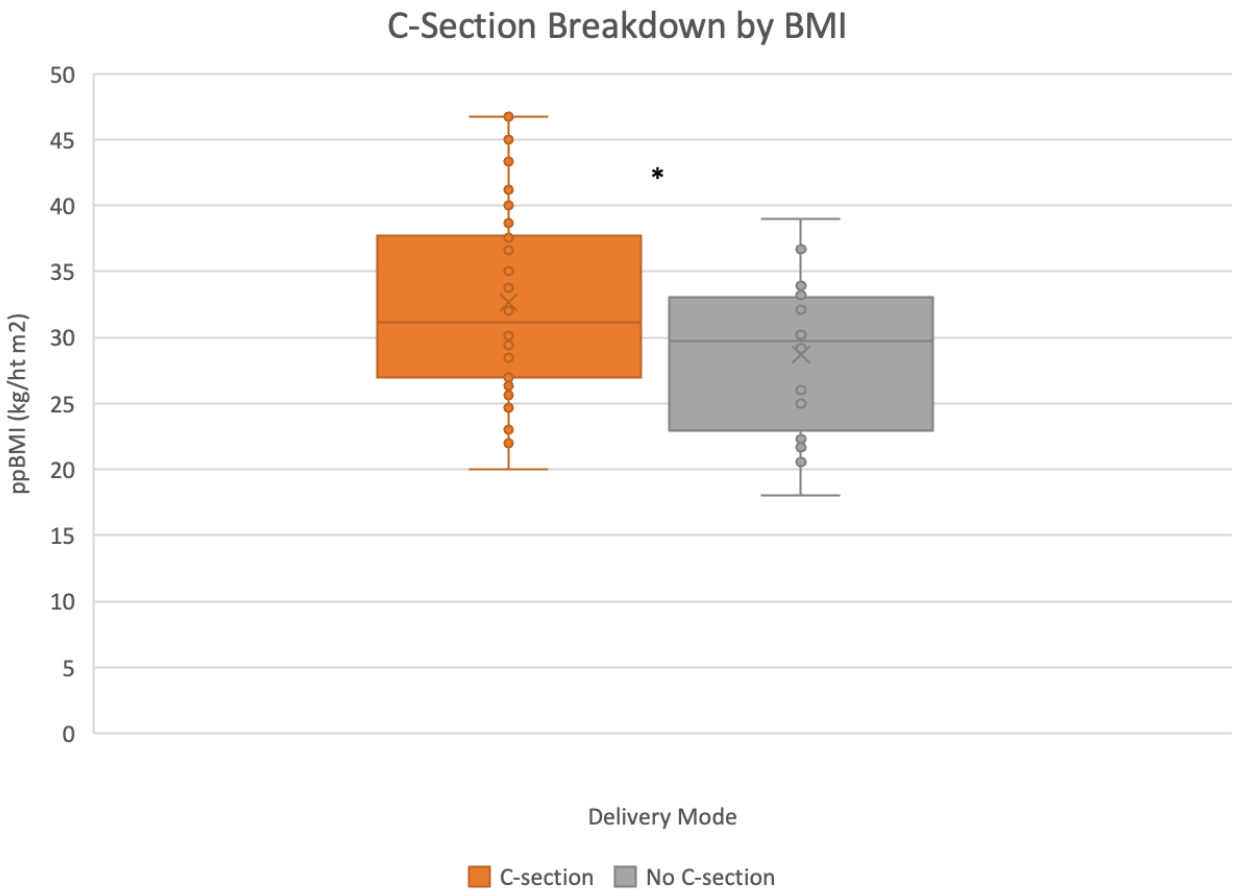

Figure 11. Box and Whisker plot of maternal pre-pregnancy BMI (y-axis) versus delivery mode (x-axis) for groups C-section (n=42) and no C-section (n=16). T-test results in a p-value of 0.0499

Race/Ethnicity

Alpha diversity differences were not statistically significant ($p>0.05$) between race/ethnicity groups of Black, White, Hispanic, and Other (Figure 12).

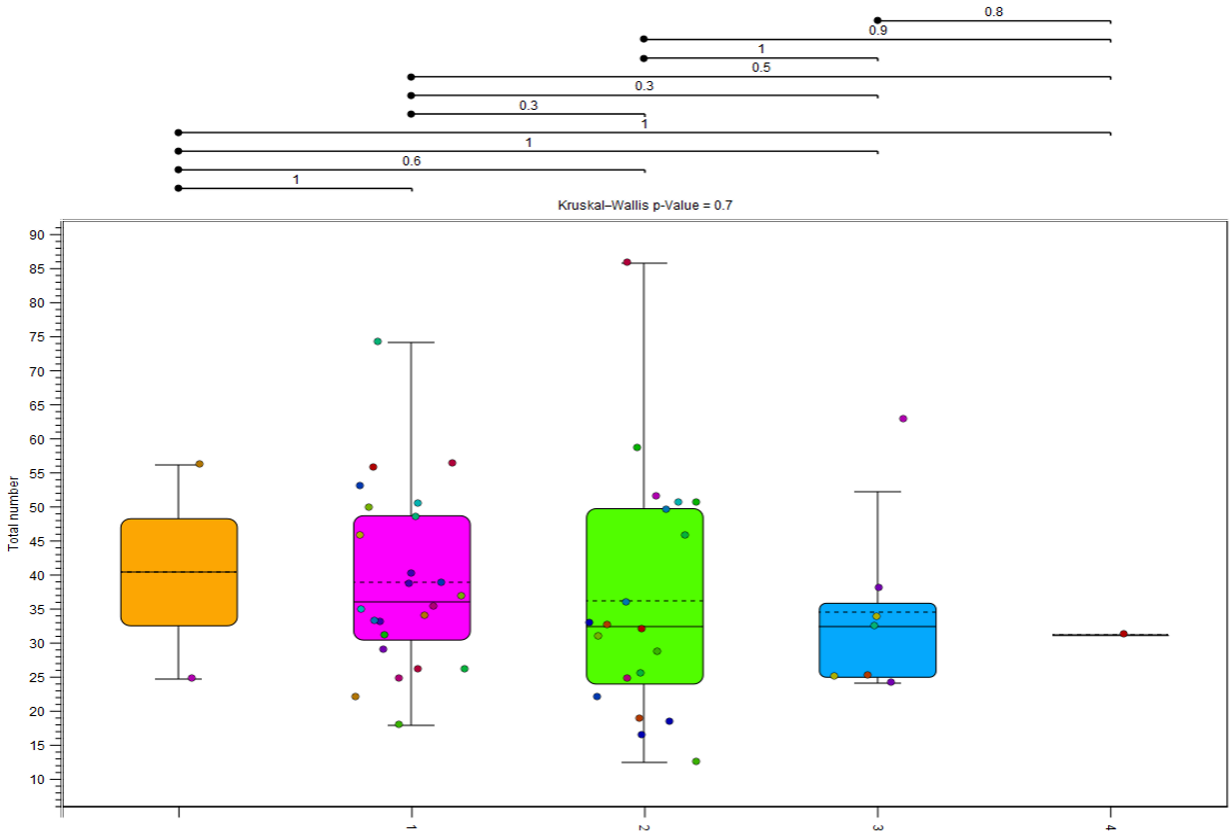

Figure 12. Box plot diagram of total number alpha diversity for race/ethnicity (x-axis) plotted against total number (y-axis) for unknown (n=1),1) Black (n= 34), 2) White (n=29), 3) Hispanic (n=7), and 4) Other (n=7). Kruskal-Wallis test for p-value= 0.7

The alpha diversity for BMI for race/ethnicity groups Black, White, and Hispanics showed no statistical significance for White vs. Hispanic BMI ($p>0.05$), no statistical significance for Black vs. Hispanic BMI ($p>0.05$), but did display significance for Black vs. White BMI ($p=0.013$) (Figure 13).

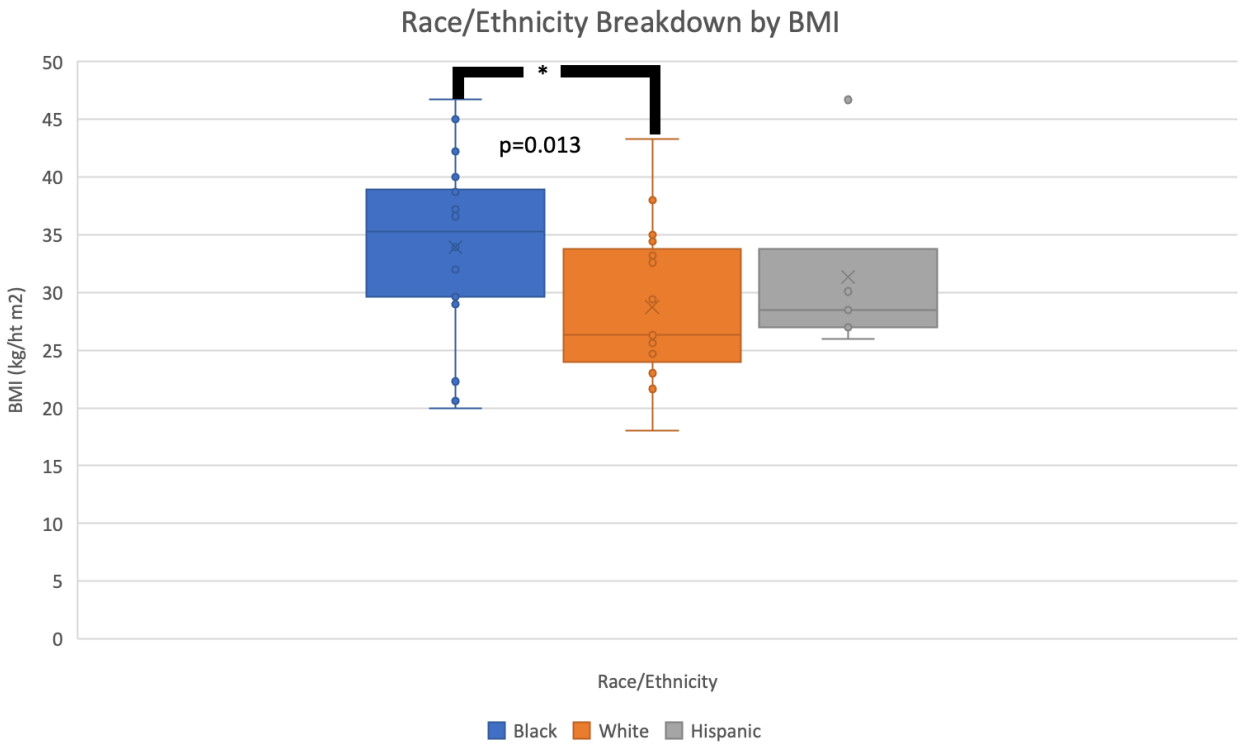

Figure 13. Box and Whisker plot of alpha diversity of BMI for race/ethnicity groups Black (n=24), White (n=21), and Hispanics (n=7)

Beta Diversity

Maternal BMI

No significant differences ($p>0.05$) were observed for pre-pregnancy maternal BMI by beta diversity metrics of Bray-Curtis dissimilarity or Jaccard similarity (Figure 14 and 15) for normal weight vs. overweight, normal weight vs. obese, or overweight vs. obese.

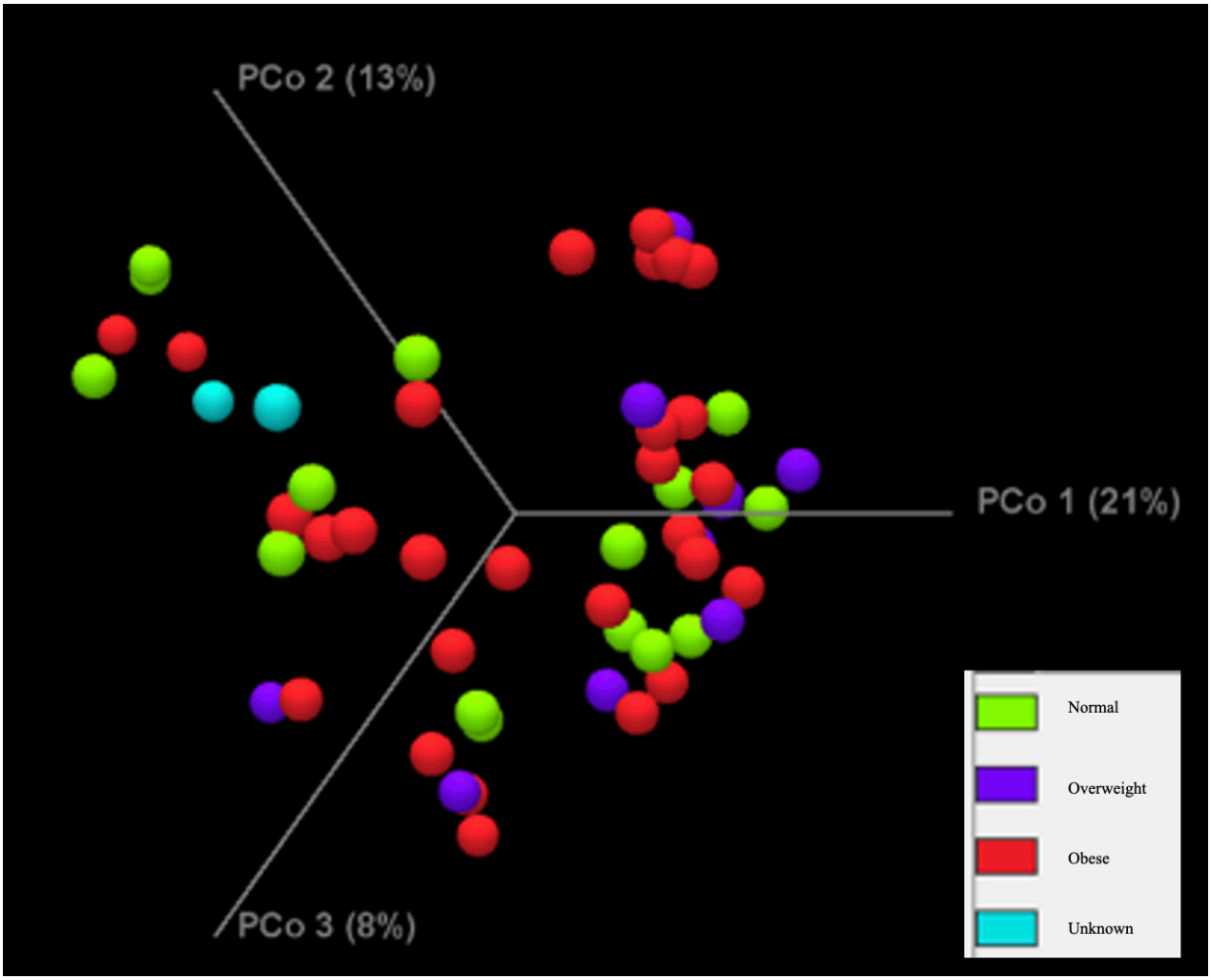

Figure 14. Bray-Curtis dissimilarity matrix PCoA plot stratified by ppBMI groups normal weight (n=12), overweight (n=21), obese (n=40), and unknown (n=2). One point represents one sequencing sample. Distances between points represent Euclidean distance. Axes are chosen to represent maximum variation. Proximity between points directly correlates to similarity

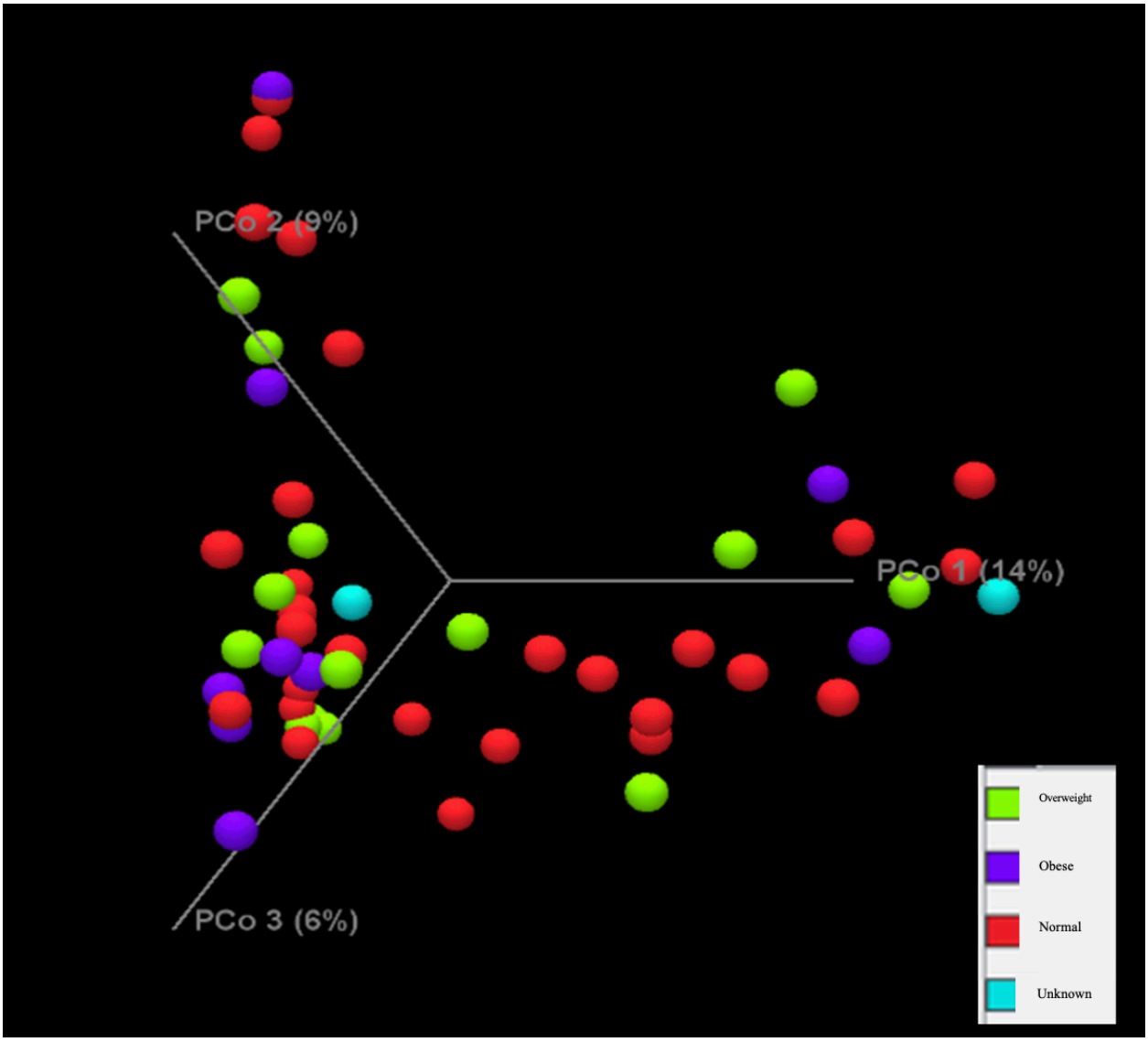

Figure 15. Jaccard dissimilarity matrix PCoA plot stratified by ppBMI. One point represents one sequencing sample. Distances between points represent Euclidean distance. Axes are chosen to represent maximum variation. Proximity between points directly correlates to similarity

Gestation Time

No significant differences were observed between >28-week gestation time, 28-32 week gestation time, or 33-36 week gestation time by beta diversity metric of Bray-Curtis dissimilarity (Figure 16).

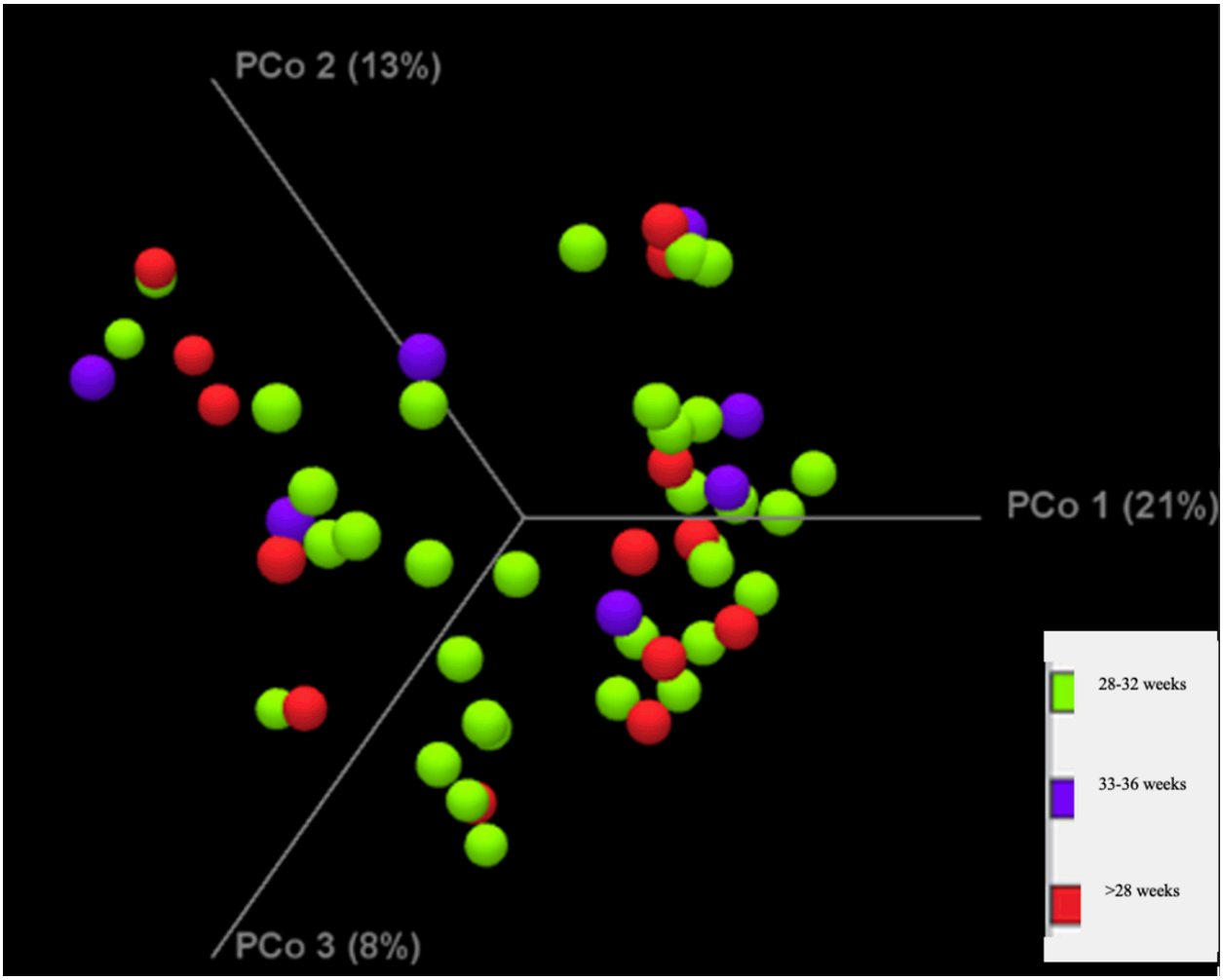

Figure 16. Bray-Curtis dissimilarity matrix PCoA plot stratified by gestation time groups <28 weeks (n=17), 28-32 weeks (n=47), and 33-36 weeks (n=10). One point represents one sequencing sample. Distances between points represent Euclidean distance. Axes are chosen to represent maximum variation. Proximity between points directly correlates to similarity

Day of Sample Collection

No significant differences were observed between day of sample collection groups 1-7 days of life, 8-14 days of life, 15-21 days of life, 22-28 days of life, or >28 days of life by beta diversity metric of Bray-Curtis dissimilarity (Figure 17).

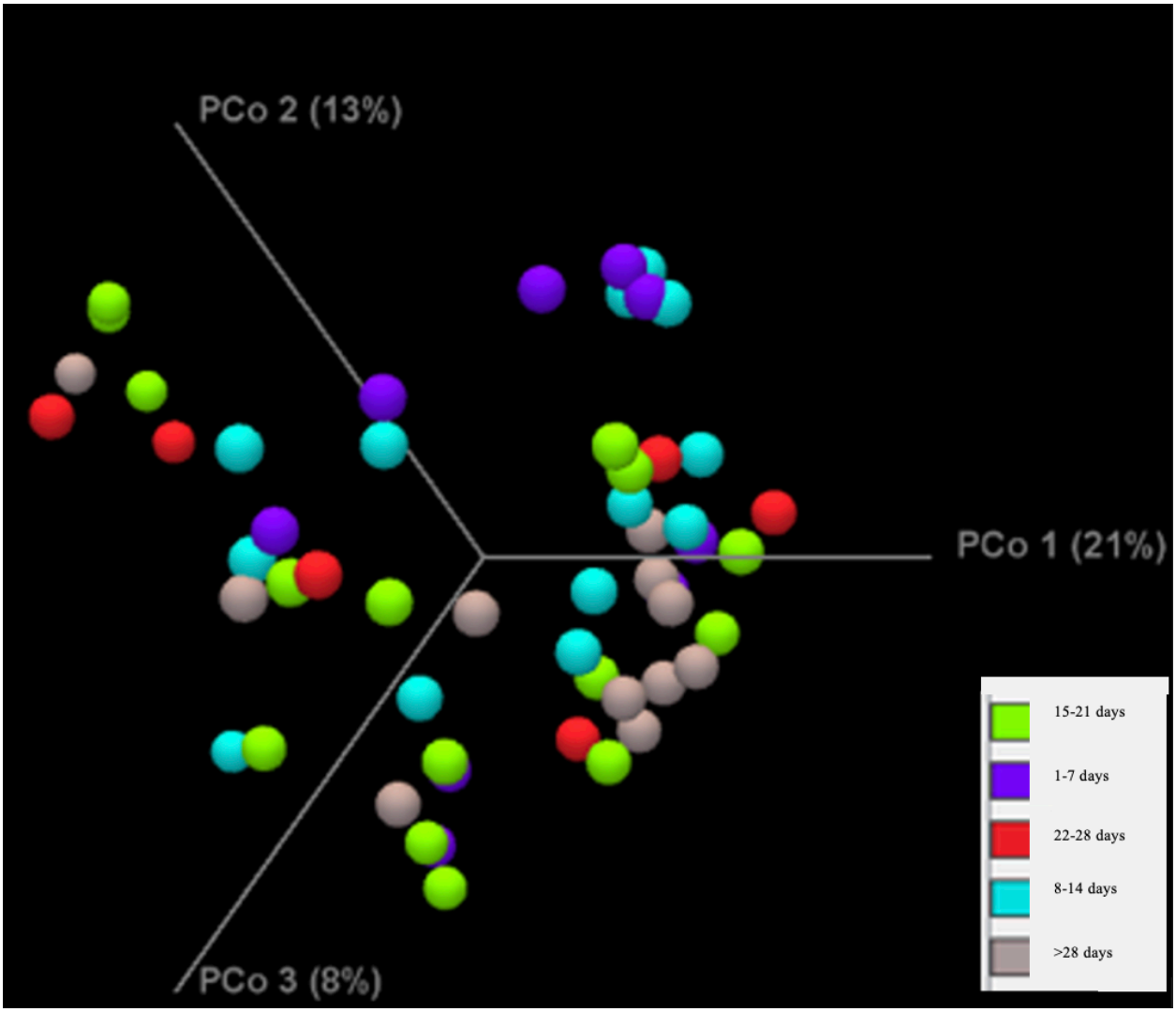

Figure 17. Bray-Curtis dissimilarity matrix PCoA plot stratified by day of life of sample collection groups 1-7 days of life (n=17), 8-14 days of life (n=17), 15-21 days of life (n=17), 22-28 days of life (n=8), and >28 days of life (n=16). One point represents one sequencing sample. Distances between points represent Euclidean distance. Axes are chosen to represent maximum variation. Proximity between points directly correlates to similarity

Delivery Mode

No significant differences were observed between delivery mode groups no c-section vs. c-section by beta diversity metric of Bray-Curtis dissimilarity (Figure 18).

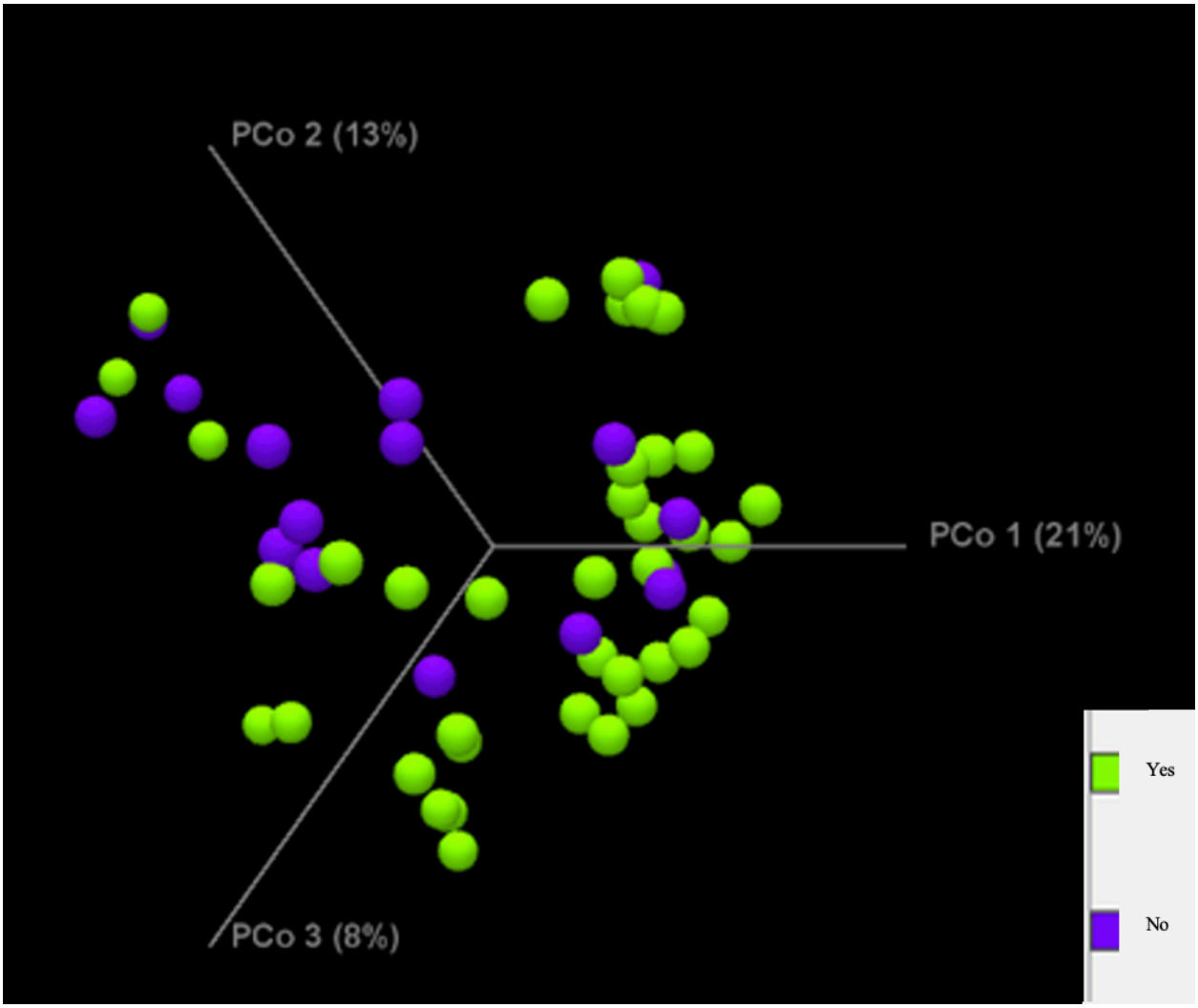

Figure 18. Bray-Curtis dissimilarity matrix PCoA plot stratified by delivery mode groups no c-section (n=27) and c-section (n=48). One point represents one sequencing sample. Distances between points represent Euclidean distance. Axes are chosen to represent maximum variation. Proximity between points directly correlates to similarity

Race/Ethnicity

No significant differences were observed between race/ethnicity groups Black, White, Hispanic, and Other by beta diversity metric of Bray-Curtis dissimilarity (Figure 19).

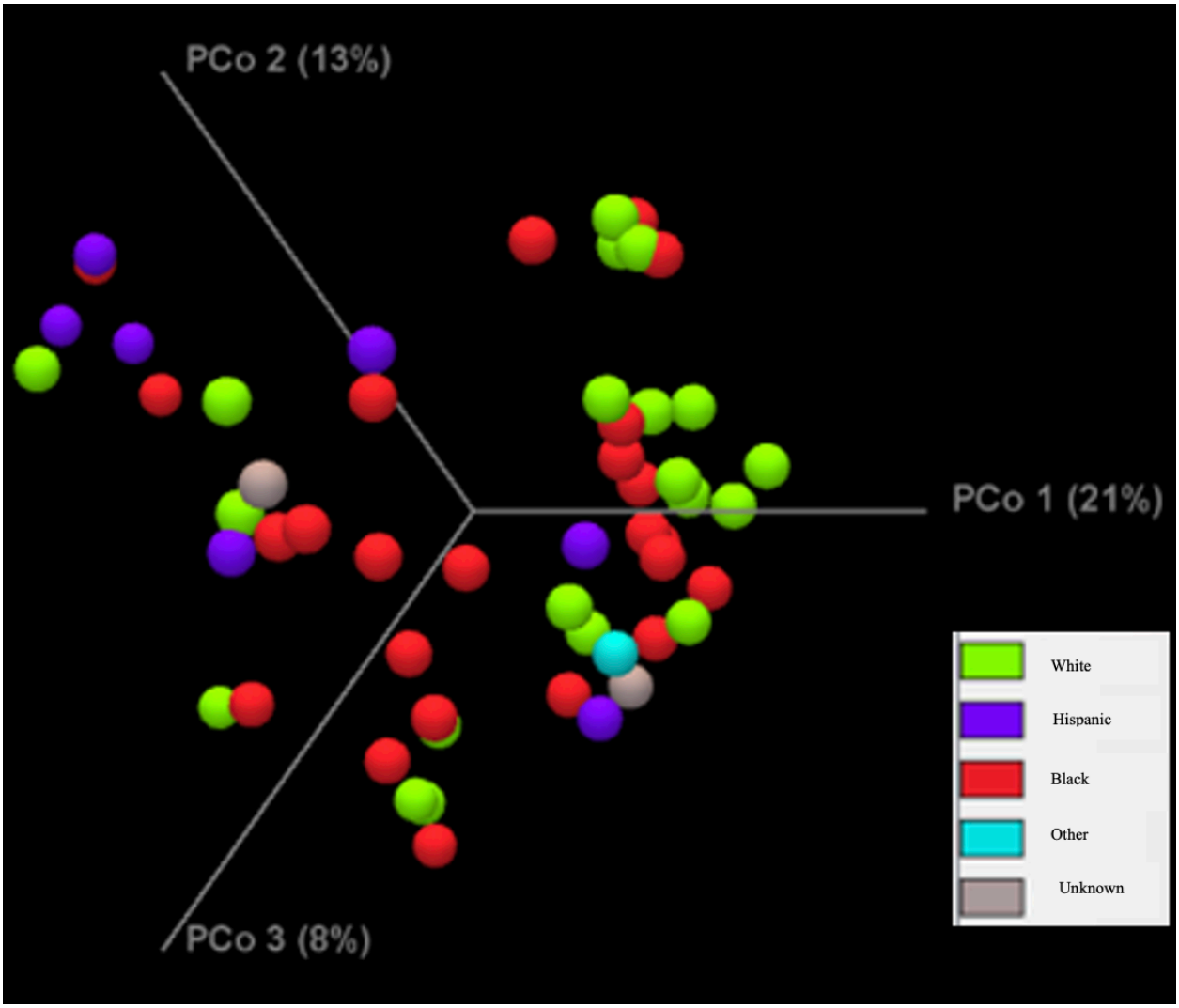

Figure 19. Bray-Curtis dissimilarity matrix PCoA plot stratified by race/ethnicity groups Black (n=34), White (n=29), Hispanic (n=7), Other (n=7), and Unknown (n=1). One point represents one sequencing sample. Distances between points represent Euclidean distance. Axes are chosen to represent maximum variation. Proximity between points directly correlates to similarity

Differential Abundance

The following taxonomical bar plot (Figure 20) was generated with CLC Genomics Workbench ver. 23 for maternal BMI groups normal weight, overweight, and obese:

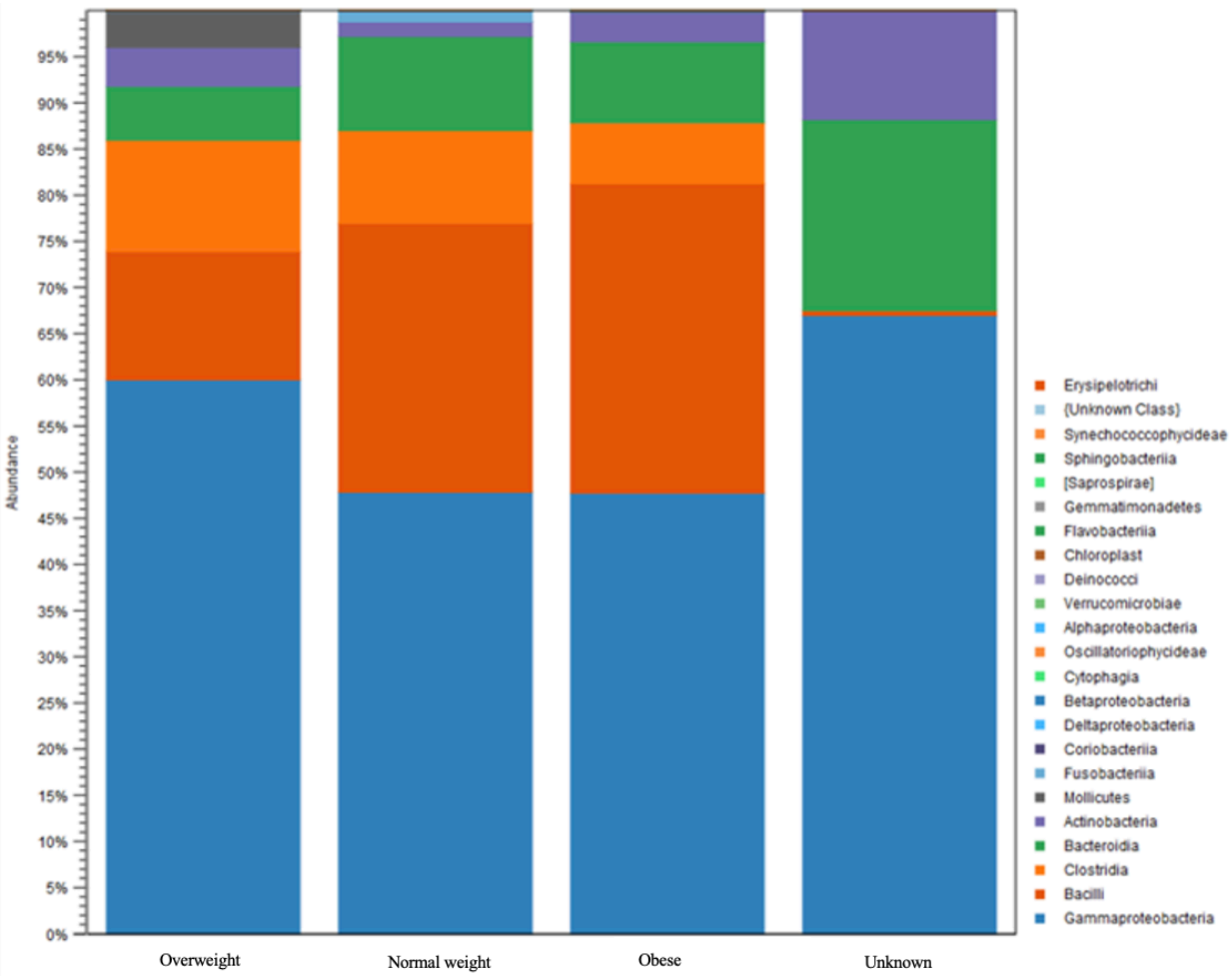

Figure 20. Relative abundance of maternal pre-pregnancy BMI microbial population at the phylum level for groups normal weight (n=12), overweight (n=21), obese (n=40), and unknown (n=2)

There was no significant difference between differential abundances of the microbial taxa identified in the ppBMI samples, as visualized by the lack of diversity in the heatmap (Figure 21) of differential abundance of the microbial taxa.

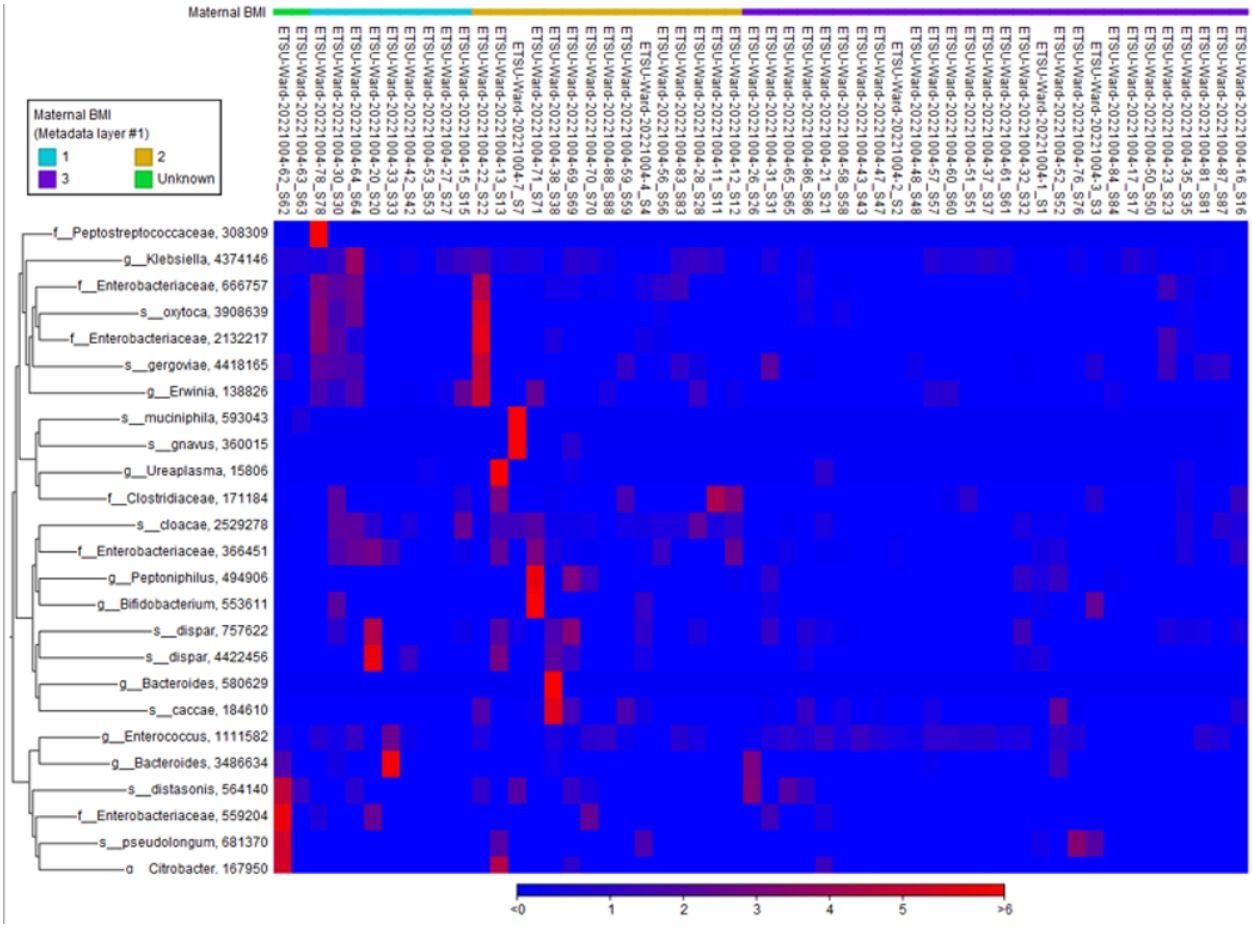

Figure 21. Heatmap of relative abundance of microbial taxa identified in premature neonates born to women of differing BMI groups 1) normal weight, 2) overweight, and 3) obese. Bright blue represents no difference, bright red represents maximum variation, colors in between the two are proportional to the percent of variation

CHAPTER 5. DISCUSSION

Maternal BMI

Due to the small sample size and lack of longitudinal sampling, a conclusion could not be made regarding alpha diversity's correlation to BMI, however the literature reports that alpha diversity tends to be negatively correlated with BMI [79,80,81]. In Zhong et al's 2020 correlational study about the gut microbiota's association with obesity status, it was determined that alpha diversity was significantly higher (p=.038) in non-obese adults than obese adults [81]. It is difficult to draw the same conclusions for the affect of maternal BMI on neonatal alpha diversity due to the immaturity of the neonatal gut microbiome. It is known that acetic, butyric,

and propionic acids are decreased in individuals with higher BMI, however, our results showed a lack of alpha diversity of SCFAs, likely due to the low sample size, lack of environmental diversity, and the immaturity of the microbiome [82,83]. These studies represent the SCFA profiles of individuals with fully developed microbiomes, which has not been previously studied in premature neonates. An accurate BMI cannot be obtained by weight and height alone for infants until two years of age [84] so it is possible that after two years, there may be greater differences. It is more likely, however, that alpha diversity differences will increase due to the food they eat and the differing environments that they live in. We observed no difference in beta diversity metrics for Bray-Curtis dissimilarity or Jaccard dissimilarity. Again, these results contrast with the literature which shows that differing BMI groups tend to also have differing and less diverse microbiome profiles as BMI increases, whereas our study showed no differences [85,86,87]. This is likely due to the small sample size of this study as well as in the inability to control for confounding factors. No general trends could be discovered from the data collected for the relative abundance of microbial taxa as BMI increased. BMI was slightly negatively correlated with % acetic acid and slightly positively correlated with % propionic acid. The literature supports the negative correlation with % acetic acid, but not % propionic acid [88,89], however the correlation between these SCFA's and BMI is not significant enough to draw any conclusions. The link between maternal pre-pregnancy BMI, neonatal SCFA profiles, and offspring health outcomes remains to be elucidated.

Gestational Time

Gestation time had no significant difference for total number alpha diversity and had no correlation to Shannon's diversity index metric for alpha diversity. Preterm infants with less

gestational time than term infants display delayed colonization, less diversity, and less abundance in their gut microbiome [90]. It is likely that our study did not find any significant difference in alpha diversity as gestation time increased because of the small sample size and because all of the neonates studied were preterm, resulting in delayed colonization. We observed no significant difference in beta diversity of gestation time groups as measured by Bray-Curtis dissimilarity. Gestation time was negatively correlated with % acetic acid and % butyric acid and positively correlated with % propionic acid. It makes sense that gestation time was positively correlated with % propionic acid, because as gestation time increases so should the diversity and abundance of the microbiome. It is likely that the negative correlation displayed between gestation time and % butyric and acetic acid is due to the small sample size, age of the newborns, lack of term infant control, and identical environment that they are all kept in.

Day of Sample Collection

The day of sample collection had no significant difference when measured by total number alpha diversity, however, was positively correlated with Shannon's diversity index. Gut microbiome develops over time as neonates are exposed to skin flora, HBM microbes, and environmental microbes so there should be some difference between the gut microbiome of infants that have a sample taken within the first week of life compared to later weeks. Shannon's diversity index was likely positively correlated with day of sample collection because the older neonates had richer and more even microbiomes than their younger counterparts. Total number alpha diversity. Total number alpha diversity most likely had less significance because it does not place as much emphasis on abundances and just considers the presence or absence of species. There was no significant difference for beta diversity metric Bray-Curtis dissimilarity for day of sample collection groups. For SCFA, day of sample collection was negatively correlated to %

acetic acid and positively correlated with % butyric acid. Levels of SCFAs increase as the microbiome develops and as gut health increases [91,92]. The positive correlation between day of life and butyric acid agrees with the literature because the older neonates displayed higher levels of butyric acid than the younger neonates did. It is likely that there was not a positive correlation observed between acetic acid levels and day of life because of the small sample size, age of neonates, lack of term control, and lack of longitudinal sampling.

Delivery Mode

There was no significant difference between c-section versus no c-section as calculated by total number alpha diversity. Neonates born vaginally get inoculated with commensal bacteria (*Bifidobacterium* and Lactobacillus) which are generally associated with healthier gut microbiome and a reduction in allergies and asthma, whereas neonates birthed by c-section exhibit a microbiome more similar to the mother's skin flora and the hospital environment (*Staphylococcus, Streptococcus, and Clostridium*). Babies born by c-section also exhibit less diverse gut microbiota than those that are vaginally birthed [93]. It is likely that there was no significant difference between neonates born via c-section and no c-section because of the small sample size, lack of longitudinal sampling, and identical living environment. Bray-Curtis dissimilarity plotted by PCoA displayed no significant difference. When delivery mode was broken down by pre-pregnancy BMI there was a significant difference between the two groups meaning that there was a significant difference between the BMI of the mother's that had a c-section and did not. The pre-pregnancy BMI tended to be higher in individuals that gave birth via c-section. If done again, it would be advantageous to have a sample set without significant difference between BMI and delivery mode.

Race/Ethnicity

There was no significant difference for total number alpha diversity between race/ethnicity. Different ethnic groups tend to have differing diets and live in different environments which confer differential gut microbial communities [94]. It is likely that no difference was observed between racial/ethnic groups because the neonates are on the same diet and in the same environment, rather than eating the ethnic foods and living in environments that would result in microbiome variability. Differences may be observed after longitudinal sampling as the microbiome develops, infants are breastfed longer, or when they begin to eat solid foods. Bray-Curtis dissimilarity plotted by PCoA displayed no significant difference. When broken down by BMI, there was significant difference between Black and White individuals, but not between Black and Hispanic or White and Hispanic. This means that there were significantly higher BMIs for Black mothers than White mothers. If repeating this study, it would be advantageous to increase the amount of normal BMI Black mothers, as well as increasing the number of Hispanic mothers.

Limitations

This study was limited by a small sample size of 75 subjects. Also, all of the samples were taken from neonates that reside in the same neonatal intensive care unit. Each infant would have been exposed to the same environmental microbiome as well as receiving the same standardized nutrition plans. Limitations of microbiome analysis should also be considered. Although microbiome analysis has progressed over the years, 16S rRNA sequencing can be limited and biased based on how complete and accurate reference databases are and differing PCR amplification frequencies due to various affinities for the primer [95].

Future Directions

Future directions for this study include increasing the sample size, evaluating neonates from various hospitals, standardizing the time of sample collection, and following the neonates up to three years, when the microbiome stabilizes. Increasing the sample size of the cohort studied would allow for better analysis for each of the subgroups. Increasing the cohort to different hospitals would allow for more randomness in environmental microbiome. Standardizing the time of stool sample collection would control for the rapid development of the microbiome so that each neonate is at the same level of development when the sample is studied. A longitudinal study would provide more insight into how the microbiome develops throughout time and allow for a better grasp of the health outcomes associated with maternal health before and at the time of birth.

CHAPTER 6. CONCLUSION

Overall, no significant differences were found for metrics of alpha diversity for microbiome between groups of BMI, gestational time, day of life of sample collection, delivery mode, or race/ethnicity. Acetic acid levels were negatively correlated with pre-pregnancy BMI, gestation time and day of life of sample collection. Propionic acid levels were positively correlated with pre-pregnancy BMI and gestation time. Butyric acid levels were slightly positively correlated to pre-pregnancy BMI, positively correlated to day of sample collection, and negatively correlated to gestation time. Shannon's diversity index was positively correlated with day of life of sample collection. Beta diversity metrics showed no statistical significance for any groups. Additionally, there was no significance in the differential abundance of microbial taxa when stratified by pre-pregnancy BMI. The results of the SCFA profiles and 16S rRNA analysis suggest that further study be conducted longitudinally, with larger sample size, differing

environments, differing diets, and with more even distributions among each grouping so that each group is fairly represented.

APPENDIX

Table of Figures

REFERENCES

[1] Ainamo, J., Paloheimo, L., Nordblad, A., & Murtomaa, H. (1986). Gingival recession in schoolchildren at 7,12 and 17 years of age in Espoo, Finland. *Community Dentistry and Oral Epidemiology*, *14*(5), 283–286. https://doi.org/10.1111/j.1600-0528.1986.tb01073.x

[2] Perez, M., Robbins, M. E., Revhaug, C., & Saugstad, O. D. (2019). Oxygen radical disease in the newborn, revisited: Oxidative stress and disease in the newborn period. *Free Radical Biology and Medicine*, *142*, 61–72. https://doi.org/10.1016/j.freeradbiomed.2019.03.035

[3] Zhang, Z., Jiang, J., Li, Z., & Wan, W. (2022). The change of cytokines and gut microbiome in preterm infants for bronchopulmonary dysplasia. *Frontiers in Microbiology*, *13*. https://doi.org/10.3389/fmicb.2022.804887

[4] KC, A., Wrammert, J., Nelin, V., Ewald, U., Clark, R., & Målqvist, M. (2015). Level of mortality risk for babies born preterm or with a small weight for gestation in a tertiary hospital of Nepal. *BMC Public Health*, *15*(1). https://doi.org/10.1186/s12889-015-2232-1

[5] Calcaterra, V., Cena, H., Verduci, E., Bosetti, A., Pelizzo, G., & Zuccotti, G. V. (2020). Nutritional surveillance for the best start in life, promoting health for neonates, infants and children. *Nutrients*, *12*(11), 3386. https://doi.org/10.3390/nu12113386

[6] De Costa, A., Moller, A.-B., Blencowe, H., Johansson, E. W., Hussain-Alkhateeb, L., Ohuma, E. O., Okwaraji, Y. B., Cresswell, J., Requejo, J. H., Bahl, R., Oladapo, O. T., Lawn, J. E., & Moran, A. C. (2021). Study protocol for WHO and UNICEF estimates of Global, regional, and national preterm birth rates for 2010 to 2019. *PLOS ONE*, *16*(10). https://doi.org/10.1371/journal.pone.0258751

[7] Warner, B. B., & Tarr, P. I. (2016). Necrotizing enterocolitis and preterm infant gut bacteria. *Seminars in Fetal and Neonatal Medicine*, *21*(6), 394–399. https://doi.org/10.1016/j.siny.2016.06.001

[8] Callaghan, W. M., MacDorman, M. F., Rasmussen, S. A., Qin, C., & Lackritz, E. M. (2006). The contribution of preterm birth to infant mortality rates in the United States. *Pediatrics*, *118*(4), 1566–1573. https://doi.org/10.1542/peds.2006-0860

[9] Walani, S. R. (2020). Global burden of preterm birth. *International Journal of Gynecology & Obstetrics*, *150*(1), 31–33. https://doi.org/10.1002/ijgo.13195

[10] Platt, M. J. (2014). Outcomes in preterm infants. *Public Health*, *128*(5), 399–403. https://doi.org/10.1016/j.puhe.2014.03.010

[11] Blencowe, H., Cousens, S., Oestergaard, M. Z., Chou, D., Moller, A.-B., Narwal, R., Adler, A., Vera Garcia, C., Rohde, S., Say, L., & Lawn, J. E. (2012). National, regional, and worldwide estimates of preterm birth rates in the year 2010 with Time Trends since 1990

for selected countries: A systematic analysis and implications. *The Lancet*, *379*(9832), 2162–2172. https://doi.org/10.1016/s0140-6736(12)60820-4

[12] Liu, L., Oza, S., Hogan, D., Chu, Y., Perin, J., Zhu, J., Lawn, J. E., Cousens, S., Mathers, C., & Black, R. E. (2016). Global, regional, and national causes of under-5 mortality in 2000–15: An updated systematic analysis with implications for the sustainable development goals. *The Lancet*, *388*(10063), 3027–3035. https://doi.org/10.1016/s0140-6736(16)31593-8

[13] Goldenberg, R. L., Culhane, J. F., Iams, J. D., & Romero, R. (2008). Epidemiology and causes of preterm birth. *The Lancet*, *371*(9606), 75–84. https://doi.org/10.1016/s0140-6736(08)60074-4

[14] Di Renzo, G. C., Tosto, V., & Giardina, I. (2018). The biological basis and prevention of Preterm Birth. *Best Practice & Research Clinical Obstetrics & Gynaecology*, *52*, 13–22. https://doi.org/10.1016/j.bpobgyn.2018.01.022

[15] Hereward, J. P., Werth, J. A., Thornby, D. F., Keenan, M., Chauhan, B. S., & Walter, G. H. (2018). Gene expression in response to glyphosate treatment in fleabane (*conyza bonariensis*) - glyphosate death response and candidate resistance genes. *Pest Management Science*, *74*(10), 2346–2355. https://doi.org/10.1002/ps.4804

[16] Kwon, M. R., Cress, E., Clark, W. A., Alamian, A., Lu, Y., & Peterson, J. M. (2018). The adipokine C1Q TNF related protein 3 (CTRP3) is elevated in the breast milk of obese mothers. *PeerJ*, *6*. https://doi.org/10.7717/peerj.4472

[17] MacKay, H., Khazall, R., Patterson, Z. R., Wellman, M., & Abizaid, A. (2013). Rats perinatally exposed to food restriction and high-fat diet show differences in adipose tissue gene expression under chronic caloric restriction. *Adipocyte*, *2*(4), 237–245. https://doi.org/10.4161/adip.24752

[18] Oosting, A., Verkade, H. J., Kegler, D., van de Heijning, B. J., & van der Beek, E. M. (2015). Rapid and selective manipulation of milk fatty acid composition in mice through the maternal diet during lactation. *Journal of Nutritional Science*, *4*. https://doi.org/10.1017/jns.2015.13

[19] Palou, M., Priego, T., Sánchez, J., Torrens, J. M., Palou, A., & Picó, C. (2010). Moderate caloric restriction in lactating rats protects offspring against obesity and insulin resistance in later life. *Endocrinology*, *151*(3), 1030–1041. https://doi.org/10.1210/en.2009-0934

[20] Sun, B., Purcell, R. H., Terrillion, C. E., Yan, J., Moran, T. H., & Tamashiro, K. L. K. (2012). Maternal high-fat diet during gestation or suckling differentially affects offspring leptin sensitivity and obesity. *Diabetes*, *61*(11), 2833–2841. https://doi.org/10.2337/db11-0957

[21] Del Prado, M., Delgado, G., & Villalpando, S. (1997). Maternal lipid intake during pregnancy and lactation alters milk composition and production and litter growth in rats . *The Journal of Nutrition*, *127*(3), 458–462. https://doi.org/10.1093/jn/127.3.458

[22] Matsuno, A. Y., Esrey, K. L., Perrault, H., & Koski, K. G. (1999). Low intensity exercise and varying proportions of dietary glucose and fat modify milk and mammary gland compositions and pup growth . *The Journal of Nutrition*, *129*(6), 1167–1175. https://doi.org/10.1093/jn/129.6.1167

[23] TSUDUKI, T., KITANO, Y., HONMA, T., KIJIMA, R., & IKEDA, I. (2013). High dietary fat intake during lactation promotes development of diet-induced obesity in male offspring of mice. *Journal of Nutritional Science and Vitaminology*, *59*(5), 384–392. https://doi.org/10.3177/jnsv.59.384

[24] Isganaitis, E., Venditti, S., Matthews, T. J., Lerin, C., Demerath, E. W., & Fields, D. A. (2019). Maternal obesity and the human milk metabolome: Associations with infant body composition and postnatal weight gain. *The American Journal of Clinical Nutrition*, *110*(1), 111–120. https://doi.org/10.1093/ajcn/nqy334

[25] Paul, H. A., Collins, K. H., Bomhof, M. R., Vogel, H. J., & Reimer, R. A. (2018). Potential impact of metabolic and gut microbial response to pregnancy and lactation in lean and diet-induced obese rats on offspring obesity risk. *Molecular Nutrition & Food Research*, *62*(4), 1700820. https://doi.org/10.1002/mnfr.201700820

[26] Enstad, S., Cheema, S., Thomas, R., Fichorova, R. N., Martin, C. R., O'Tierney-Ginn, P., Wagner, C. L., & Sen, S. (2020). The impact of maternal obesity and breast milk inflammation on developmental programming of infant growth. *European Journal of Clinical Nutrition*, *75*(1), 180–188. https://doi.org/10.1038/s41430-020-00720-5

[27] Isganaitis, E. (2021). Milky ways: Effects of maternal obesity on human milk composition and childhood obesity risk. *The American Journal of Clinical Nutrition*, *113*(4), 772–774. https://doi.org/10.1093/ajcn/nqab009

[28] Koyuncuoğlu Güngör, N. (2014). Overweight and obesity in children and adolescents. *Journal of Clinical Research in Pediatric Endocrinology*, 129–143. https://doi.org/10.4274/jcrpe.1471

[29] Han, J. C., Lawlor, D. A., & Kimm, S. Y. S. (2010). Childhood obesity. *The Lancet*, *375*(9727), 1737–1748. https://doi.org/10.1016/s0140-6736(10)60171-7

[30] Yanovski, J. A. (2015). Pediatric obesity. an introduction. *Appetite*, *93*, 3–12. https://doi.org/10.1016/j.appet.2015.03.028

[31] TS;, O. (n.d.). *Maternal nutrition, infant health, and subsequent fertility*. Philippine journal of nutrition. Retrieved March 9, 2023, from https://pubmed.ncbi.nlm.nih.gov/12266149/

[32] Mate, A., Reyes-Goya, C., Santana-Garrido, Á., & Vázquez, C. M. (2020). Lifestyle, maternal nutrition and Healthy Pregnancy. *Current Vascular Pharmacology*, *19*(2), 132–140. https://doi.org/10.2174/1570161118666200401112955

[33] Gernand, A. D., Schulze, K. J., Stewart, C. P., West, K. P., & Christian, P. (2016). Micronutrient deficiencies in pregnancy worldwide: Health effects and prevention. *Nature Reviews Endocrinology*, *12*(5), 274–289. https://doi.org/10.1038/nrendo.2016.37

[34] van Gool, J. D., Hirche, H., Lax, H., & De Schaepdrijver, L. (2018). Folic acid and primary prevention of neural tube defects: A review. *Reproductive Toxicology*, *80*, 73–84. https://doi.org/10.1016/j.reprotox.2018.05.004

[35] Bloomfield, F. H. (2011). How is maternal nutrition related to preterm birth? *Annual Review of Nutrition*, *31*(1), 235–261. https://doi.org/10.1146/annurev-nutr-072610-145141

[36] Zerfu, T. A., Pinto, E., & Baye, K. (2018). Consumption of dairy, fruits and dark green leafy vegetables is associated with lower risk of adverse pregnancy outcomes (APO): A prospective cohort study in rural Ethiopia. *Nutrition & Diabetes*, *8*(1). https://doi.org/10.1038/s41387-018-0060-y

[37] Williams, Christine B., Mackenzie, Kusaynyonon C., & Gahagan, Sheila (2014). The effect of maternal obesity on The offspring. *Clinical Obstetrics & Gynecology*, *57*(3), 508–515. https://doi.org/10.1097/grf.0000000000000043

[38] ECW;, D. A. K. G. (n.d.). *Increases in prepregnancy obesity: United States, 2016-2019*. NCHS data brief. Retrieved March 9, 2023, from https://pubmed.ncbi.nlm.nih.gov/33270551/

[39] Ramakrishnan, U., Grant, F., Goldenberg, T., Zongrone, A., & Martorell, R. (2012). Effect of women's nutrition before and during early pregnancy on maternal and infant outcomes: A systematic review. *Paediatric and Perinatal Epidemiology*, *26*, 285–301. https://doi.org/10.1111/j.1365-3016.2012.01281.x

[40] Pinheiro, T. V., & Goldani, M. Z. (2018). Maternal pre-pregnancy overweight/obesity and gestational diabetes interaction on delayed breastfeeding initiation. *PLOS ONE*, *13*(6). https://doi.org/10.1371/journal.pone.0194879

[41] Aimukhametova G, Ukybasova T, Hamidullina Z, Zhubanysheva K, Harun-Or-Rashid M, Yoshida Y, Kasuya H, Sakamoto J. The impact of maternal obesity on mother and neonatal health: study in a tertiary hospital of Astana, Kazakhstan. Nagoya J Med Sci. 2012 Feb;74(1-2):83-92. PMID: 22515114; PMCID: PMC4831253.

[42] Valentine CJ, Wagner CL. Nutritional management of the breastfeeding dyad. Pediatr Clin North Am. 2013 Feb;60(1):261-74. doi: 10.1016/j.pcl.2012.10.008. PMID: 23178069.

[43] Abu-Saad K, Kaufman-Shriqui V, Freedman LS, Belmaker I, Fraser D. Preconceptional diet quality is associated with birth outcomes among low socioeconomic status minority women in a high-income country. Eur J Nutr. 2021 Feb;60(1):65-77. doi: 10.1007/s00394-020-02221-4. Epub 2020 Mar 17. PMID: 32185478.

[44] Mitku AA, Zewotir T, North D, Jeena P, Naidoo RN. Modeling Differential Effects of Maternal Dietary Patterns across Severity Levels of Preterm Birth Using a Partial Proportional Odds Model. Sci Rep. 2020 Mar 26;10(1):5491. doi: 10.1038/s41598-020-62447-4. PMID: 32218503; PMCID: PMC7099014.

[45] Rasool A, Alvarado-Flores F, O'Tierney-Ginn P. Placental Impact of Dietary Supplements: More Than Micronutrients. Clin Ther. 2021 Feb;43(2):226-245. doi: 10.1016/j.clinthera.2020.11.017. Epub 2020 Dec 23. PMID: 33358257; PMCID: PMC8026523.

[46] Moossavi S, Sepehri S, Robertson B, Bode L, Goruk S, Field CJ, Lix LM, de Souza RJ, Becker AB, Mandhane PJ, Turvey SE, Subbarao P, Moraes TJ, Lefebvre DL, Sears MR, Khafipour E, Azad MB. Composition and Variation of the Human Milk Microbiota Are Influenced by Maternal and Early-Life Factors. Cell Host Microbe. 2019 Feb 13;25(2):324-335.e4. doi: 10.1016/j.chom.2019.01.011. PMID: 30763539.

[47] Bode L, McGuire M, Rodriguez JM, Geddes DT, Hassiotou F, Hartmann PE, McGuire MK. It's alive: microbes and cells in human milk and their potential benefits to mother and infant. Adv Nutr. 2014 Sep;5(5):571-3. doi: 10.3945/an.114.006643. PMID: 25469400; PMCID: PMC4188237.

[48] Collado MC, Isolauri E, Laitinen K, Salminen S. Distinct composition of gut microbiota during pregnancy in overweight and normal-weight women. Am J Clin Nutr. 2008 Oct;88(4):894-9. doi: 10.1093/ajcn/88.4.894. PMID: 18842773.

[49] Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, Bewtra M, Knights D, Walters WA, Knight R, Sinha R, Gilroy E, Gupta K, Baldassano R, Nessel L, Li H, Bushman FD, Lewis JD. Linking long-term dietary patterns with gut microbial enterotypes. Science. 2011 Oct 7;334(6052):105-8. doi: 10.1126/science.1208344. Epub 2011 Sep 1. PMID: 21885731; PMCID: PMC3368382.

[50] Cabrera-Rubio R, Collado MC, Laitinen K, Salminen S, Isolauri E, Mira A. The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery. Am J Clin Nutr. 2012 Sep;96(3):544-51. doi: 10.3945/ajcn.112.037382. Epub 2012 Jul 25. PMID: 22836031.

[51] Verduci E, Giannì ML, Vizzari G, Vizzuso S, Cerasani J, Mosca F, Zuccotti GV. The Triad Mother-Breast Milk-Infant as Predictor of Future Health: A Narrative Review. Nutrients. 2021 Feb 2;13(2):486. doi: 10.3390/nu13020486. PMID: 33540672; PMCID: PMC7913039.

[52] Bzikowska-Jura A, Sobieraj P, Szostak-Węgierek D, Wesołowska A. Impact of Infant and Maternal Factors on Energy and Macronutrient Composition of Human Milk. Nutrients. 2020 Aug 26;12(9):2591. doi: 10.3390/nu12092591. PMID: 32858897; PMCID: PMC7551856.

[53] Lemas DJ, Young BE, Baker PR 2nd, Tomczik AC, Soderborg TK, Hernandez TL, de la Houssaye BA, Robertson CE, Rudolph MC, Ir D, Patinkin ZW, Krebs NF, Santorico SA, Weir T, Barbour LA, Frank DN, Friedman JE. Alterations in human milk leptin and insulin are associated with early changes in the infant intestinal microbiome. Am J Clin Nutr. 2016 May;103(5):1291-300. doi: 10.3945/ajcn.115.126375. PMID: 27140533; PMCID: PMC4841936.

[54] Nommsen LA, Lovelady CA, Heinig MJ, Lönnerdal B, Dewey KG. Determinants of energy, protein, lipid, and lactose concentrations in human milk during the first 12 mo of lactation: the DARLING Study. Am J Clin Nutr. 1991 Feb;53(2):457-65. doi: 10.1093/ajcn/53.2.457. PMID: 1989413.

[55] De Luca A, Frasquet-Darrieux M, Gaud MA, Christin P, Boquien CY, Millet C, Herviou M, Darmaun D, Robins RJ, Ingrand P, Hankard R. Higher Leptin but Not Human Milk Macronutrient Concentration Distinguishes Normal-Weight from Obese Mothers at 1-Month Postpartum. PLoS One. 2016 Dec 22;11(12):e0168568. doi: 10.1371/journal.pone.0168568. PMID: 28005966; PMCID: PMC5179069.

[56] Fields DA, Demerath EW. Relationship of insulin, glucose, leptin, IL-6 and TNF-α in human breast milk with infant growth and body composition. Pediatr Obes. 2012 Aug;7(4):304-12. doi: 10.1111/j.2047-6310.2012.00059.x. Epub 2012 May 10. PMID: 22577092; PMCID: PMC3393795.

[57] Saben JL, Sims CR, Abraham A, Bode L, Andres A. Human Milk Oligosaccharide Concentrations and Infant Intakes Are Associated with Maternal Overweight and Obesity and Predict Infant Growth. Nutrients. 2021 Jan 29;13(2):446. doi: 10.3390/nu13020446. PMID: 33572881; PMCID: PMC7911788.

[58] Perez PF, Doré J, Leclerc M, Levenez F, Benyacoub J, Serrant P, Segura-Roggero I, Schiffrin EJ, Donnet-Hughes A. Bacterial imprinting of the neonatal immune system: lessons from maternal cells? Pediatrics. 2007 Mar;119(3):e724-32. doi: 10.1542/peds.2006-1649. PMID: 17332189.

[59] LeMay-Nedjelski L, Butcher J, Ley SH, Asbury MR, Hanley AJ, Kiss A, Unger S, Copeland JK, Wang PW, Zinman B, Stintzi A, O'Connor DL. Examining the relationship between maternal body size, gestational glucose tolerance status, mode of delivery and ethnicity on human milk microbiota at three months post-partum. BMC Microbiol. 2020 Jul 20;20(1):219. doi: 10.1186/s12866-020-01901-9. PMID: 32689933; PMCID: PMC7372813.

[60] Sugino KY, Ma T, Kerver JM, Paneth N, Comstock SS. Human Milk Feeding Patterns at 6 Months of Age are a Major Determinant of Fecal Bacterial Diversity in Infants. J Hum Lact. 2021 Nov;37(4):703-713. doi: 10.1177/0890334420957571. Epub 2020 Sep 14. PMID: 32926654.

[61] Prentice PM, Schoemaker MH, Vervoort J, Hettinga K, Lambers TT, van Tol EAF, Acerini CL, Olga L, Petry CJ, Hughes IA, Koulman A, Ong KK, Dunger DB. Human Milk Short-Chain Fatty Acid Composition is Associated with Adiposity Outcomes in Infants. J Nutr. 2019 May 1;149(5):716-722. doi: 10.1093/jn/nxy320. PMID: 31050748.

[62] Stinson LF, Gay MCL, Koleva PT, Eggesbø M, Johnson CC, Wegienka G, du Toit E, Shimojo N, Munblit D, Campbell DE, Prescott SL, Geddes DT, Kozyrskyj AL. Human Milk From Atopic Mothers Has Lower Levels of Short Chain Fatty Acids. Front Immunol. 2020 Jul 21;11:1427. doi: 10.3389/fimmu.2020.01427. PMID: 32903327; PMCID: PMC7396598.

[63] Lapidaire W, Lucas A, Clayden JD, Clark C, Fewtrell MS. Human milk feeding and cognitive outcome in preterm infants: the role of infection and NEC reduction. Pediatr Res. 2022 Apr;91(5):1207-1214. doi: 10.1038/s41390-021-01367-z. Epub 2021 Jun 24. PMID: 34168271; PMCID: PMC9122812.

[64] Taylor SN, Basile LA, Ebeling M, Wagner CL. Intestinal permeability in preterm infants by feeding type: mother's milk versus formula. Breastfeed Med. 2009 Mar;4(1):11-5. doi: 10.1089/bfm.2008.0114. PMID: 19196035; PMCID: PMC2932544.

[65] Taylor SN, Basile LA, Ebeling M, Wagner CL. Intestinal permeability in preterm infants by feeding type: mother's milk versus formula. Breastfeed Med. 2009 Mar;4(1):11-5. doi: 10.1089/bfm.2008.0114. PMID: 19196035; PMCID: PMC2932544.

[66] Cristofalo EA, Schanler RJ, Blanco CL, Sullivan S, Trawoeger R, Kiechl-Kohlendorfer U, Dudell G, Rechtman DJ, Lee ML, Lucas A, Abrams S. Randomized trial of exclusive human milk versus preterm formula diets in extremely premature infants. J Pediatr. 2013 Dec;163(6):1592-1595.e1. doi: 10.1016/j.jpeds.2013.07.011. Epub 2013 Aug 20. PMID: 23968744.

[67] Ford SL, Lohmann P, Preidis GA, Gordon PS, O'Donnell A, Hagan J, Venkatachalam A, Balderas M, Luna RA, Hair AB. Improved feeding tolerance and growth are linked to increased gut microbial community diversity in very-low-birth-weight infants fed mother's own milk compared with donor breast milk. Am J Clin Nutr. 2019 Apr 1;109(4):1088-1097. doi: 10.1093/ajcn/nqz006. PMID: 30982856; PMCID: PMC6462428.

[68] Lin YH, Hsu YC, Lin MC, Chen CH, Wang TM. The association of macronutrients in human milk with the growth of preterm infants. PLoS One. 2020 Mar 26;15(3):e0230800. doi: 10.1371/journal.pone.0230800. PMID: 32214387; PMCID: PMC7098608.

[69] Nommsen-Rivers LA, Chantry CJ, Peerson JM, Cohen RJ, Dewey KG. Delayed onset of lactogenesis among first-time mothers is related to maternal obesity and factors associated with ineffective breastfeeding. Am J Clin Nutr. 2010 Sep;92(3):574-84. doi: 10.3945/ajcn.2010.29192. Epub 2010 Jun 23. PMID: 20573792.

[70] Chetta KE, Schulz EV, Wagner CL. Outcomes improved with human milk intake in preterm and full-term infants. Semin Perinatol. 2021 Mar;45(2):151384. doi: 10.1016/j.semperi.2020.151384. Epub 2021 Jan 2. PMID: 33494913.

[71] Järvinen KM, Martin H, Oyoshi MK. Immunomodulatory effects of breast milk on food allergy. Ann Allergy Asthma Immunol. 2019 Aug;123(2):133-143. doi: 10.1016/j.anai.2019.04.022. Epub 2019 Apr 29. PMID: 31048004; PMCID: PMC6693634.

[72] *Alpha diversity*. One Codex Docs. (n.d.). Retrieved March 13, 2023, from https://docs.onecodex.com/en/articles/4136553-alpha-diversity

[73] Faith, D. P. (1992). Conservation evaluation and phylogenetic diversity. *Biological Conservation*, *61*(1), 1–10. https://doi.org/10.1016/0006-3207(92)91201-3

[74] Jaccard, P. (1912). The distribution of the flora in the alpine zone.1. *New Phytologist*, *11*(2), 37–50. https://doi.org/10.1111/j.1469-8137.1912.tb05611.x

[75] *Beta diversity*. One Codex Docs. (n.d.). Retrieved March 13, 2023, from https://docs.onecodex.com/en/articles/4150649-beta-diversity

[76] Korstanje, J. (2021, July 9). *Principal coordinates analysis*. Medium. Retrieved April 2, 2023, from https://towardsdatascience.com/principal-coordinates-analysis-cc9a572ce6c

[77] Schwiertz A, Taras D, Schafer K, et al. Microbiota and SCFA in lean and overweight healthy subjects. Obesity (Silver Spring) 2010;18:190-5.

[78] Schaefer K. Analysis of Short Chain Fatty Acids from different intestinal samples by capillary gas chromatograph. Chromatographia. 1995;40(9):550-6.

[79] Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic acids research, 41(1), e1

[80] Gao X, Zhang M, Xue J, Huang J, Zhuang R, Zhou X, Zhang H, Fu Q, Hao Y. Body Mass Index Differences in the Gut Microbiota Are Gender Specific. Front Microbiol. 2018 Jun 22;9:1250. doi: 10.3389/fmicb.2018.01250. PMID: 29988340; PMCID: PMC6023965.

[81] Zhong, X.; Harrington, J.M.; Millar, S.R.; Perry, I.J.; O'Toole, P.W.; Phillips, C.M. Gut Microbiota Associations with Metabolic Health and Obesity Status in Older Adults. *Nutrients* **2020**, *12*, 2364. https://doi.org/10.3390/nu12082364

[82] Heimann E, Nyman M, Pålbrink AK, Lindkvist-Petersson K, Degerman E. Branched short-chain fatty acids modulate glucose and lipid metabolism in primary adipocytes. Adipocyte. 2016 Oct 28;5(4):359-368. doi: 10.1080/21623945.2016.1252011. PMID: 27994949; PMCID: PMC5160390.

[83] Lin HV, Frassetto A, Kowalik EJ Jr, Nawrocki AR, Lu MM, Kosinski JR, Hubert JA, Szeto D, Yao X, Forrest G, Marsh DJ. Butyrate and propionate protect against diet-induced obesity and regulate gut hormones via free fatty acid receptor 3-independent mechanisms. PLoS One. 2012;7(4):e35240. doi: 10.1371/journal.pone.0035240. Epub 2012 Apr 10. PMID: 22506074; PMCID: PMC3323649.

[84] Centers for Disease Control and Prevention. (2015, April 15). *Using the who growth charts*. Centers for Disease Control and Prevention. Retrieved April 3, 2023, from https://www.cdc.gov/nccdphp/dnpao/growthcharts/who/using/index.htm

[85] Yun Y, Kim HN, Kim SE, Heo SG, Chang Y, Ryu S, Shin H, Kim HL. Comparative analysis of gut microbiota associated with body mass index in a large Korean cohort. BMC Microbiol. 2017 Jul 4;17(1):151. doi: 10.1186/s12866-017-1052-0. PMID: 28676106; PMCID: PMC5497371.

[86] Turnbaugh, P., Hamady, M., Yatsunenko, T. *et al.* A core gut microbiome in obese and lean twins. *Nature***457**, 480–484 (2009). https://doi.org/10.1038/nature07540

[87] Peters BA, Shapiro JA, Church TR, Miller G, Trinh-Shevrin C, Yuen E, Friedlander C, Hayes RB, Ahn J. A taxonomic signature of obesity in a large study of American adults. Sci Rep. 2018 Jun 27;8(1):9749. doi: 10.1038/s41598-018-28126-1. PMID: 29950689; PMCID: PMC6021409.

[88] Kondo T, Kishi M, Fushimi T, Ugajin S, Kaga T. Vinegar intake reduces body weight, body fat mass, and serum triglyceride levels in obese Japanese subjects. Biosci Biotechnol Biochem. 2009 Aug;73(8):1837-43. doi: 10.1271/bbb.90231. Epub 2009 Aug 7. PMID: 19661687.

[89] van Deuren T, Blaak EE, Canfora EE. Butyrate to combat obesity and obesity-associated metabolic disorders: Current status and future implications for therapeutic use. Obes Rev. 2022 Oct;23(10):e13498. doi: 10.1111/obr.13498. Epub 2022 Jul 20. PMID: 35856338; PMCID: PMC9541926.

[90] Cuna A, Morowitz MJ, Ahmed I, Umar S, Sampath V. Dynamics of the preterm gut microbiome in health and disease. Am J Physiol Gastrointest Liver Physiol. 2021 Apr 1;320(4):G411-G419. doi: 10.1152/ajpgi.00399.2020. Epub 2021 Jan 13. PMID: 33439103; PMCID: PMC8238167.

[91] Rios-Covian, D., González, S., Nogacka, A. M., Arboleya, S., Salazar, N., Gueimonde, M., & de los Reyes-Gavilán, C. G. (2020). An overview on fecal branched short-chain fatty acids along human life and as related with body mass index: Associated Dietary and anthropometric factors. *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/fmicb.2020.00973

[92] Wang, X., Li, J., Li, N., Guan, K., Yin, D., Zhang, H., Ding, G., & Hu, Y. (2021). Evolution of intestinal gases and fecal short-chain fatty acids produced in vitro by preterm infant gut microbiota during the first 4 weeks of life. *Frontiers in Pediatrics*, *9*. https://doi.org/10.3389/fped.2021.726193

[93] Coelho GDP, Ayres LFA, Barreto DS, Henriques BD, Prado MRMC, Passos CMD. Acquisition of microbiota according to the type of birth: an integrative review. Rev Lat Am Enfermagem. 2021 Jul 19;29:e3446. doi: 10.1590/1518.8345.4466.3446. PMID: 34287544; PMCID: PMC8294792.

[94] Borrello K, Lim U, Park SY, Monroe KR, Maskarinec G, Boushey CJ, Wilkens LR, Randolph TW, Le Marchand L, Hullar MA, Lampe JW. Dietary Intake Mediates Ethnic Differences in Gut Microbial Composition. Nutrients. 2022 Feb 4;14(3):660. doi: 10.3390/nu14030660. PMID: 35277019; PMCID: PMC8840192.

[95] Jo JH, Kennedy EA, Kong HH. Research Techniques Made Simple: Bacterial 16S Ribosomal RNA Gene Sequencing in Cutaneous Research. J Invest Dermatol. 2016 Mar;136(3):e23-e27. doi: 10.1016/j.jid.2016.01.005. PMID: 26902128; PMCID: PMC5482524.